Design and Optimization of Sulfuric Acid Pretreatment of Extracted Olive Tree Biomass Using Response Surface Methodology

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Olive tree biomass (OTB) represents an interesting feedstock for bioethanol production. In this study, olive tree pruning was water extracted and pretreated by dilute sulfuric acid to achieve high sugar recoveries from cellulosic and hemicellulosic fractions. Temperature (160 to 200 °C), acid concentration (0 to 8 g acid/100 g extracted raw material), and solids loading (15% to 35% w/v) were selected as operation variables and modified according to a Box-Behnken experimental design. The optimal conditions for the acid pretreatment were 160 °C, 4.9 g sulfuric acid/100 g biomass, and 35% solids loading (w/v), according to multiple criteria that considered the maximization of both the hemicellulosic sugars concentration in prehydrolysate and the overall sugar yield. These optimized conditions yielded a sugar concentration of 79.8 g/L, corresponding to an overall yield of 39.8 g total sugars/100 g extracted OTB. The fermentability of hemicellulosic sugars prehydrolysates from the acid pretreatment was evaluated by *Escherichia coli* after a detoxification stage by overliming. The prehydrolysates with lower concentrations of toxic compounds were fermented and achieved ethanol yields higher than 80% of the theoretical ethanol yield.

Keywords: Olive tree pruning; Acid pretreatment; High solids loading; E. coli; Bioethanol

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INTRODUCTION

Olive tree (*Olea europaea*) biomass is an agricultural residue generated primarily in Mediterranean countries. This lignocellulosic material is produced every two years after fruit harvesting and includes leaves and wood in varying proportions (Martínez-Patiño *et al.* 2015). According to the Food and Agriculture Organization of the United Nations (FAO 2016), over 10 million ha worldwide were occupied by olive tree cultivation in 2014, and some 30 million T of olive tree pruning biomass were generated according to an average production of 3 T per ha (Cara *et al.* 2006). This renewable material resulting from the pruning labor is usually eliminated from fields by burning, consequently contributing to climate change. The content of sugars in this biomass, approximately 50%, makes the material a suitable feedstock for the production of bioethanol (Romero-García *et al.* 2014).

The biorefinery concept uses plants with integrated processes that maximize the utilization of whole lignocellulosic feedstock by fractionation of the three major components (cellulose, hemicellulose, and lignin) and conversion into valuable bioproducts (Carvalheiro *et al.* 2008). The complex structure of lignocellulosic biomass,

with physical and chemical barriers, hinders second-generation ethanol production, which has not yet been commercialized (Galbe and Zacchi 2012; Buruiana *et al.* 2014; Brown 2015). Pretreatment plays an essential role in the biochemical processes of bioethanol (Carvalheiro *et al.* 2008; Chiaramonti *et al.* 2012; Leitner and Lindofer 2016) and is considered the most expensive stage (Chiaramonti *et al.* 2012). Its objective is to maximize the recovery of fermentable sugars using low energy input and inhibitory co-products (Ben Chaabane and Marchal 2013).

Pretreatment technologies for lignocellulosic materials include physical processes and thermochemical and biological treatments (Taherzadeh and Karimi 2008). Acid pretreatments involve the removal of hemicellulose components and reduction of the recalcitrance of the lignocellulosic biomass. In these processes, acid acts as a catalyst, partially solubilizing hemicelluloses and lignin, leaving a cellulose-rich substrate that is more accessible to enzyme degradation (Silverstein *et al.* 2007; Taherzadeh and Karimi 2008; Alvira *et al.* 2010). Dilute acid is an effective and well-known technology for pretreating lignocellulosic biomass (Sassner *et al.* 2008; Taherzadeh and Karimi 2008; Alvira *et al.* 2010; Chiaramonti *et al.* 2012). Moreover, pretreatment by dilute acid is a favorable method for use on the industrial scale (Carvalheiro *et al.* 2008; Alvira *et al.* 2010).

When increasing dry matter/solids loading, acid pretreatments generate higher amounts of degradation products, such as acetic and formic acid, furfural, hydroxymethylfurfural (HMF), and phenolic compounds. However, the type and concentrations of these compounds also depend on the lignocellulosic feedstock. Therefore, it is crucial to optimize this stage for every feedstock (Koppram *et al.* 2014; Leitner and Lindofer 2016).

The aim of this work was to optimize sulfuric acid pretreatment of extracted olive tree biomass (OTB) at high dry matter, maximizing the production of fermentable sugars. Considering the importance of utilizing the whole material to improve the process economy (Sassner *et al.* 2008), this work focused on the pretreatment conditions that allowed high enzymatic digestibility of solids and high hemicellulosic sugars recoveries in prehydrolysates. The fermentability of the hemicellulosic sugar solutions by an ethanologenic *Escherichia coli* that could consume both C5 and C6 sugars was evaluated after a detoxification step.

EXPERIMENTAL

Preparation of the Lignocellulosic Feedstock

Olive tree biomass was collected in the province of Jaen (Spain), air-dried, and ground to a particle size smaller than 4 mm. Then, a water extraction in an autoclave at 120 °C for 60 min was performed in 10-L bottles containing 700 g of OTB at 10% (w/v) solid loading to remove the extractive fraction. After this step, solids were filtered, washed, and dried in an oven at 40 °C. The solid fraction of extracted OTB was characterized as described in the Analytical Methods Section.

Sulfuric Acid Pretreatment

Extracted OTB was used as a substrate in this step. Sulfuric acid pretreatment was carried out in a 1-L stirred tank reactor (Parr Instrument Company, Moline, IL, USA) made of stainless steel and then surface treated with Carpenter 20®. Three variables were

studied, *i.e.*, temperature, acid concentration, and S/L ratio. The classical experimental approach, where only a variable is modified while the other two remain constant, would require 3x3x3=27 experiments. As a favorable alternative, experimental designs, where all the variables or factors are simultaneously modified, can result in a lower number of experiments, without lacking of accuracy (Bezerra *et al*. 2008). In the present case, a Box-Behnken experimental design was performed, with a total of 17 experiments (4 of them were replicates of the center point). Agitation was set at 300 rpm. Solid loadings ranged between 15% and 35% (w/v), and the volume of liquid remained constant (400 mL). Acid concentration was varied between 0% and 8% (g of acid/100 g of biomass). The reactor was heated at a rate of 5 °C/min, and the temperature ranged from 160 to 200 °C. Residence time was 10 min once the pretreatment temperature was reached, followed by a fast cooling by placing the reactor in an ice bath until it reached 40 °C. Water-insoluble solids (WIS) and liquid fractions were separated by filtration. Sugar and inhibitor compositions of liquid fractions (prehydrolysates) were measured and, after a detoxification step, subsequently submitted to fermentability tests with *Escherichia coli* MM160. Concerning pretreated solids, they were characterized and subjected to an enzymatic hydrolysis for assessing cellulose digestibility. The results were analyzed by the statistical software Design Expert 7.0.0 (Stat-Ease Inc., Minneapolis, MN, USA).

Enzymatic Hydrolysis

Enzymatic hydrolysis (EH) of washed pretreated solids was performed in 100-mL Erlenmeyer flasks, containing 25 mL of 0.05 M sodium citrate buffer (pH 4.8) and 1.25 g of solid (5% solids concentration). The Erlenmeyer flasks were kept in a rotatory shaker at 50 °C and 150 rpm for 72 h. The cellulase enzyme loading (CellicCTec3, Bagsvaerd, Denmark) was 15 FPU/g of substrate and β-glucosidase (Novozyme 50010, Bagsvaerd, Denmark) loading was 15 international units/g of substrate, both kindly provided by Novozymes A/S (Bagsvaerd, Denmark). The experiments were performed in triplicate and samples were taken every 24 h to determine glucose concentration by high performance liquid chromatography (HPLC). The amount of glucose present in commercial enzyme solutions was measured for consideration in the final results of enzymatic hydrolysis.

Fermentability Tests

Liquid fractions obtained after pretreatment were subjected to fermentability tests to determine whether, after detoxification, the sugars present in these prehydrolysates could be fermented to ethanol by a recombinant *E. coli* MM160.

The detoxification step was performed by overliming (Martínez *et al.* 2000), to increase the fermentability of prehydrolysates by decreasing the concentrations of degradation products. Overliming consists of adding $Ca(OH)_2$ until pH 10 is reached, and shaking for 30 min at 50 °C. Afterwards, the formed precipitate was removed by centrifugation at 3500 rpm for 10 min; the pH of the supernatant solution was adjusted to 6.5 with sulfuric acid, and this solution was centrifuged again if more precipitate was formed. Prehydrolysates obtained without acid (runs 1, 4, 7, and 15) were previously submitted to a mild acid posthydrolysis (3% w/v sulfuric acid at 120 °C for 30 min) to break oligomeric components into monomeric sugars that could be fermented by *E. coli*.

The microorganism *E. coli* MM160 was kindly donated by Dr. Ingram from the University of Florida, Gainesville, FL, USA. The inoculum was prepared by adding 2 mL stock frozen in 40% glycerol to Erlenmeyer flasks containing 75 mL of AM1 culture

medium (Martínez *et al.* 2007). The bacteria were grown on a rotatory shaker at 150 rpm and 37 °C for 24 h.

The fermentability tests were carried out in the devices described in Martínez-Patiño *et al.* (2015) at 37 °C and pH 7.0. The volume of inoculum required was determined by measuring the absorbance at 620 nm to obtain an initial biomass concentration of 0.5 g/L (dry basis) in the prehydrolysate. To avoid the dilution of the prehydrolysate, the inoculum was centrifuged at 3500 rpm for 10 min. The liquid was separated, and the solid biomass was re-suspended in 150 mL of prehydrolysate. Finally, the salts of the AM1 culture medium (Martínez *et al.* 2007) were added to the prehydrolysate, which was sterilized by filtration (0.22 µm, Millipore, Ireland). Several samples were taken to determine the consumption of sugars and the production of ethanol during the time.

Analytical Methods

The composition of solid fractions (raw OTB, extracted OTB, and pretreated solids) was determined according to the National Renewable Energy Laboratory (NREL) analytical methods for biomass (2016). Determination of the extractive fraction also included a previous two-step extraction procedure with water and ethanol in the case of raw OTB and extracted OTB.

Liquid fractions (prehydrolysates and fermentation broths) were analyzed by HPLC equipped with a refractive index detector. Sugar composition in prehydrolysates (glucose, xylose, galactose, arabinose, and mannose) were determined using a carbohydrate column (CARBOSep CHO-782 Pb, Transgenomic, Inc., Omaha, NE, USA) with ultrapure water as an eluent, a flow rate of 0.6 mL/min, and a column temperature of 70 °C. Samples were previously neutralized with $CaCO_3$, centrifuged, and filtered through 0.2 µm membranes. Prehydrolysates from runs 1, 4, 7, and 15 (without sulfuric acid as a catalyst) were measured before and after a mild acid posthydrolysis (3% w/v sulfuric acid at 120 °C for 30 min) to determine oligomeric fraction.

In the case of inhibitory compounds in prehydrolysates (formic and acetic acid, HMF, and furfural) or fermentation profile (sugars, inhibitors, and ethanol), an ICSep ICE-COREGEL 87H3 column (Transgenomic, Inc., Omaha, NE, USA) was used. The mobile phase was 5 mM sulfuric acid solution at a flow rate of 0.6 mL/min flow rate, and the column temperature was 65 °C. In this column, xylose, galactose, and mannose had the same retention times. Consequently, their concentration was presented collectively as the sum, called XGM.

Total phenolic compounds, expressed as gallic acid equivalents, were measured by spectrophotometry using the Folin-Ciocalteu reagent (Singleton and Rossi 1965).

RESULTS AND DISCUSSION

Feedstock

The composition of OTB before and after water extraction is summarized in Table 1. The raw OTB contained 55% total sugars, with glucose being the main sugar, although 17% of this sugar was identified in the extractive fraction as non-structural glucose. Xylose was the main hemicellulosic sugar, accounting for 63% of the sugar content in the hemicellulose fraction. Before the acid pretreatment, raw OTB was subjected to an aqueous extraction in order to remove soluble components like extractives, avoiding the

formation of complex lignin-carbohydrates (Ballesteros *et al.* 2011). Li *et al.* (2016) also tested the effect of removing water extractives from corn stover, and determined a significant increase in the enzymatic digestibility after liquid hot water pretreatment.

The solid recovery in this extraction step was 80%, with a solubilization of 83.3% of the aqueous extractive fraction. Non-structural glucose content in extractives was also almost completely solubilized.

Table 1. Chemical Characterization of OTB (% Oven Dry Weight)

Composition	Raw OTB	Extracted OTB
Glucose	27.9 ± 1.1	34.4 ± 1.3
Hemicellulosic sugars	21.3 ± 0.8	26.5 ± 0.6
Xylose	13.5 ± 0.8	16.4 ± 0.6
Galactose	2.6 ± 0.1	3.2 ± 0.1
Arabinose	3.1 ± 0.2	4.6 ± 0.2
Mannose	2.1 ± 0.1	2.3 ± 0.1
Lignin	18.5 ± 0.3	23.2 ± 0.2
Acid insoluble lignin	16.1 ± 0.3	20.3 ± 0.3
Acid soluble lignin	2.4 ± 0.1	2.9 ± 0.1
Total extractives	24.8 ± 0.7	8.9 ± 0.5
Aqueous extractives	21.1 ± 0.5	4.4 ± 0.4
Ethanolic extractives	3.7 ± 0.2	4.5 ± 0.1
Glucose in aqueous extractives	5.7 ± 0.2	0.8 ± 0.1
Acetyl groups	2.0 ± 0.1	2.3 ± 0.1
Ash	2.9 ± 0.4	3.8 ± 0.2

Table 2. Solid Recovery and Pretreated Solids Composition

Run	*T* (°C)	Acid Concentration (g H_2SO_4/100 g biomass)	Solid Loading (%w/v)	Solid Recovery (%)	Glucose (%)	Xylose (%)	AIL* (%)
1	180	0	35	68.4	46.9	9.9	34.1
2	200	4	35	55.8	40.8	-	54.5
3	200	4	15	51.7	45.0	-	49.9
4	160	0	25	80.6	36.2	17.3	30.4
5	160	4	35	58.6	44.8	3.8	42.3
6	160	8	25	53.3	48.1	1.9	44.7
7	180	0	15	64.8	44.4	8.8	35.6
8	180	8	35	48.9	37.6	-	61.4
9	180	4	25	54.5	47.0	-	45.6
10	180	4	25	54.9	47.3	-	45.3
11	180	4	25	54.3	45.3	-	46.5
12	180	8	15	47.9	50.0	-	48.9
13	180	4	25	55.0	45.6	-	45.2
14	180	4	25	54.5	45.6	-	44.8
15	200	0	25	63.1	43.6	2.7	42.0
16	200	8	25	43.6	20.1	-	72.0
17	160	4	15	58.4	45.6	3.9	38.9

*Acid insoluble lignin

Effect of Sulfuric Acid Pretreatment

Acid pretreated solids

As expected, maximum solid recovery corresponded to the softest pretreatment conditions (run 4, 160 °C, no acid, 25% solid loading) with a value as high as 80.6% (Table 2). Only 43.6% of solids were recovered at the most severe conditions (run 16, 200 °C, 8% H_2SO_4, 25% solid loading). Because extractives were mostly solubilized in the previous aqueous extraction, the biomass solubilized by acid pretreatment corresponded mainly to the hemicellulose fraction and some part of the glucan fraction, depending on pretreatment conditions. Even though most of the glucans could be considered cellulose, approximately 20% of OTB glucan is starch, which is not solubilized in the water extraction step (Ballesteros *et al*. 2011). This starch fraction should be solubilized during the acid pretreatment.

As shown in Table 2, in the experiment performed under the mildest conditions (run 4), no hemicellulose solubilization was detected, and pretreated solid contained 17.5% xylose. However, the extreme pretreatment conditions (run 16) achieved the total solubilization of the hemicellulose fraction and an important solubilization of the cellulose fraction, leaving a pretreated solid with high lignin content and low cellulose content (18.2%).

Overall, when OTB was pretreated without acid (runs 1, 4, 7, and 15), the resulting solids maintained noticeable xylose content. These results were similar to those obtained by Cara *et al.* (2007) with OTB pretreated by hot water without previous extraction. When sulfuric acid was used in the pretreatment, a more effective solubilization of the hemicellulose fraction was observed. Only a slight presence of xylose in OTB after pretreatment was detected in the experiments conducted with sulfuric acid at 160 °C (runs 5, 6, and 17).

As shown in Table 2, pretreatment conditions at the central point (runs 9, 10, 11, 13, and 14) of the experimental design resulted in pretreated solids free of hemicellulose and with a glucose content of approximately 46%. A close result in terms of glucose content was reported by Cara *et al*. (2008), working in similar conditions with unextracted OTB (2.8 g sulfuric acid/g biomass at 180 ºC). This similarity confirmed that the extraction step did not imply an easier removal of structural sugars. Nevertheless, the effect that the water extraction of OTB had on decreasing lignin re-condensation reactions was evidenced. The lignin recovery in the case of pretreated OTB without previous extraction (Cara *et al*. 2007, 2008) reached values close to 200%. In this study, however, only a slight lignin re-condensation was detected in most of the experiments. A considerable increase in lignin recovery (approximately 150%) was observed in the experiment performed at the hardest conditions (8% sulfuric acid, 200 °C, run 16), and in the experiments conducted at 35% (w/v) solid loading (runs 2 and 8). By comparing experiments performed with the same pretreatment conditions and at lower solid loading (runs 3 and 12, respectively), lower re-condensation was observed. This observation suggested a relationship between the higher presence of solid matter and the possible occurrence of lignin re-condensation reactions during the pretreatment of biomass.

Acid prehydrolysates

One of the main objectives of the pretreatment was to achieve biomass fractionation by solubilizing the hemicellulosic fraction. The fractionation resulted in a solution rich in hemicellulosic sugars that could be fermented into ethanol, although minimal formation of inhibitory compounds was desired. Sugar and inhibitor

concentrations obtained at different pretreatment conditions are shown in Tables 3 and 4, respectively. A post-hydrolysis step (described in the Fermentability tests section) was necessary to release free sugars in pretreatment experiments conducted without acid (runs 1, 4, 7, and 15); this step produced up to 80% of sugars in oligomeric form. The sugar and inhibitor concentrations for these experiments were determined after this post-hydrolysis step (Tables 3 and 4).

Table 3. Sugar Composition of Acid Prehydrolysates and Hemicellulosic Sugar Recovery

Run	Glucose (g/L)	Xylose (g/L)	Galactose (g/L)	Arabinose (g/L)	Mannose (g/L)	Hemicellulosic Sugar Recovery (%)[a]
1*	12.56	26.51	5.66	7.94	1.73	45.0
2	12.48	5.31	3.09	3.32	1.21	13.9
3	6.34	4.53	2.12	2.57	0.87	25.3
4*	6.47	3.84	3.01	8.83	0.47	24.3
5	16.31	37.80	8.74	13.94	3.19	68.4
6	13.60	26.42	6.61	9.59	2.57	68.0
7*	5.66	13.35	2.64	4.16	0.71	52.3
8	27.91	12.79	5.02	5.92	2.34	28.1
9	13.40	24.01	6.58	9.60	2.09	63.6
10	12.84	23.91	5.75	8.43	2.03	60.4
11	13.22	24.39	5.78	8.54	2.08	61.4
12	10.51	11.54	3.20	4.34	1.21	50.9
13	12.54	22.84	5.46	7.89	1.95	57.4
14	13.57	24.60	5.78	8.25	2.12	61.3
15*	5.26	5.34	1.73	0.83	1.62	14.3
16	21.47	0.97	1.23	0.85	0.60	5.5
17	7.78	18.58	3.95	6.68	1.17	76.2

*Concentrations determined after a post-hydrolysis step

[a] Hemicellulosic sugar recovery: g hemicellulosic sugar in prehydrolysate/100 g hemicellulosic sugar in extracted OTB

Table 4. Composition of Inhibitory Compounds in Prehydrolysates

Run	Formic Acid (g/L)	Acetic Acid (g/L)	HMF (g/L)	Furfural (g/L)	Phenolic Compounds** (g/L)
1*	2.69	9.10	0.30	2.00	5.39
2	3.61	12.85	5.13	13.51	6.11
3	1.69	5.83	1.87	7.51	3.96
4*	0.74	1.82	0.08	0.33	2.91
5	3.40	10.38	0.34	2.55	6.36
6	3.81	8.64	0.40	4.19	7.46
7*	1.01	4.30	0.13	0.90	2.76
8	5.36	11.92	1.82	10.49	7.88
9	2.78	8.73	0.71	5.17	5.64
10	2.97	8.79	0.67	5.01	6.01
11	3.02	8.88	0.71	5.28	5.83
12	2.39	5.70	0.59	5.29	4.96
13	2.77	8.62	0.69	5.10	5.74
14	2.90	8.89	0.72	5.25	5.82
15*	4.42	9.87	0.64	4.02	5.95
16	5.23	9.31	4.02	9.95	6.64

*Concentrations determined after a post-hydrolysis step

**Measured as gallic acid equivalent

High pretreatment temperatures combined with high H_2SO_4 concentrations resulted in higher hemicellulose solubilization and high degradation of the solubilized carbohydrates (demonstrated in Tables 3 and 4).

In general, xylose was the main sugar in the prehydrolysates. High severity of the pretreatment, however, could mean degradation of this sugar and, therefore, lower xylose concentration in prehydrolysates. This fact was seen in runs 2, 3, 8, and 16 (Table 3), and related to high concentrations of furfural in these experiments, ranging between 7.5 and 13.5 g/L (Table 4) because this furan compound was formed by degradation of C5-sugars. Xylose was not the main sugar in the liquid fraction of run 4, although in this case the mild conditions did not allow the solubilization of a high proportion of the hemicellulose fraction. Hydroxymethylfurfural in prehydrolysates from the degradation of hexoses (glucose, galactose, and mannose) exceeded only 1% (g HMF/100 g extracted OTB) when the pretreatment was carried out at 200 °C in the presence of sulfuric acid (runs 2, 3, and 16). Degradation reactions of furan compounds resulted in the formation of formic acid. The formic acid concentration was lower than 1 g/L only in the experiment conducted at the lowest pretreatment temperature without acid (run 4). At the highest level of temperature and acid concentration (run 16), a maximum concentration of 5.3 g/L was attained (Table 4).

The presence of inhibitory compounds in prehydrolysates related to the sugar degradation produced by high acid concentrations and high temperatures, except in the case of acetic acid. In general, high concentrations of inhibitory compounds were detected in this study (Table 4), probably due to the high-solids loading used in the pretreatment (Koppram *et al*. 2014). Thus, at 25% and 35% solid loadings, concentrations of acetic acid, which is produced by the deacetylation of hemicellulose, were higher than 8 g/L, achieving a maximum value of 12.9 g/L for run 2 (200 °C, 4% H_2SO_4, 35% solid loading). However, acetic acid concentration as low as 1.8 g/L was measured in the prehydrolysate corresponding to run 4, also at high-solids loading (25% w/v). This high concentration was attributed to the low severity of this pretreatment, which did not solubilize the hemicellulose fraction (Table 2). Therefore, the acetyl groups in that fraction were not solubilized at these conditions.

Recoveries of hemicellulosic sugars less than 25% (refer to the hemicellulosic sugars content in extracted OTB) were determined for pretreatment experiments carried out at 200 °C (runs 2, 3, 15, and 16) due to sugar degradation reactions. In the central point conditions of the experimental design (180 °C, 4% H_2SO_4, 25% solid loading) the sugar recovery in the liquid fraction was approximately 60%.

However, the use of high-solid loadings during pretreatment allowed prehydrolysates rich in sugars to be obtained when the severity conditions precluded sugar degradation and hence high levels of inhibitors. Thus, the highest sugar concentration in prehydrolysate, 80 g/L, was obtained at 35% solid loading with 4% H_2SO_4 at 160 °C (run 5). This result corresponded to 68.4% of the hemicellulosic sugars in extracted OTB. The highest hemicellulosic sugar recovery, 76.2%, was achieved at the same conditions, although at 15% solid loading (run 17) and consequently the sugar concentration was only of 38 g/L. This maximum recovery compared favorably with that obtained previously using the same raw material and phosphoric acid as a catalyst during pretreatment. In that instance, hemicellulosic sugar recovery of less than 70% was reported (Martínez-Patiño *et al*. 2015).

Enzymatic Hydrolysis

In order to study the improvement of the enzymatic hydrolysis of OTB after the sulfuric acid pretreatment, enzymatic hydrolysis tests were carried out under standard conditions with a solid loading of 5% for all the pretreated solids.

Table 5 shows that glucose concentrations by enzymatic hydrolysis ranged between 10 g/L and 20 g/L, except in the pretreatment carried out at the lowest temperature and without acid (run 4). In this experiment, a glucose solution of only 4.8 g/L was attained, corresponding to the lowest Y_{EH} (27.8%) and glucose recovery by EH (23%). This fact could be explained because the mild pretreatment conditions did not alter the cellulose structure enough. Furthermore, the presence of 17.3% hemicellulose in this pretreated solid (Table 2) could have hindered enzymatic access to the cellulose chains, consequently causing the poor performance of enzymatic hydrolysis.

Table 5. Glucose Production by Enzymatic Hydrolysis of Pretreated OTB

Run	Glucose Concentration by EH (g/L)	EH Yield (%)	Glucose Recovery by EH (%)
1	12.41 ± 0.40	55.1	50.3
2	16.78 ± 0.07	85.2	55.2
3	19.94 ± 0.71	91.8	60.9
4	4.77 ± 0.03	27.8	23.0
5	14.14 ± 0.26	65.5	48.9
6	16.24 ± 0.25	70.2	51.1
7	13.69 ± 0.47	64.2	52.5
8	14.15 ± 0.29	78.0	40.8
9	18.68 ± 1.04	82.1	60.0
10	16.85 ± 0.45	73.6	54.3
11	17.79 ± 0.19	81.1	56.7
12	17.93 ± 0.09	73.9	50.4
13	17.43 ± 0.14	79.0	56.4
14	17.14 ± 0.55	77.8	54.9
15	18.32 ± 1.43	86.6	67.8
16	9.50 ± 0.33	97.7	24.3
17	13.52 ± 0.29	61.3	46.5

EH yield: g glucose by enzymatic hydrolysis/100 g glucose in pretreated OTB
Glucose recovery by EH: g glucose by enzymatic hydrolysis/100 g glucose content in extracted OTB

The maximum values of EH yields were attained at the highest temperature (200 °C) with yields above 85% (runs 2, 3, 15, and 16), although the glucose recovery by EH decreased when the acid concentration decreased (because of cellulose solubilization). Thus, cellulose was almost completely hydrolyzed when OTB was pretreated at the highest temperature and acid concentration (run 16), achieving an EH yield of 97.7%, although the glucose recovery by EH was as low as 24.3%. On the other hand, the highest glucose concentration (19.9 g/L) was achieved at 15% solid loading with 4% H_2SO_4 and 200 °C (run 3), corresponding to an EH yield of 91.8% and a glucose recovery of 60.9%. As can be observed in Table 5, when OTB was pretreated at 200 °C without acid (run 15), better results in terms of glucose recovery were obtained: an EH yield of 86.6% was

attained, and the glucose recovery by EH was 7% higher (67.8%). These results can be explained because the presence of acid in the pretreatment increased the enzymatic digestibility of OTB, although it also resulted in higher cellulose solubilization. These results accorded with previously reported results that examined unextracted OTB pretreated by hot water (Cara *et al*. 2007). The present results compare favorably with those achieved by dilute sulfuric acid pretreatment on un-extracted OTB (Cara *et al*. 2008).

Evaluation of Key Factors on Sugar Solubilization

Considering that hemicellulosic sugars C5 and C6 represent more than 43% of the total sugars in raw OTB, it is crucial to find alternative uses, or to utilize microorganisms that can ferment this sugar stream to reduce the cost of the biological conversion of this feedstock (Carvalheiro *et al*. 2008). The overall sugar yield ($Y_{overall}$) represents the recovery of fermentable sugars from the extracted OTB, and it may be used to evaluate the performance of both pretreatment and enzymatic hydrolysis. This parameter was determined by taking into account sugar contents in the prehydrolysate and glucose released by enzymatic hydrolysis.

Sulfuric acid pretreatment was carried out based on the experimental design obtained by Design Expert 7.0.0 software. Because this work focused on maximizing the production of fermentable sugars, sugar concentration in prehydrolysate (C_{sugars}) and overall sugar yield ($Y_{overall}$) were chosen as model responses. Second-order polynomial equations were used to express these responses as functions of the coded independent factors (Eqs. 1 and 2), where T, C_A, and SL represented the temperature, sulfuric acid concentration, and solid loading, respectively.

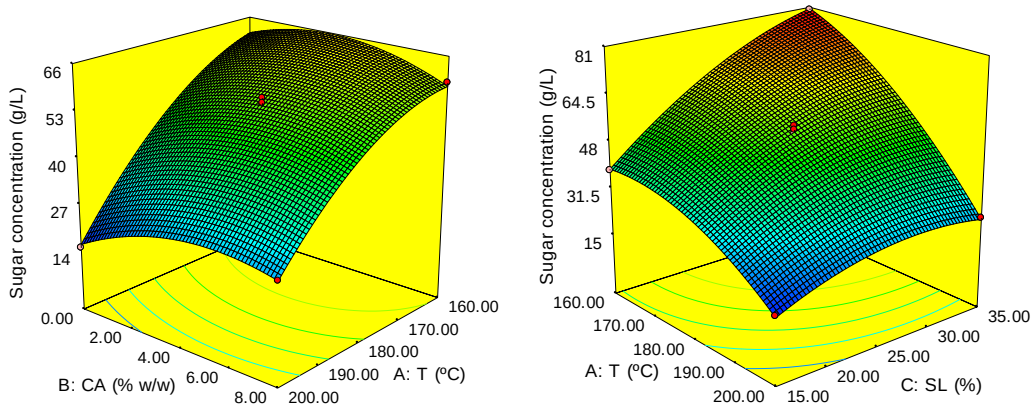
$$\text{Sugar concentration in prehydrolysate (g/L)} = 53.53 - 19.57T + 1.45C_A + 12.73SL + 3.23TC_A - 8.21TSL - 7.48T^2 - 6.05C_A^2 - 6.06SL^2 \quad (1)$$

$$R^2 = 0.9953 \quad R^2\,adjust = 0.9900$$

$$\text{Overall sugar yield (\%)} = 40.94 - 5.64T - 0.028C_A - 2.36SL - 5.84TC_A - 1.40C_A SL - 5.35T^2 - 6.21C_A^2 \quad (2)$$

$$R^2 = 0.9774 \quad R^2\,adjust = 0.9576$$

As can be seen in Eqs. 1 and 2, according to the values of the coefficients of determination R^2 and R^2_{adjust}, the models were highly predictive for both responses. The negative effect of the temperature factor was the most significant in both cases. The acid concentration and solid loading exerted a positive influence on sugar concentration in liquids. When also considering the solubilization of glucose by enzymatic hydrolysis, the influence of both factors on the response $Y_{overall}$ was negative, although less significant (Eq. 2).



(a) **(b)**

Fig. 1. The combined effects of different factors on the sugar concentration of the prehydrolysate: (a) pretreatment temperature and sulfuric acid concentration at 25% solids loading, and (b) pretreatment temperature and solids loading at 4% (w/v) sulfuric acid

Figure 1a shows the response surface for the sugar concentration of prehydrolysates (C_{sugars}) as a function of temperature and sulfuric acid concentration at 25% solid loading. C_{sugars} decreased with increasing pretreatment temperature because of the degradation of sugars at those conditions. This response increased slightly, however, when the acid concentration increased as high as 4%, and decreased slightly thereafter at all pretreatment temperatures. Regarding solid loading, C_{sugars} was highest, as expected, when this parameter was maintained at a high level and temperature at the low level. The influence of solid loading on C_{sugars} was, however, less significant at the highest pretreatment temperature (Fig. 1b), possibly because of sugar degradation processes.

The response surface for the combined effects of pretreatment temperature (T) and sulfuric acid concentration (C_A) on the overall sugar yield is depicted in Fig. 2a. Both factors exerted a positive effect up to 180 °C and 4%, respectively, while an increase of both factors resulted in lower values of $Y_{overall}$. This fact can be explained by sugar degradation at highest temperatures and acid concentrations (because of the high severity of the pretreatment) involving lower sugar recoveries. The effect of solid loading on this response is shown in Fig. 2b, with a negative influence at the highest acid concentrations, achieving values of $Y_{overall}$ similar to those experiments conducted without acid. This fact was explained because the absence of acid in the pretreatment did not allow a high sugar recovery of OTB to be obtained, while high acid concentrations involved reactions of solubilized sugar degradation.

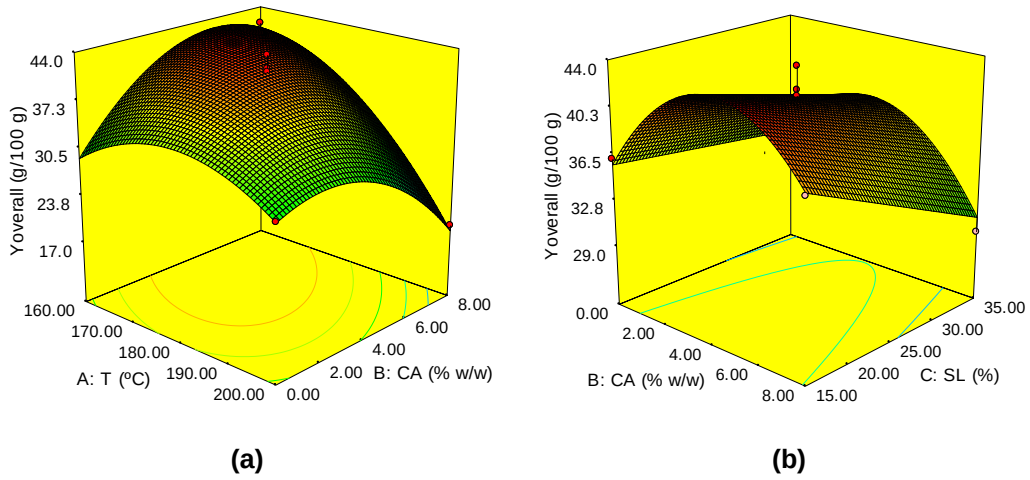


Fig. 2. The combined effects of different factors on the overall sugar yield: (a) pretreatment temperature and sulfuric acid concentration at 25% solids loading, and (b) sulfuric acid concentration and solids loading at 180 °C

The highest values of $Y_{overall}$ were achieved at central point conditions (runs 9, 10, 11, 13, and 14) at 160 °C (runs 5, 6, and 17) with values above 40 g sugars/100 g extracted OTB. This yield corresponded to more than 65% of sugars in extracted OTB, achieving a maximum value of 70.3% (run 9). This maximum overall yield was similar to that obtained by Martínez-Patiño (2015) from extracted OTB pretreated by phosphoric acid.

Optimization of Sulfuric Acid Pretreatment of Extracted OTB

The optimization of the sulfuric acid pretreatment of OTB was performed according to two different criteria (Table 6). Considering only the maximization of $Y_{overall}$, the optimal conditions for sulfuric acid pretreatment of OTB were found at 164 °C, 5.92% H_2SO_4 (expressed as g H_2SO_4/100 g extracted OTB), and 15% solid loading (Opt. 1). These conditions yielded a sugar solution of 37 g/L and an overall yield of 45.9 g sugars/100 g extracted OTB according to the model. This yield corresponded to 75% sugars content in extracted OTB.

Table 6. Optimization Criteria and Results for the Sulfuric Acid Pretreatment of Extracted OTB at Optimized Conditions

Optimization Criterion	Conditions			Predicted Responses		Experimental Responses	
Maximize	T (°C)	C_A (%)	SL (%)	C_{sugars} (g/L)	$Y_{overall}$ (g/100 g)	C_{sugars} (g/L)	$Y_{overall}$ (g/100 g)
$Y_{overall}$ (Opt. 1)	164	5.9	15	37.1	45.9	36.1 ± 1.0	41.8 ± 0.8
C_{sugars} and $Y_{overall}$ (Opt.2)	160	4.9	35	79.8	39.8	78.8 ± 2.5	39.8 ± 0.7

C_{sugars}: sugar concentration in prehydrolysate
$Y_{overall}$: sum of the glucose released by EH and total sugars content in prehydrolysate/100 g extracted OTB

The aim of the second optimization criterion was to make more viable the fermentation of the hemicellulosic sugar solution after pretreatment, with high initial sugar concentration. As a compromise to maximize both the sugar concentration in liquid and the overall sugar yield, optimal conditions for sulfuric acid pretreatment of OTB were found to be 160 °C, 4.9% H_2SO_4 (expressed as g H_2SO_4/100 g extracted OTB), and 35% solid loading (Opt. 2). These optimal conditions yielded a sugar solution as concentrated as 79.8 g/L that corresponded to an overall yield of 39.8 g total sugars/100 g extracted OTB according to the model.

To validate the model, two new experiments were conducted in triplicate, setting the process factors to the optimum ones given for each optimization criterion. As can be seen in Table 6, the experimental values for responses were highly adjusted to the predicted values.

The use of high dry matter in the acid pretreatment (Opt. 2) allowed for a highly concentrated sugar solution to be obtained without a remarkable drop in the yield. It has been reported that high sugar concentrations are required in order to achieve ethanol solutions with concentration above 4% (w/v), allowing an efficient distillation stage (Larsen *et al*. 2008; Koppram *et al*. 2014).

Figure 3 shows the entire process and material balance data for acid pretreatment at optimal conditions corresponding to Opt. 2 (160 °C, 10 min, 4.94 g H_2SO_4/100 g extracted OTB, 35% solid loading). A total of 69.3% of initial sugars (55.7 g/100 g of raw OTB) was recovered in the different steps of the process. Non-structural sugars present in the extractives, mainly glucose, were easily solubilized in water extraction, accounting for 11.3% of initial sugars.

The acid pretreatment step achieved a sugar recovery in prehydrolysate of 32.1%, resulting from the hemicellulose solubilization. On the other side, 25.9% of the initial sugars were released by enzymatic hydrolysis. The rest of the sugars from extracted OTB either remained in the final solid without being hydrolyzed, or were degraded and converted to inhibitory compounds detected in the prehydrolysate.

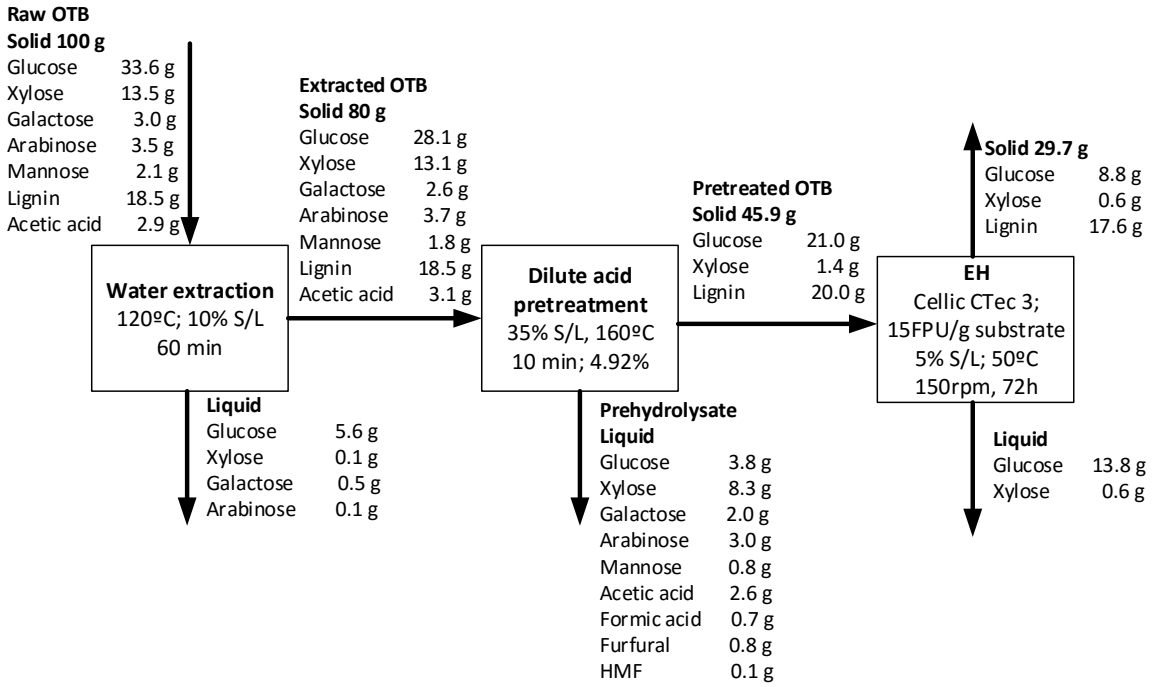


Fig. 3. Material balance flow diagram of the overall process for fermentable sugar production from extracted OTB by dilute sulfuric acid pretreatment at optimal conditions (160 °C, 10 min, 4.94 g H_2SO_4/100 g extracted OTB, 35% solid loading)

Fermentability Tests of Prehydrolysates

Escherichia coli has been shown to be an efficient fermentation strain for mixed hexose-pentose solutions (Fernández-Sandoval *et al*. 2012). In this work, sulfuric acid prehydrolysates of OTB were submitted to fermentability tests by an ethanologenic *E. coli*. In order to overcome the drawbacks of high levels of inhibitory compounds in hemicellulosic sugar solution, a detoxification step was necessary. The aim of this step was to achieve a more readily fermentable broth (Koppram *et al*. 2014). Overliming was chosen as the detoxification method because it has been recognized as an efficient method to remove inhibitory compounds, such as furans, although no effect has been produced on acetic acid (Martínez *et al*. 2000). The *E. coli* strain used in this study, however, is highly tolerant to acetic acid. Furthermore, acetate can be used as a carbon source because it can be metabolized by *E. coli* (Zaldivar and Ingram 1999).

Only five prehydrolysates (runs 4, 5, 6, 7, and 17) were fermented with ethanol production; the rest were toxic for *E. coli* and could not be fermented. Their inability to ferment can likely be attributed to the high levels of furfural in these prehydrolysates, even after overliming (data not shown). The higher toxic effect of this inhibitor compound on *E. coli* has been tested in previous works reporting a tolerance limit of furfural for this microorganism of approximately 2 g/L (Geddes *et al*. 2011).

Table 7 shows the composition of the fermented prehydrolysates, before and after overliming. As can be seen, sugar losses were not remarkable. In general, overliming was able to reduce concentrations of all sugar degradation products (formic acid, furans, and phenolic compounds) in the prehydrolysates. As expected, however, acetic acid concentrations were unaltered.

Table 7. Composition of Prehydrolysates Before and After Overliming, and Ethanol Production by *E. coli*

Run	Total Sugars (g/L)		Inhibitors (g/L)					Ethanol Production	
			Formic acid	Acetic acid	HMF	Furfural	Phenols	C_E (g/L)	Y_E (%)
4	BO	23.59	0.75	1.83	0.10	0.30	2.91	10.1[b]	85
	AO	23.18	0.73	2.09	0.07	0.21	1.27		
5	BO	75.64	2.29	10.84	0.41	2.25	5.35	24.9[a]	70
	AO	76.72	1.06	11.07	0.06	0.45	3.15		
6	BO	54.04	2.12	8.99	0.50	3.65	5.40	20.4[a]	77
	AO	51.85	1.09	8.89	0.16	1.41	2.59		
7	BO	25.75	1.42	4.49	0.16	1.00	2.76	10.0[b]	81
	AO	23.27	1.60	4.48	0.00	0.10	1.27		
17	BO	33.13	1.30	4.66	0.14	0.72	2.30	14.5[b]	88
	AO	31.21	0.58	4.85	0.05	0.32	1.10		

BO: Before overliming; AO: After overliming; Total sugars: Sum of glucose, xylose, mannose, galactose, and arabinose

Y_E: Ethanol yield, referred to the theoretical ethanol yield (0.51 g ethanol/g glucose)

[a] after 120 h fermentation time

[b] after 48 h fermentation time

During the fermentation of prehydrolysates in runs 5 and 6, simultaneous sugar consumption was observed and glucose was depleted before the rest of sugars. After 96 h, neither glucose nor arabinose were detected in the fermentation broth. However, in both experiments, approximately 25% of the XGM was not metabolized by *E. coli*. Ethanol concentrations of 25 and 20 g/L were achieved after 120 h, corresponding to 70% and 77%, respectively, of the theoretical ethanol yield (referred to the sugars present in the fermentation broth). These low yields were explained by the organism's incomplete consumption of XGM, leaving approximately 10 g/L of these sugars (xylose, galactose, and mannose) in the fermentation broth. This behavior can be seen in Fig. 4, corresponding to the fermentation of the prehydrolysate from run 5 (shown as an example).

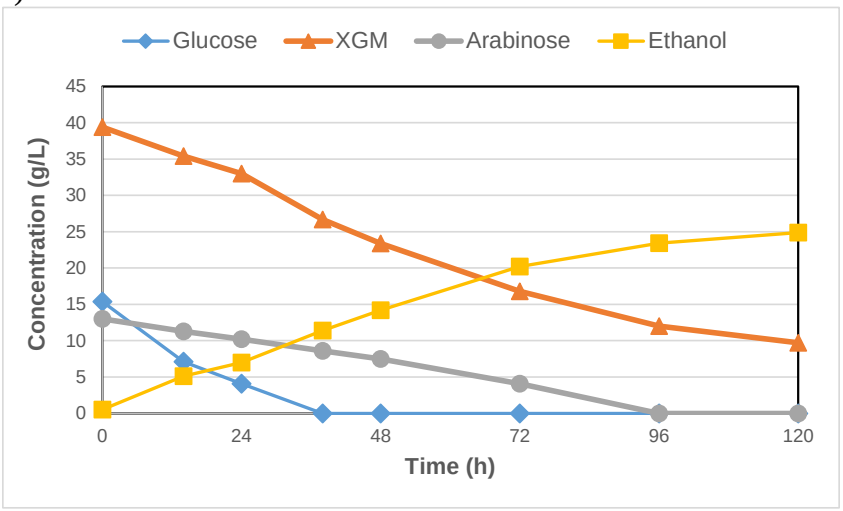


Fig. 4. The time course during the *E. coli* MS04 fermentation of acid OTB prehydrolysate (run 5) detoxified by overliming

Incomplete utilization of xylose was observed by Geddes *et al*. (2011). They reported that an increase of inhibitory compound levels, particularly furfural, caused one third of the xylose in sugarcane bagasse hydrolysate to remain unfermented into ethanol by *E. coli* MM160 after 240 h. Likewise, Nieves *et al*. (2011) improved the xylose consumption by *E. coli* MM 160's parent, *E. coli* MM170, by injecting air into the headspace of the fermentation vessel, achieving near complete fermentation after 96 h.

Concerning prehydrolysates from runs 4, 7, and 17, with lower initial sugar concentrations, simultaneous sugar consumption also occurred. Glucose disappeared only after 12 h of fermentation and no sugars were identified in the fermenting broth after 36 h of fermentation. As an example, Fig. 5 shows the time course during the fermentation of prehydrolysate from run 17. This improved performance of fermentation resulted from the lowest level of inhibitory compounds in the medium, mainly furfural and phenolic compounds. Sugars present in these prehydrolysates were completely consumed. Therefore, higher ethanol yields (referred to as the "sugars content" in the fermenting broths) than those corresponding to the fermentation of prehydrolysates from runs 5 and 6 were achieved, although ethanol concentrations remained lower than 15 g/L.

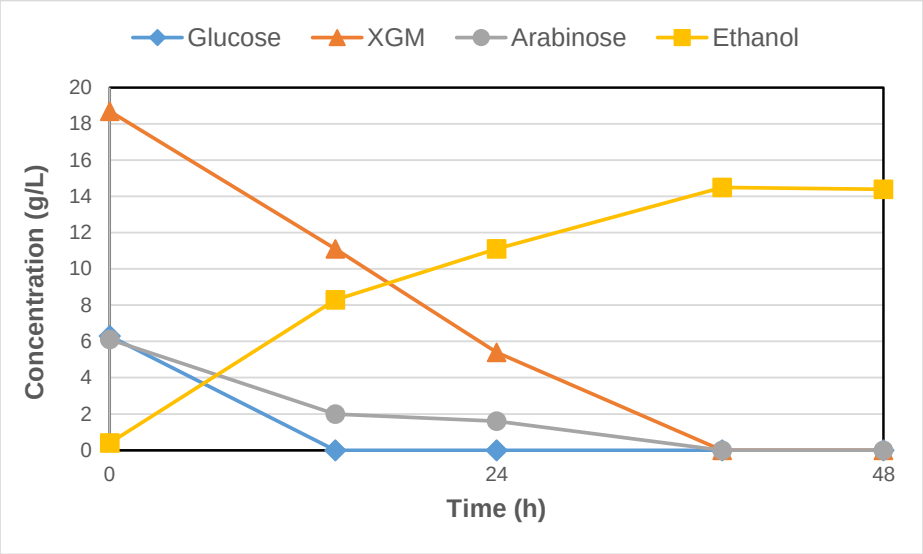


Fig. 5. The time course during *E. coli* MS04 fermentation of acid OTB prehydrolysate (run 17) detoxified by overliming

As expected, the highest ethanol concentration from hemicellulosic sugars (25 g/L) was obtained when the pretreatment was carried out at 35% solid loading (run 5). The use of high dry matter in the pretreatment usually resulted in lower yields because of the high formation of toxic compounds. Thus, in this study, the highest ethanol yields were achieved for prehydrolysates from lower solid loadings with the complete utilization of sugars present in the medium and values higher than 80% of the theoretical ethanol yield.

CONCLUSIONS

1. The effect that water extraction had on olive tree biomass in decreasing lignin recondensation reactions during the acid pretreatment was evidenced without implying the loss of structural sugars. Likewise, a relationship between the higher presence of solid matter in the pretreatment reactor and the possible occurrence of lignin recondensation reactions was determined.
2. Sulfuric acid pretreatment of extracted OTB at optimized conditions (35% solids loading, 160 °C, and 1.7% w/v acid concentration) produced a hemicellulosic sugar solution as concentrated as 79 g/L, corresponding to an overall yield of sugars of 40 g/100 g extracted OTB.
3. *Escherichia coli* was an efficient ethanologenic microorganism. It was able to ferment hemicellulosic prehydrolysates from extracted OTB pretreated by dilute sulfuric acid with yields higher than 70% (referred to total sugars in prehydrolysate) after detoxification by overliming.

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