Carbohydrate Polymers 99 (2014) 791–799

Contents lists available at ScienceDirect

Carbohydrate Polymers

journal homepage: www.elsevier.com/locate/carbpol

Enhanced enzymatic hydrolysis of pretreated almond-tree prunings for sugar production

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ARTICLE INFO

Article history:
Received 6 July 2013
Received in revised form 15 August 2013
Accepted 28 August 2013
Available online 5 September 2013

Keywords:
Almond-tree prunings
Delignification
Dilute acid pretreatment
Enzymatic hydrolysis
Hydrothermal pretreatment

ABSTRACT

Almond-tree prunings (ATP), an agricultural residue largely available in Mediterranean countries, were pretreated with either hot water or dilute sulphuric acid at 180–230 °C. Solids derived from hot water pretreatments were further submitted to alkaline peroxide delignification. In addition, all solids obtained from the three mentioned processes were hydrolysed by cellulases and β-glucosidases to investigate their enzymatic digestibilities. Hot water pretreatment led to high oligosaccharide yields (18.2 g/100 g ATP at 190 °C) while dilute acid pretreatment provided the highest monosaccharide yields (24.0 g/100 g ATP at 190 °C) along with low concentrations of fermentation inhibitors. Glucose yields from enzymatic hydrolysis were strongly affected by both pretreatment type and pretreatment temperature. The highest temperature assayed for both hydrothermal and dilute sulphuric acid pretreatment maximized the glucose recovery (49.2% and 72.8%, respectively) while solids derived from alkaline peroxide treatment achieved maximal glucose concentrations (41.9 g/L, 58.4% of potential yield).

1. Introduction

Increasing concerns about global warming and declining fossil fuels reserves have led to the development of green processes that use sustainable resources to produce renewable energy. In this context, agricultural residues are regarded as potential substrates for bioenergy industries in numerous countries (Cornelissen, Koper, & Deng, 2012). Almond [*Prunus amygdalus* Batsch], which belongs to the *Rosaceae* family, is a mid-size tree widely under cultivation in the Mediterranean region, California and Australia that can grow to a height of 6–9 m. Over 1.5 million hectares of almond trees are cultivated worldwide, most of them (547,822 ha) in Spain (FAO, 2013). The almond-tree grove in Spain produces 1.34 t/ha annual residual biomass after pruning (Velázquez-Martí, Fernández-González, López-Cortés, & Salazar-Hernández, 2011), and it can be estimated that the annual Spanish production of this material is approximately 7.3×10^8 kg.

In most cases, almond-tree prunings are left on the land to be incinerated or ploughed into the soil, after grinding, with the disadvantages that this can result in (soil mineralization, air pollution and fire risks). There are only scarce reports on the conversion of their chemical energy into heat, mechanic or electric energy (González et al., 2006). The production of liquid fuels, such as bioethanol, through biochemical conversion technology has never been assayed for almond-tree prunings but it has provided promising results for other green biomasses, such as hardwoods (Romaní, Garrote, Alonso, & Parajó, 2010), softwoods (Galbe & Zacchi, 2002) or agricultural residues (Cuevas et al., 2010; García Martín, Cuevas, Bravo, & Sánchez, 2010).

Enzymatic hydrolysis followed by fermentation is one of the most common methods employed to produce ethanol from lignocellulose materials. Enzymatic hydrolysis of cellulose to glucose is carried out by cellulase enzymes that are highly specific catalysts. For this process, the raw material needs to be pretreated so that the cellulose in the plant fibres is exposed to enzyme attack. Among the great variety of techniques used in the pretreatment stage, in recent years hydrothermal pretreatment (also called liquid hot water pretreatment, LHW) has been comprehensively studied as it is capable of completely solubilizing the hemicelluloses using only water as reagent (Feria et al., 2011). The residence time for LHW process is usually 5–15 min at temperatures in the range 200–230 °C, and approximately 40–60% of the total biomass is solubilized, including 4–22% and 35–60% of cellulose and lignin content, respectively (Kumar, Barrett, Delwiche, & Stroeve, 2009). In hydrothermal pretreatment, hydronium ions (derived from the autoionisation of water) and acetic acid (derived from the breakdown of acetyl groups) cause the catalytic depolymerisation of hemicelluloses to oligosaccharides and sugars, but monosaccharides yields tend to be limited (Xiao et al., 2013). Since monomeric xylose can be also used as substrate for microbial production of fuel-ethanol (Zhao, Zhang, & Tan, 2008) complete xylan depolymerisation to xylose by dilute acid hydrolysis could improve the

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http://dx.doi.org/10.1016/j.carbpol.2013.08.089

feasibility of the biorefinery scheme. Dilute acid can convert xylan to xylose with a maximum yield of 70–90%. The main drawback of dilute acid hydrolysis is the formation of sugar degradation compounds (furfural, 5-hydroxy-methyl-furfural . . .).

Results from hydrothermal and dilute acid pretreatments illustrate that the improvement of enzyme digestibility of pretreated cellulose, pentoses production and inhibitors release are dependent on the type of raw material used and operational conditions (reaction time, acid concentration, and mainly temperature) applied for pretreatment. Therefore, a study of process parameters is essential to maximize glucose and hemicellulosic sugars release from biomass and to diminish the production of yeasts inhibitors from thermal degradation of sugars.

In order to improve the enzyme digestibility of lignin-rich residues remaining after pretreatments, additional delignification steps are reported as beneficial. Alkaline peroxide delignification (APD) of biomass is commonly used under mild operating conditions because hydrogen peroxide is well known as an oxidant that reacts with lignin under alkaline conditions. This technique has been evaluated with good results on rapeseed straw (Karagöz, Rocha, Özkan, & Angelidaki, 2012), bamboo (Yamashita, Shomo, Sasaki, & Nakamura, 2010), softwood (Yang, Boussaid, Mansfield, Gregg, & Saddler, 2002), and hardwood substrates (Ramos, Breuil, & Saddler, 1992).

The objectives of the present study were (a) to examine the chemical composition of almond-tree prunings, (b) to identify the most favourable temperatures for the hydrothermal and dilute sulphuric acid pretreatments of raw material in terms of both maximizing the fermentable sugar yields in the prehydrolysates and improving the enzymatic digestibility of pretreated solids, and (c) to analyze whether a delignification stage is convenient, regarding the susceptibility of cellulose from hydrothermal pretreated residues to enzymatic hydrolysis.

2. Materials and methods

2.1. Raw material

Almond-tree prunings (ATP) were obtained from an almond [*Prunus dulcis* (Mill) D. A. Webb cv Tuono] orchard near the town of Alhama de Granada (southern Granada province, SE Spain, UTM coordinates: 37°01′46.53″N, 3°56′16.64″W), air-dried at room temperature to equilibrium moisture content, milled by using a laboratory blade mill (Retsch GMBH, Germany) to achieve 0.125–0.300 mm particle size, homogenized in a single lot and stored until used.

2.2. Hydrothermal and dilute sulphuric-acid pretreatments

Liquid hot water (LHW) and dilute sulphuric acid (DSA) pretreatments were performed in a 2-L Parr reactor Series 4522 (Moline, IL, USA). The reactor was loaded with 40 g of dry raw material and 400 mL of water or dilute (0.025 M) sulphuric acid solution so the solid/liquid ratio was 1/10. The equipment was closed hermetically and stirred at 250 rpm. Afterwards, the suspension was heated by a system of electric coils surrounding the reactor until a given operating temperature (180, 190, 200, 210, 220 or 230 °C) was reached. After 5 min at this temperature, the reactor was removed from the heating system and cooled in a water bath to about 30 °C in less than 10 min (Fig. 1). The pretreated material was separated into solid residue and liquid prehydrolysate by vacuum filtration. The solid was washed with distilled water, air-dried at room temperature to equilibrium moisture content of about 6%, weighed and analyzed for sugars and lignin composition. The water-soluble extract was analyzed for sugars, acetic acid and 5-hydroxy-methyl-furfural composition.

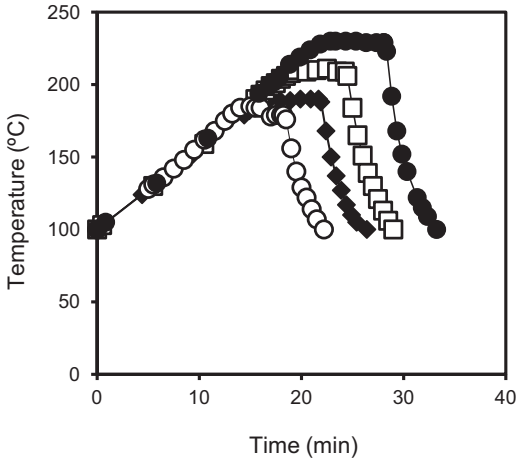

Fig. 1. Temperature profile of four hydrothermal pretreatments carried out at 180 °C (○), 190 °C (♦), 210 °C (□), and 230 °C (●), respectively.

2.3. Alkaline peroxide delignification

Alkaline peroxide delignification (APD) was performed in 500 mL round-bottom flask connected to a total reflux condenser (Dimroth type) to prevent evaporation losses. Six grams (dry weight) of hydrothermal pretreated material were added to 120 mL of 1% (w/v) hydrogen peroxide solution. The pH was adjusted to 11.5 using 4 M NaOH. Alkaline peroxide pretreatments were performed at boiling temperature for two incubation times (30 and 60 min). After delignification, solid and liquid fractions were separated by filtration. The solid fraction was washed with distilled water several times, dried at room temperature to equilibrium moisture content of about 7%, weighed and analyzed for sugars and lignin composition.

2.4. Enzymatic hydrolysis

Water-insoluble fibres from hydrothermal and dilute sulphuric acid pretreatments, and from alkaline peroxide delignification, were enzymatically hydrolysed by a cellulolytic complex (Celluclast 1.5 L) kindly provided by Novo Nordisk Bioindustrial (Madrid, Spain). Experiments were carried out with cellulase loadings of 15 FPU per gram of dry solids. Fungal β-glucosidase (Novozyme 188) was used to supplement the β-glucosidase activity with an enzyme loading of 30 international units (IU) per gram of dry solids. Enzymatic hydrolysis was performed in 0.05 M sodium citrate buffer (pH 4.8) at 50 °C on a rotary shaker at 150 rpm for 72 h and at 10% (w/v) treated material concentration. Samples were withdrawn from the reaction media at 24, 48 and 72 h for glucose concentration determination. All assays were performed in duplicate.

2.5. Analytical methods

Extractives in raw material were determined gravimetrically using a two-step sequential extraction process by Soxhlet to remove water soluble material and ethanol soluble material according to a procedure adapted from Sluiter, Ruiz, Scarlata, Sluiter, and Templeton (2008). Determinations of structural carbohydrates and acid-insoluble lignin in raw material (ATP) and pretreated (LHW, DSA or APD) solids were carried out using two-step acid hydrolysis based on the procedure published by the National Renewable Energy Laboratory (NREL, 2011). Firstly, solids

(300 mg) were treated with 3 mL 72% (v/v) H_2SO_4 at 30 °C for 60 min. Subsequent, the samples were diluted to 3% (v/v) H_2SO_4 and autoclaved for 1 h at 120 °C. The resulting suspensions were filtered on glass filters. The solid fraction, corresponding to acid-insoluble lignin (AIL), was dried and weighed. The liquid fraction, after dilution, was filtered through a 0.22 μm nylon membrane (Millipore) and its monosaccharides (L-arabinose, D-galactose, D-xylose, D-glucose and D-mannose) were separated using a Dionex ICS-3000 chromatography system (Dionex corporation, Sunnyvale, CA, USA) equipped with a CarboPac™ PA20 (3 × 150 mm) analytical column, CarboPac™ PA20 (3 × 30 mm) guard column, gradient pump and pulsed amperometric detector. Elution took place at 30 °C. The eluent was 2 mM NaOH, at a flow rate of 1 mL/min. Acid-soluble lignin was determined from absorbance at 205 nm. Ash was determined according to TAPPI Standard Method T 15 os-58.

The sugar and 5-hydroxy-methyl-furfural content of the liquid fraction after LHW or DSA pretreatments was determined by high performance ionic liquid chromatography (HPILC) using the above-described system. Acetic acid was quantified following the enzymatic method described by Bergmeyer and Möllering (1974). Oligosaccharides were measured by an indirect method based on quantitative acid hydrolysis of the liquors with 3% (v/v) H_2SO_4 at 121 °C for 60 min. Glucose concentration from enzymatic hydrolysis samples was measured by an enzymatic determination glucose assay kit (Chemelex LABKIT Glucose-TR, Barcelona, Spain).

All the analyses were carried out in duplicate and average results are shown.

3. Results and discussion

3.1. Raw material composition

The compositional data of the raw material used in this work are listed in Table 1. Cellulose was the main structural component (31.3%), followed by acid-insoluble lignin (AIL, 28.7%) and hemicellulose (23.1%, calculated as the sum of xylan, arabinan, galactan, mannan and acetyl groups). Acetyl groups represented about 3% of the initial biomass. Including the acid-soluble lignin (ASL) fraction, total lignin value was 31.2%.

Extractives which include non-structural sugars, tannins and chlorophyll were present at levels of 12.3% and the ash content was 2.8%. The composition in lignin and ash is in agreement with data previously reported (Ververis, Georghiou, Christodoulakis, Santas, & Santas, 2004), but those authors obtained higher cellulose content (about 40%).

Table 1
Almond tree prunings composition.

Component	% (dry matter basis)[a]
Moisture	6.7 ± 0.1
Cellulose	31.3 ± 1.6
Hemicellulose	
Xylan	15.2 ± 0.7
Arabinan	2.3 ± 0.1
Galactan	1.3 ± 0.1
Mannan	1.2 ± 0.1
Acetyl groups	3.0 ± 0.2
Acid insoluble lignin (AIL)	28.7 ± 1.6
Acid soluble lignin (ASL)	2.5 ± 0.2
Extractives	
In water	11.0 ± 0.4
Glucose	1.2 ± 0.1
In ethanol	1.3 ± 0.0
Ash	2.8 ± 0.2

[a] Average of at least three determinations.

The total monosaccharides content (59.1 g/100 g raw material) illustrates the potential of this biomass as substrate for ethanol production, although its AIL content is higher than those reported for other agricultural residues such as corncobs, wheat straw, barley straw, rice husk and olive pruning debris (García Martín et al., 2010; Mateo, Puentes, Sánchez, & Moya, 2013; Nabarlatz, Ebringerová, & Montané, 2007).

3.2. Hydrothermal and dilute sulphuric acid pretreatments

The efficiency of liquid hot water (LHW) and dilute sulphuric acid (DSA) pretreatments in the hydrolysis of almond-tree prunings was tested by performing each pretreatment at six different temperatures for 5 min. Table 2 shows the total gravimetric recovery (TGR) and the chemical composition of the water-insoluble solids resulting from LHW and DSA assays. TGR was calculated as the dry weight of pretreated solid divided by the dry weight of raw material before pretreatment, and it was expressed as percentage.

The pretreatments resulted in TGR percentages between 58.4% and 68.2% for LHW and between 54.5% and 59.5% for DSA, indicating that almost half of the material was solubilized in the most severe DSA experiment. Data of the main fractions in the remaining solids revealed that cellulose and lignin were in general well preserved, whereas hemicellulose was mostly hydrolysed during pretreatments. It was found that the increase of temperature or sulphuric acid concentration encouraged solid solubilization. However, this trend started to decrease when treatment temperature was above 210 °C, at which the hemicelluloses was totally solubilized (hemicellulose content < 1%). The hemicellulose removal from solids led to an apparent increase of the percentages of cellulose and lignin in pretreated solids, although at the highest temperatures (from 210 °C for LHW and from 200 °C for DSA) the percentage of cellulose decreased (Table 2), which can be explained by the partial solubilization of its easily hydrolysable fraction (García Martín et al., 2010; Jacobsen & Wyman, 2010).

The acid-insoluble lignin (AIL) fraction was well preserved and its levels in the pretreated solids increased with temperature from 38.5% to 47.1% in LHW pretreatments and 44.7% to 54.0% in DSA hydrolysis. The percentages of acid-soluble lignin (ASL) in the resulting solids varied from 1.7% to 1.1% and from 1.4% to 0.9% for LHW and DSA pretreatments, respectively (Table 2). The increases observed were apparent in most cases, as the dry matter recovery from pretreatments were low due to solubilization of hemicelluloses, ASL, etc. and hence the computation on 100 g dry matter could magnify the values.

The composition of the liquid prehydrolysates is shown in Fig. 2. LHW pretreatments did achieve high yields neither for sugars nor for inhibitors under the conditions tested. However, important oligosaccharides amounts were recovered when working at temperatures below 210 °C (Fig. 2a). The highest yields in glucose (1.8 g/100 g ATP), arabinose (1.7 g/100 g ATP), xylose (2.0 g/100 g ATP), galactose plus mannose (0.6 g/100 g ATP) and 5-HMF (1.1 g/100 g ATP) were achieved at 180 °C, 200 °C, 210 °C, 220 °C and 230 °C, respectively. The highest acetic acid yield, resulting from the splitting of acetyl groups of hemicellulose, was 0.3 g/100 g ATP (data not shown). The optimal temperature for oligosaccharide production (18.2 g/100 g ATP) was found to be 190 °C. Due to the high amounts of oligosaccharides in hydrolysates from LHW pretreatments, these filtrates can be regarded as promising feedstock for the production of oligomeric sugars. Consistent with the results, hydrothermal pretreatment of olive stones at 200 °C for 2 min produced solutions containing 16 g oligosaccharides per 100 g raw material (Cuevas, Sánchez, Bravo, Cruz, & García, 2009). The oligosaccharide composition (% weight) of the hydrolysate from LHW pretreatment at 190 °C was: 67.7% xylan, 18.7% glucan, 6.4% arabinan, 5.2% galactan, and 2.1% mannan.

Table 2
Composition (%, dry matter) and total gravimetric recovery (TGR, %) of water insoluble fibre resulting from hydrothermal (LHW) or dilute sulphuric-acid (DSA) pretreatments at different temperature conditions.

	T (°C)	TGR	Hemicellulose	Cellulose	AIL	ASL
LHW	180	68.2	10.8 (6.9)	42.4 (29.0)[a]	38.5 (26.2)	1.7 (1.2)
	190	64.1	5.6 (3.6)	45.1 (28.9)	41.0 (26.3)	1.4 (0.9)
	200	60.7	3.1 (1.9)	47.0 (28.5)	44.5 (27.0)	1.2 (0.7)
	210	58.9	1.1 (0.7)	50.4 (29.7)	46.1 (27.2)	1.1 (0.6)
	220	58.7	0.4 (0.2)	49.9 (29.2)	47.2 (27.7)	1.1 (0.6)
	230	58.4	0.0 (0.0)	48.7 (28.4)	47.1 (27.5)	1.2 (0.7)
DSA	180	59.5	6.6 (3.9)	46.7 (27.8)	44.7 (26.6)	1.4 (0.8)
	190	57.6	3.0 (1.7)	49.9 (28.7)	45.7 (26.3)	1.3 (0.7)
	200	56.5	2.0 (1.1)	51.4 (29.1)	48.2 (27.3)	1.2 (0.7)
	210	54.8	0.8 (0.4)	51.2 (28.0)	51.0 (27.9)	1.0 (0.5)
	220	54.8	0.2 (0.1)	48.0 (26.4)	53.1 (29.1)	0.9 (0.5)
	230	54.5	0.0 (0.0)	35.5 (19.4)	54.0 (29.4)	1.1 (0.6)

[a] Data are expressed in parentheses as a percentage based on dry weight of raw material.

DSA pretreatments led to a strong hydrolysis of oligosaccharides, so the oligosaccharides yield in prehydrolysates rapidly decreased from 7.2 g/100 g ATP at 180 °C to complete depletion at 200 °C (Fig. 2b). Oligosaccharides hydrolysis improved sugar yields, mainly at mild temperatures (190–200 °C) due to the lower thermal sugar degradation. Thus, maximum yields in arabinose (2.8 g/100 g ATP) and glucose (5.1 g/100 g ATP) were achieved at 180 °C and 190 °C, respectively, while the highest yields in xylose (13.3 g/100 g ATP) and galactose plus mannose (2.4 g/100 g ATP) were found at 200 °C. Xylose yield was 77% of the maximum yield attainable. This value is similar to that obtained by Torget, Werdene, Himmel, and Grohmann (1990), who tested the dilute sulphuric acid (0.5%) pretreatment of rice straw at 201 °C (10 min process time) and found a maximum yield of 80.8% in the depolymerisation of xylan to xylose. At higher temperatures, depolymerisation reactions competed with sugar degradation reactions. As can be seen in

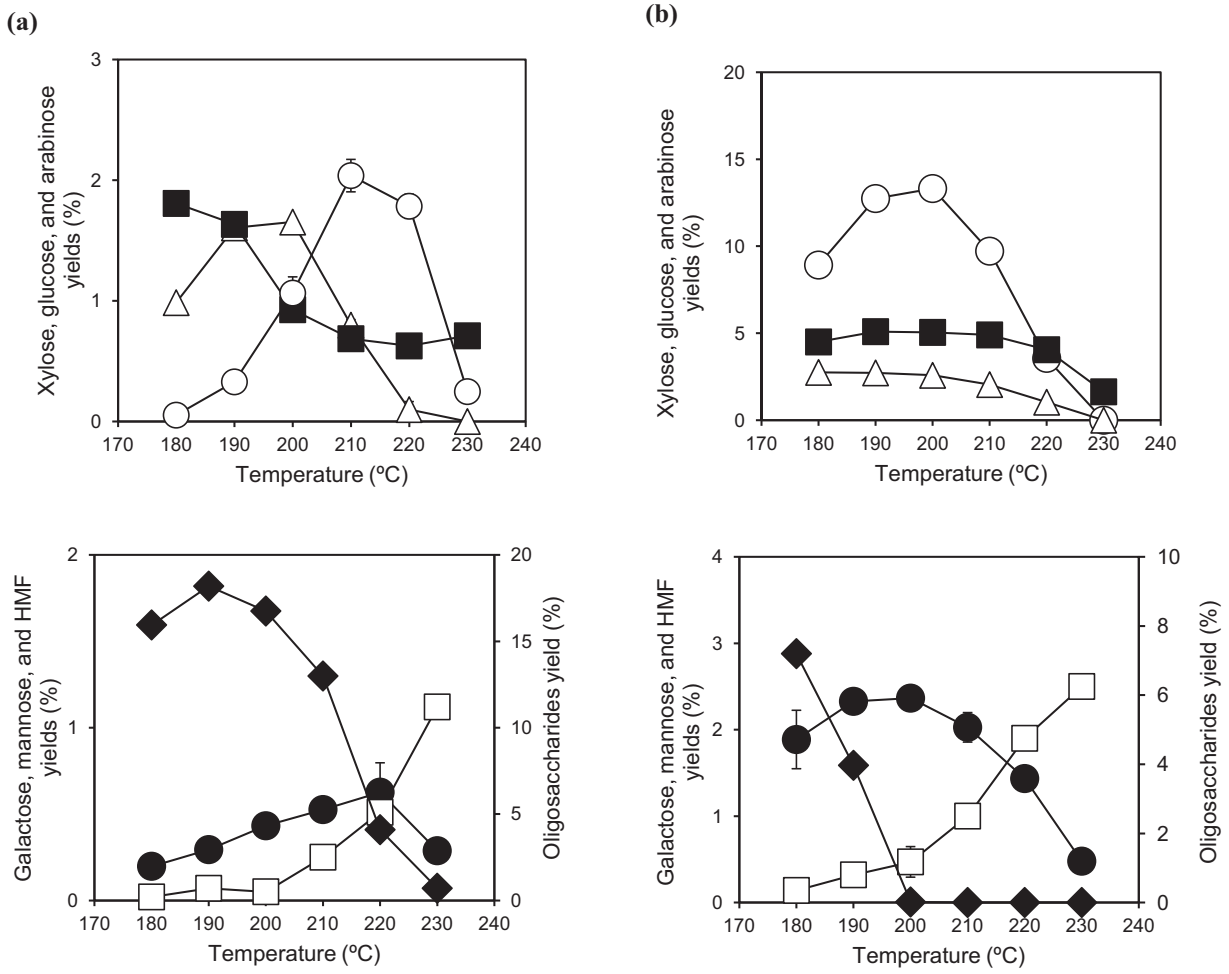

Fig. 2. Release of monomeric sugars (○, xylose; ■, glucose; △, arabinose; ●, galactose + mannose), oligosaccharides (♦), and 5-hydroxy-methyl-furfural (□) from almond-tree prunings after hydrothermal pretreatment (a) or dilute sulphuric acid pretreatment (b).

Table 3
Composition[a] of filtrates and water insoluble solids resulting from liquid-hot-water and dilute sulphuric acid pretreatments of almond-tree prunings.

Pretreatments	T (°C)	Glucose (%)				Hemicellulosic sugars[b] (%)			
		A	B	C	SD[c]	A	B	C	SD[c]
LHW	180	91.3	5.0	9.4	0.0	30.0	13.1	54.2	2.6
	190	91.2	4.5	9.3	0.0	15.5	15.8	64.2	4.6
	200	90.0	2.6	8.3	0.0	8.1	17.3	59.6	15.0
	210	93.7	1.9	6.7	0.0	2.8	16.5	45.7	34.9
	220	92.2	1.7	3.6	2.5	1.0	12.5	12.1	74.4
	230	89.6	1.9	1.5	7.0	0.0	4.4	0.7	94.9
DSA	180	87.6	12.4	0.8	0.0	17.0	65.0	5.9	12.1
	190	90.6	14.0	0.4	0.0	7.5	81.8	3.3	7.4
	200	91.7	13.9	0.0	0.0	4.9	79.2	0.0	15.9
	210	88.5	13.5	0.0	0.0	1.8	59.8	0.0	38.4
	220	83.1	11.1	0.0	5.8	0.6	26.6	0.0	72.8
	230	61.1	4.5	0.0	34.4	0.0	3.0	0.0	97.0

[a] Sugars yield in the pretreated solid (A), and monomers (B) and oligomers yields (C) in the prehydrolysate, expressed in relation to the initial content of sugars from almond-tree prunings.
[b] Considering the sum of xylose, arabinose, galactose, and mannose.
[c] Sugars degradation.

Fig. 2b, temperatures above 200 °C led to decreases in monosaccharides concentrations in prehydrolysates due to thermal degradation reactions. The inhibitors production also increased with temperature; in the experiment carried out at 230 °C, 5-HMF and acetic acid yields reached 2.5 g/100 g ATP and 2.1 g/100 g ATP, respectively.

Glucose and hemicellulosic sugars (as the sum of xylose, arabinose, galactose and mannose) formed as a result of the DSA and LHW pretreatments, in both resulting solids and prehydrolysates, are shown in Table 3. In addition, we calculated the total carbohydrate content for resulting solids. On the contrary, we made a distinction between monosaccharides and oligosaccharides for prehydrolysates. Recovery yields were expressed as the percentage of each component in relation with its content in the initial material.

Glucose was almost completely preserved in the cellulose matrix in LHW pretreatments and significant degradations were found only at 220 °C (2.5%) and 230 °C (7.0%). In contrast, DSA pretreatments led to higher glucose degradation (5.8% at 220 °C and 34.4% at 230 °C) as well as to nil production of oligosaccharides containing glucose in prehydrolysates. Hemicellulosic sugars were thermally degraded faster than glucose. Thus, more than 38% hemicellulosic sugars were degraded during DSA pretreatment at 210 °C. Despite of this sugar degradation, 1.8% of hemicellulosic sugars still remained in the solid residue at the end of the pretreatment (Table 3). Therefore, it is difficult to achieve simultaneously complete hemicellulose hydrolysis and high sugars yield in prehydrolysates, which is in agreement with other reports (Hernández et al., 2013).

3.3. Alkaline peroxide delignification

LHW pretreated solids were submitted to another pretreatment (alkaline peroxide delignification, APD) to increase the cellulose content by removing the lignin fraction, so improving the posterior enzymatic digestibility. Two process times were chosen: 30 and 60 min. The delignified solid recovery (DSR) as well its cellulose and lignin content is shown in Table 4. DSR was calculated as the dry weight of solids remaining after APD divided by the dry weight of solids after LHW pretreatments. Delignified solid recovery was significantly high in all the experiments, varying between 69.6% and 77.6%. DSR values, along with lignin percentages in delignified solids, decreased with the increase of the temperature of the LHW pretreatment and the APD process time. Lignin percentage in the delignified solid increased with the increase of lignin content in the LHW pretreated solid (Table 2). Lignin recovery data from APD pretreatments carried out for 60 min revealed that between 55.7% and 60.9% (referred to LHW pretreated material) or between 57.5% and 63.0% (referred to raw material) of lignin was solubilized (Table 4).

The cellulose percentages in delignified solids were related to the temperature of the LHW pretreatment. When the temperature

Table 4
Delignified solid recovery (DSR) and lignin and cellulose content[a] of water insoluble solids after alkaline peroxide delignification of LHW pretreated material.

T (°C)[b]	t (min)[c]	DSR	Lignin (%)			Cellulose (%)		
			A	B	C	A	B	C
180	30	77.6	21.3	39.2	42.9	51.7	86.4	94.6
190		76.0	25.2	42.8	46.7	54.0	83.0	91.0
200		75.0	27.0	42.8	45.4	56.5	81.1	90.1
210		74.0	30.3	46.1	48.7	60.7	83.5	89.1
220		73.6	30.5	45.8	47.5	66.6	90.7	98.2
230		72.9	32.5	48.2	50.3	64.0	85.9	95.9
180	60	73.5	21.3	37.1	40.6	56.0	88.6	97.0
190		72.8	23.3	37.8	41.3	58.5	86.1	94.5
200		71.6	25.6	38.8	41.2	60.4	82.8	92.0
210		71.1	25.4	37.0	39.1	65.6	86.7	92.5
220		70.3	27.7	39.7	41.2	68.7	89.4	96.8
230		69.6	30.0	42.5	44.3	67.0	85.8	95.8

[a] Percentage in the pretreated solid (A), and recoveries expressed in relation to the content in the raw material (B) or in the pretreated material (C).
[b] Hydrothermal pretreatment temperature.
[c] Alkaline peroxide delignification process time.

Table 5
Glucose concentrations (g/L) obtained by enzymatic hydrolysis of pretreated almond-tree prunings by hydrothermal pretreatment (LHW), dilute sulphuric acid pretreatment (DSA), or hydrothermal pretreatment plus alkaline peroxide delignification (LHW + APD). Influence of pretreatment temperature.

Pretreatment		Delignification	Enzymatic hydrolysis time (h)		
Type	T (°C)		24	48	72
Untreated	–		–	–	8.9 ± 0.4
LHW	180	–	8.3 ± 0.1	9.3 ± 1.1	9.8 ± 0.9
	190		10.0 ± 0.1	10.8 ± 2.0	12.1 ± 1.5
	200		15.9 ± 0.4	17.5 ± 1.2	20.4 ± 0.2
	210		19.2 ± 0.1	23.7 ± 0.1	23.8 ± 0.6
	220		25.4 ± 0.1	26.5 ± 3.0	23.3 ± 3.1
	230		24.3 ± 1.0	28.8 ± 0.4	27.9 ± 3.4
DSA	180	–	12.2 ± 0.1	13.7 ± 0.8	13.8 ± 0.4
	190		16.5 ± 0.3	17.5 ± 0.5	18.9 ± 2.0
	200		21.2 ± 1.5	22.4 ± 0.7	22.7 ± 1.5
	210		25.6 ± 0.2	25.9 ± 1.5	32.1 ± 0.3
	220		26.4 ± 0.1	27.2 ± 2.2	28.4 ± 0.8
	230		29.9 ± 1.3	35.0 ± 4.5	31.7 ± 3.9
LHW	180	APD (60 min)	25.0 ± 1.6	30.0 ± 2.3	35.5 ± 1.6
	190		28.4 ± 0.8	33.2 ± 2.0	35.5 ± 2.1
	200		31.5 ± 2.1	32.9 ± 1.9	39.9 ± 1.6
	210		33.3 ± 1.6	38.6 ± 1.1	41.9 ± 0.0
	220		28.8 ± 2.6	37.7 ± 3.1	41.0 ± 2.4
	230		32.9 ± 0.8	35.9 ± 3.7	32.5 ± 3.7

in the hydrothermal pretreatment increased from 180 °C to 220 °C, the cellulose content in delignified solids augmented up to 66.6% and 68.7% after 30-min and 60-min APD pretreatment, respectively. By contrast, solids pretreated at 230 °C provided lower cellulose percentages after delignification (64.0% and 67.0% after 30 min and 60 min APD pretreatment, respectively). The maximum increase in cellulose content was attained for material pretreated at 220 °C, increasing from 49.9% (after LHW pretreatment) to 68.7% (after APD pretreatment carried out for 60 min). The high cellulose recovery after the alkaline delignification with peroxide was a major finding. The recovery of cellulose for both process times were alike, being 93.2% the average value for the ADP carried out for 30 min and 94.8% for the pretreatment performed for 60 min. Results revealed that less than 15% of the initial cellulose content in almond-tree prunings and less than 10% of the cellulose in the resulting solids from LHW pretreatment were removed after the delignification process (Table 4). Therefore, a high cellulose percentage from the initial almond-tree prunings is available for further enzymatic hydrolysis.

3.4. Enzymatic hydrolysis

Enzymatic hydrolysis was performed to assess the efficiency of the 3 pretreatments assayed, namely LHW pretreatment, DSA pretreatment, and LHW pretreatment combined with alkaline peroxide delignification, for enhancing the enzymatic hydrolysability of the cellulose contained in almond-tree prunings. In general glucose concentration increased with time, except in some experiments in which the parameter dropped at 72 h (Table 5). As no anti-bacterial agent was added to the enzymatic hydrolysis, long incubations could lead to a decrease in monosaccharides as result of bacterial growth after 48 h. High glucose concentration (8.9 g/L) was achieved when the enzymatic hydrolysis was performed directly on almond prunings. Glucose concentrations from solids pretreated with LHW increased from 9.8 g/L to 28.8 g/L when increasing the pretreatment temperature from 180 °C to 230 °C. The same trend was observed in the enzymatic hydrolysis of solids obtained from DSA pretreatments, however the use of sulphuric acid led to higher glucose concentrations (e.g., 13.8 g/L at 180 °C and 35.0 g/L at 230 °C). Solids from LHW pretreatment followed by alkaline peroxide delignification provided glucose concentrations between 35.5 g/L (LHW pretreatment at 180 °C) and 41.9 g/L (LHW pretreatment at 210 °C). Above this latter temperature (210 °C), the glucose concentrations dropped (e.g., 35.9 g/L at 230 °C), which could indicate that the temperature during the LHW pretreatment should not overcome this value to obtain solids with high enzymatic digestibility.

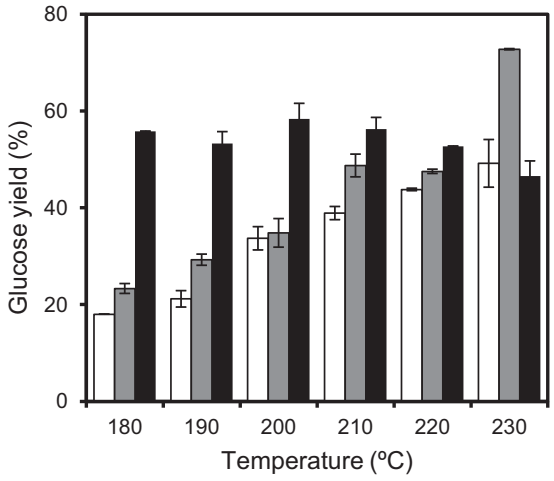

Fig. 3. Enzymatic hydrolysis yields (%) after hydrothermal pretreatment (white bar), dilute sulphuric acid pretreatment (grey bar) and hydrothermal pretreatment followed by alkaline peroxide delignification (black bar).

Enzymatic hydrolysis yields, expressed in percentage as glucose obtained in the bioprocess divided by the potential glucose in pretreated solids, are shown in Fig. 3. Results illustrated that LHW pretreatments at low temperature led to the lowest enzymatic yields (18.0% at 180 °C and 21.2% at 190 °C), some of them even lower than that obtained in the enzymatic hydrolysis of non-pretreated almond prunings (24.4%) while enzymatic hydrolysis of solids pretreated with DSA provided higher yields. When the temperature of the DSA pretreatment was 230 °C, the subsequent enzymatic yield reached 72.8%. This value could be indicated that a deeper breakdown of lignocellulosic structure was provoked at the highest temperature assayed in DSA pretreatments, thus facilitating the enzymatic hydrolysis of cellulose. The results are in agreement with those reported by Wei, Wu, and Liu (2012), who achieved enzymatic yields close to 76% of theoretical in the dilute (0.75%) acid pretreated (160 °C for 10 min) of eucalyptus chips. Furthermore, Brodeur-Campbell, Klinger, and Shonnard (2012) reported 70% glucose recovery for acid pretreated switchgrass.

The enzymatic hydrolysis of solids from LHW pretreatment combined with 60 min alkaline peroxide delignification further increased the sugar yields (e.g., 58.4% when the hydrothermal pretreatment was performed at 200 °C). However, from 210 °C to 230 °C the enzymatic hydrolysis yield dropped from 56.3% to 46.6%, which could be related to the increasing lignin content in these solids (Table 4). At this latter temperature, the extent of enzymatic hydrolysis and sugar yield were lower than those from LHW or DSA as single pretreatments (Fig. 3). Our results are in agreement with earlier findings (Cara, Ruiz, Ballesteros, Negro, & Castro, 2006). Working with olive pruning debris, these authors (Cara et al., 2006) reported maximum enzymatic yields of about 35% when the raw material was steam-explosion pretreated at 230 °C. Nevertheless, when the pretreated solid was submitted to alkaline peroxide delignification, the highest sugar yield (61.9%) was obtained from solids pretreated at 190 °C.

3.5. Overall sugars yields

Apart from glucose generated from pretreated solids in the enzymatic hydrolysis step, significant amounts of glucose, hemicellulosic monosaccharides (xylose, arabinose, galactose, and mannose), and oligosaccharides were released to the prehydrolysates during LHW or DSA pretreatments. Therefore, to assess the overall process for ethanol production, it is necessary to consider the sum of sugars from different process steps (overall sugars yield, OSY). Fig. 4 summarizes the total glucose and hemicellulosic sugars in the pretreatment and enzymatic hydrolysis steps. The yields reported were determined as percentage of the total dry weight of raw material.

LHW pretreatments provided prehydrolysates with low sugars content and solids with low enzymatic digestibility. Therefore, overall sugars yields from hydrothermal pretreatments were low (Fig. 4a), varying from 8.8% and 17.3% for pretreatments performed at 180 °C and 220 °C, respectively. APD led to enhanced digestibility of solids pretreated with LHW along with an improvement of the overall sugars yield (Fig. 4b). The OSY was about 20.6% and remained constant when LHW pretreatments were carried out between 180 °C and 220 °C, and decreased up to 17.2% for the highest temperature assayed (230 °C). The OSY from hydrothermal pretreatments increased by submitting the filtrates obtained by LHW pretreatment to post-hydrolysis with 3% sulphuric acid to convert their oligosaccharides into monosaccharides. In this case, OSY reached a maximum value of 40.6% when LHW pretreatment was conducted at 200 °C (Fig. 4c). The above data implies a recovery of simple sugars equivalent to 68.7% of the theoretical sugars in raw material.

Finally, Fig. 4d shows OSY values after pretreatments with dilute sulfuric acid (0.025 M) and enzymatic hydrolysis of the resulting solids. Increasing pretreatment temperature from 180 °C to 200 °C rose sugars production in both prehydrolysates and enzymatic hydrolysates. However, above 200 °C the enhancement of enzymatic hydrolysis yields was not enough to compensate the decrease of monosaccharides yields in prehydrolysates due to thermal degradation, and OSY values sharply dropped.

Ethanol production attainable from each pretreatment studied in this work is shown in Table 6. The theoretical conversion yield

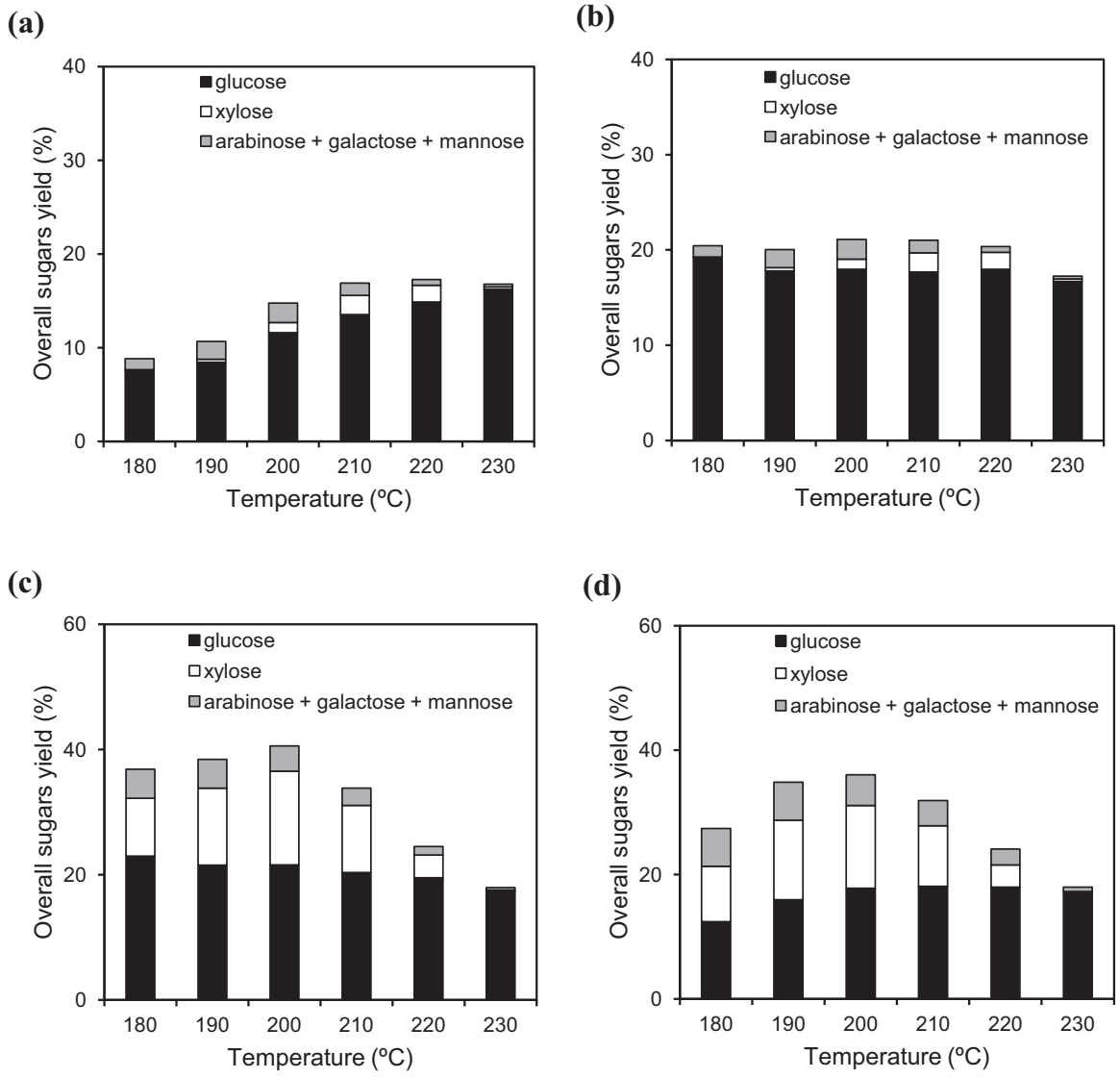

Fig. 4. Monomeric carbohydrates production from solid and liquid fractions obtained when almond-tree pruning was submitted to four different processes: (a) hydrothermal pretreatment, (b) hydrothermal pretreatment followed by alkaline peroxide delignification of remaining solids, (c) hydrothermal pretreatment followed by alkaline peroxide delignification of remaining solids and acid post-hydrolysis of prehydrolizates, (d) dilute sulphuric acid pretreatment. All pretreated solids were submitted to enzymatic hydrolysis.

Table 6
Theoretical ethanol yield (Y_E) from four different sugars production schemes.[a]

Process type		Y_E (kg/100 kg raw material)		
Solid	Prehydrolysate	Solid fraction[b]	Liquid fraction[c]	Total
LHW (220 °C)	–	7.3	1.2	8.5
LHW (220 °C) + APD (60 min)	–	8.8	1.2	10.1
LHW (200 °C) + APD (60 min)	Acid posthydrolysis	8.7	10.0	18.7
DSA (200 °C)	–	6.5	9.4	15.9

LHW: liquid hot water; APD: alkaline peroxide delignification; DSA: dilute sulphuric acid.
[a] Theoretical yield of ethanol was defined as 0.51 g of ethanol per g of glucose or xylose.
[b] Considering free glucose from enzymatic hydrolysis.
[c] Considering glucose and xylose in the prehydrolysate.

of glucose or xylose to ethanol was defined as 0.51 g ethanol per g of sugar. The combination of hydrothermal pretreatment (200 °C, 5 min) and alkaline peroxide delignification followed by enzymatic hydrolysis of the remaining solids, plus the post-acid hydrolysis of the prehydrolysate obtained from LHW treatment gave ethanol yields of 18.7 g/100 g of raw material. Alternatively, dilute sulphuric acid hydrolysis (200 °C for 5 min) plus enzymatic hydrolysis led to slightly lower ethanol yields (15.9 g/100 g), so this technique could be used as an individual prehydrolysis stage for the recovery of sugars (mainly glucose and xylose) from almond-tree prunings. Perceptively, this latter technique could increase the feasibility of the production of bioethanol in an industrial scheme.

4. Conclusions

This preliminary study confirms that almond-tree prunings can be regarded as a potential feedstock for sugars production as a first stage towards fuel ethanol generation, due its high polysaccharides content (59.1 g monosaccharides per 100 g raw material), and low cost. However, the high lignin content of this biomass could hinder the enzymatic hydrolysis of the cellulosic fraction. The operating temperature and the nature of the pretreatment applied to the raw material significantly affected the composition of both prehydrolysates and solid residues. Hydrothermal pretreatment led to prehydrolysates with noticeably higher yields of oligosaccharides (18.2 g/100 g raw materials at 190 °C). In contrast, dilute sulphuric acid (0.025 M) pretreatment resulted in high monosaccharides recovery (24.0 g/100 g raw materials at 190 °C). The production of the fermentation inhibitor 5-HMF was almost nil in both prehydrolysates.

Solids pretreated by liquid hot water or dilute sulphuric acid gave maximum glucose yields (49.2% and 72.8% respectively) at the highest pretreatment temperature assayed (230 °C). On the other hand, the enzymatic digestibility of liquid hot water pretreated solids was further enhanced by means of alkaline peroxide delignification.

The most suitable scheme for sugar production from almond-tree prunings consisted of dilute sulphuric acid hydrolysis at temperatures about 200 °C followed by enzymatic hydrolysis, and thereby 36.0 g of sugars could be generated per 100 g of raw material, including 31.2 g glucose and xylose (59%of the potential glucose and xylose present in raw material).

Acknowledgements

The authors would like to acknowledge Novo Nordisk Bioindustrial (Madrid, Spain) and 'Finca *El Boje*' (Alhama de Granada, Spain) for supplying enzymes and almond-tree prunings, respectively. The authors also wish to thank to Andalusia Regional Government (Spain) for financial support.

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