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High Solids Loading Pretreatment of Olive Tree Pruning with Dilute Phosphoric Acid for Bioethanol Production by *Escherichia coli*

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ABSTRACT

This paper deals with a new approach for using olive tree pruning biomass as raw material for ethanol and other chemical production. This process includes a water extraction step, followed by a high solids loading dilute phosphoric acid pretreatment and an ethanologenic *Escherichia coli* fermentation for the conversion of all of the sugars released. Results show that the operational conditions leading to the maximal hemicelullosic sugar recovery in the liquid fraction (near 70%) are 170 °C and 0.5% (w/v) phosphoric acid concentration. The fermentation of the mixed sugars found in the detoxified prehydrolysates by ethanologenic *E. coli* MS04 led to an ethanol concentration of 23 g/L and an ethanol yield of 0.46 g of ethanol/g of sugars consumed. When the ethanol production from the pretreated solids after enzymatic hydrolysis is also taken into account, 13.2 g of ethanol/100 g of original material was obtained. The production of other interesting compounds is also considered.

1 Introduction

Olive tree cultivation, traditionally considered one of the main crops in Mediterranean countries, has spread worldwide because of the healthy properties attributed to olive oil consumption. Today, more than 10 million hectares are cultivated in more than 20 countries, of which a quarter is located in Spain. (1) As an essential step of cultivation, olive trees are usually pruned every 2 years to remove old branches and increase production. Most of the olive tree pruning (OTP) is eliminated by burning or grinding and spreading across the field. Because of the large amount of biomass that is generated yearly, OTP has been proposed as raw material for producing second-generation bioethanol within the biorefinery concept. (2) This approach also allows for the production of high value-added products, chemicals, and energy, using all of the components of biomass.

Pretreatment is a key step in the production of ethanol from lignocellulosic materials. Different pretreatments have been applied to OTP, e.g., liquid hot water, (3, 4) steam explosion, (5, 6) dilute acid, (7-9) inorganic salts, (10) and organosolvent pretreatments.

(3, 11, 12) Dilute acid pretreatment aims to solubilize the hemicellulose fraction and improve the digestion of cellulose by enzymes. Sulfuric acid is the most commonly used acid, but its replacement by other acids, such as phosphoric acid, can present some advantages. The decrease in the generation of inhibitors during pretreatment, the lower corrosiveness, which reduces material costs of equipment, and the possibility of using the distillation residues as fertilizer are some of them. (13)

To reduce energy costs during distillation, it is important to obtain concentrated sugar solutions that might be converted into ethanol concentrations above 4% (v/v) in the fermentation step. (14) Pretreatment with high solids loading allows for the achievement of higher concentrations of hemicellulosic sugars in the prehydrolysates, which makes the fermentation of pentoses more interesting. Also, this configuration has a lower consumption of energy because it requires a lower addition of water, which is heated during pretreatment. (15)

Concerning fermentation, the microorganism selected for ethanol production should exhibit high fermentation yields (>90% of theoretical), high product tolerance (>40 g/L of ethanol) and productivity, low requirements for growth, inhibitor resistance, genetic stability, and broad pH and temperature range of use. (16) Previous works have considered different yeasts for fermentation of OTP prehydrolysates as *Pachysolen tannophilus* (8, 17) and *Scheffersomyces stipitis* (*Pichia stipitis*). (6, 8) Both microorganisms have been described as able to convert both glucose and pentose to ethanol, which is a major issue in taking full advantage of all sugars present in the olive tree biomass. In addition to yeasts, bacteria have also been widely studied for ethanol production by means of genetic engineering techniques, such as expression of the genes responsible for sugar fermentation coming from other microorganisms. Another common practice consists of cell adaptation to the hydrolysate components. (18) As a prominent example, modified *Escherichia coli* has been successfully applied to the fermentation of hydrolysates produced from a number of raw materials, including sugar cane bagasse, (19) eucalyptus, (13) wheat straw, (20) and corn stover, (21) exhibiting the ability to produce ethanol at high yields from both xylose and glucose. Additionally, the performance of ethanologenic *E. coli* was improved by metabolic evolution, resulting in an increased acetate tolerance, (22) which constitutes a significant advantage because acetate is usually found in hydrolysates as a consequence of pretreatment.

In an attempt to improve the previously reported results from olive tree biomass, in this work, genetically modified *E. coli* was studied for the first time on hydrolysates obtained by dilute phosphoric acid pretreatment. High solids loadings (the highest to date with OTP) in the pretreatment step were employed to obtain concentrated fermentable sugar solutions. Also, the advantages of performing a preliminary step of extraction were evaluated. Finally, mass balances of the whole process are determined to compare the results to previous works dealing with OTP.

2 Materials and Methods

2.1 Raw Material

OTP was locally collected after olive-harvesting, air-dried to an equilibrium moisture content of about 7%, and milled to a particle size smaller than 4 mm with a laboratory hammer mill (Retsch, SM 100). This lignocellulosic biomass was subjected to aqueous extraction at 120 °C for 60 min. The extraction was carried out in an autoclave using 1 L glass bottles (working volume of 0.5 L) with water and OTP at 10% (w/v). As a general rule, the extracted solids obtained after filtration (extracted OTP) were used as raw material for pretreatment. This process configuration is based on previous works, (6, 23) which have shown that water extraction before OTP pretreatment significantly improved the overall sugar recovery. Both initial OTP and extracted OTP materials were characterized using the National Renewable Energy Laboratory (NREL) standard biomass analytical procedures, (24) as described in section 2.8.

2.2 Pretreatment

The solid material obtained from the extraction step was pretreated in a 1 L agitated tank reactor (Parr Instrument Company, Moline, IL). A total of 500 mL of dilute phosphoric acid solution was mixed with 150 g of extracted OTP, 30% dry basis (db) solids concentration. The experimental variables investigated were temperature (in the range of 170–210 °C) and phosphoric acid concentration (from 0.5 to 1.5%, w/v). The agitation rate was kept at 350 rpm, and the residence time was established in 10 min once the desired temperature was reached. The heating time lasted from 30 to 40 min, while the cooling time took about 10 min. The slurry obtained after each experiment was filtered for solid and liquid separation. The water-insoluble solids (WIS) were washed with water and subjected to enzymatic hydrolysis (EH) assays after drying in oven at 40 °C. The liquid fractions (prehydrolysates) in selected conditions were used for fermentation. WIS and prehydrolysates characterization was made to determine the sugar content and other components as acid-insoluble lignin in solids and inhibitory compounds in the liquid fractions.

As described above, the effect of operational conditions in the pretreatment was evaluated using extracted OTP as a raw material. Alternatively, in the pretreatment conditions selected for fermentation tests, the pretreatment step was performed directly with OTP, without an extraction step, to compare the effect of the process configuration on the fermentability of the prehydrolysates.

2.3 EH

EH tests were conducted to evaluate cellulose digestibility in WIS after the different pretreatment conditions assayed. EH tests of extracted OTP (without pretreatment) were also performed as a control. Experiments were carried out on a shaking incubator (Certomat-R, B-Braun, Germany) in 100 mL Erlenmeyer flasks with 25 mL of 0.05 M sodium citrate buffer (pH 4.8) and 1.25 g (db) of WIS (5% solids concentration). The temperature was keep at 50 °C, and agitation speed was maintained at 150 rpm. Cellulosic-hydrolyzing enzymes (CellicCTec3, kindly provided by Novozymes A/S, Denmark) was loaded at 15 filter paper units (FPU)/g of WIS. Β-glucosidase (Novozyme 50010, Novozymes A/S) was supplemented with 15 international units (IU)/g of WIS. The experiments were carried out for 72 h, with sampling every 24 h to determine the glucose concentration. Glucose present in commercial enzyme solutions was also determined and taken into account by subtracting the corresponding value to the results. The experiments were performed by triplicate, and average results and standard deviations are determined.

2.4 Microorganisms and Growth Conditions

E. coli strain MS04 was kindly donated by Dr. Martínez from the Institute of Biotechnology [National Autonomous University of Mexico (UNAM), Mexico]. (22) The microorganism was stored at −80 °C as frozen stocks in 40% glycerol. Before the fermentation experiments, inoculum of *E. coli* MS04 was prepared by transferring the microorganisms to 75 mL of AM1 culture medium (25) modified with 2 g/L sodium acetate, 0.1 g/L citric acid, 22.8 g/L xylose, and 21 g/L glucose. The bacteria were grown at 37 °C on the shaking incubator at 150 rpm for 24 h.

2.5 Detoxification of Prehydrolysates

The liquid fractions (prehydrolysates) obtained from pretreatment conditions selected to reach the highest hemicelulosic sugar recoveries were subjected to a detoxification procedure prior to fermentation experiments. After a mild acid posthydrolysis [4% (w/v) sulfuric acid at 120 °C for 30 min] to release the monomeric sugars present in oligomeric form, a detoxification step was performed by overliming, (26) consisting basically of the addition of $Ca(OH)_2$ until pH 10, maintaining it on a shaking incubator for 30 min at 50 °C. Then, sulfuric acid was added to pH 6.5, and the precipitate was removed by centrifugation at 3500 rpm for 10 min (Rotina 420, Hettich Zentrifugen, Germany). Inhibitory compound and sugar compositions were analyzed before and after the detoxification step.

2.6 Fermentation of Prehydrolysates

Before starting the fermentation with *E. coli* MS04, prehydrolysates were supplemented with salts as in the growth medium (section 2.4) and the liquor was sterilized by filtration (Millipore GP, 0.22 μm, Millipore, Ireland). The initial cell biomass concentration was approximately 0.5 g/L (db). The volume of inoculum was estimated by measuring the absorbance at 620 nm. The determined volume was centrifuged at 3500 rpm for 10 min, and the biomass is harvested while the liquid is discarded. Finally, the cells are resuspended in the hydrolysate. Fermentation was performed at 37 °C and pH 7. Fermentation devices consisted of 300 mL glass flasks, with 150 mL of fermentation liquor provided with the pH probe and agitation by a magnetic stirring. The temperature was maintained by a water bath, and pH was monitored and automatically corrected by the addition of 2 M KOH. Each flask was stopped by a robber cap, with three holes for venting, sampling, and pH monitoring. The experiments were made by triplicate for 72 h. Several samples were taken to determine sugar, inhibitory compound, and ethanol concentrations.

2.7 Fermentation of Enzymatic Hydrolysates

Enzymatic hydrolysates obtained in selected pretreatment conditions were subjected to fermentation. The microorganism and experimental conditions were the same as described for the prehydrolysate fermentation (see section 2.6), with an experimental time of 24 h. The experiments were made in triplicate, and sugar and ethanol concentrations were determined. Ethanol production from glucose present in the enzyme solution was subtracted.

2.8 Analytical Methods

The composition of raw materials and WIS was determined according to NREL analytical methods for biomass, (24) including previous extraction with water and ethanol in the case of raw material. Prehydrolysates were centrifuged and filtered through 0.20 μm membranes (Sartorius Stedim, Goettingen, Germany) and analyzed by high-performance liquid chromatography (HPLC) for sugar and inhibitory compound determinations. The HPLC system (Waters, Milford, MA) was equipped with a refractive index detector (model 2414). A CARBOSep CHO-782 Pb (Transgenomic, Inc., Omaha, NE) carbohydrate analysis column was used for the monomeric sugars (glucose, xylose, galactose, arabinose, and mannose) determinations. Ultrapure water was used as a mobile phase at a flow rate of 0.6 mL/min and a temperature of 70 °C. Samples were previously submitted to an acid posthydrolysis treatment [4% (w/v) sulfuric acid at 120 °C for 30 min] to hydrolyze oligomeric sugars into monomers. Furfural, hydroxymethylfurfural (HMF), and acetic and formic acid concentrations were analyzed by HPLC in a Hewlett-Packard 1100 system (Palo Alto, CA) equipped with a refractive index detector. An ICSep ICE-COREGEL 87H3 column (Trasnsgenomic, Inc., Omaha, NE) was used with 5 mM sulfuric acid as the eluent at a flow rate of 0.6 mL/min and a temperature of 65 °C. Fermentation samples were also analyzed with this column (sugars, inhibitors, and ethanol). In the case of sugars, xylose, galactose, and mannose are presented as a mixture, and the sum of them is called XGM. The total phenolic content was determined by a colorimetric procedure using the Folin–Ciocalteu reagent. (27) The values were expressed as gallic acid equivalents.

3 Results and Discussion

3.1 Water Extraction of OTP

The composition of OTP before and after the water extraction step (Table 1) shows that the only component significantly affected was the extractive fraction. When it was taken into account that the total solids mass recovery in this step was 81%, it can be noted that sugars, lignin, and ash remained almost unaltered, whereas over 70% of the extractives were solubilized. The extracted solids contained nearly 50% of structural sugars, which may be converted into ethanol after pretreatment.

Table 1

The resulting aqueous solution after extraction also contains soluble sugars, mainly glucose. A total of 1.5% of free glucose (g/100 g of raw material) was found in this liquid fraction, reaching 5.5% after a posthydrolysis step. Small quantities of other sugars, including xylose (0.2%), galactose (0.6%), and arabinose (0.3%), and a non-negligible amount of mannitol (3.7%) were also present. Phenolic compounds were also found in this aqueous solution (3.1 g of gallic acid equiv/L), which could be a potentially usable high-value co-product. (28)

3.2 Effect of the Phosphoric Acid Concentration and Temperature in the Pretreatment Step

The composition of WIS obtained after pretreatment of extracted OTP at different temperatures and phosphoric acid concentrations is shown in Table 2. WIS total gravimetric recoveries between 58 and 67% were obtained in the assayed ranges. Raw material solubilization increased as long as the temperature and acid concentration were increased during pretreatment. WIS are mainly composed of cellulose and lignin, whereas the hemicellulose was entirely solubilized in experiments performed at 190 and 210 °C.

Table 2

The sugar content in prehydrolysates is shown in Table 3. Data show the percentage of sugars that were detected as oligomers at each operation condition set and the final sugar composition of the liquid once the posthydrolysis was performed. Because of the elevated solids loadings employed during pretreatment (30%), prehydrolysates with relatively high sugar concentrations were obtained (>50 g/L for 170 °C pretreatment). Some of these sugars are found in the liquid fraction in oligomeric form, especially at the lowest phosphoric acid concentration assayed (0.5%). At 170 °C and 0.5% acid concentration, 52% of oligomers were obtained, requiring a posthydroysis step to obtain the monomeric sugar form before fermentation.

Table 3

Xylose is the main sugar present in the prehydrolysates, followed by glucose, arabinose, galactose, and mannose. As the pretreatment temperature increased from 170 to 210 °C, the sugar content declined significantly. This could be the result of a partial degradation of carbohydrates during pretreatment at higher temperatures. Table 4 shows the composition of some inhibitory compounds found in the prehydrolysates, including hydroxymethylfurfural (HMF) and furfural, resulting from glucose and xylose degradation, respectively, (29) formic acid, phenolic compounds, and acetic acid. As a general rule, the concentrations of degradation compounds increased with the acid concentration and the pretreatment temperature. These compounds have been reported as potential inhibitors for microbial growth; (30) thus, minimizing their presence in the prehydroysates could be considered as a criterion to select the more suitable pretreatment conditions. (31)

Table 4

To visualize the mass recovery of sugars during pretreatment, taking into account both liquid and solids fractions, a bar chart is represented in Figure 1. Concerning glucose mass recoveries, Figure 1A shows that some glucose was found in the liquid fractions, mainly because of the glucose present at the remaining extractive fraction and the solubilization of some structural glucans. No significant glucose losses were detected for experiments performed at 170 and 190 °C, regardless of the acid concentration, whereas significant cellulose solubilization was detected at 210 °C, exceeding 25% of glucose degradation at 1.5% phosphoric acid. Concerning the hemicellulose fraction (Figure 1B), maximum recoveries in prehydrolysates (near 70%) were obtained for experiments performed at 170 °C without a significant difference, regardless of the acid concentration. These results are within range of those obtained in other works with the same raw material pretreated by autohydrolysis (4, 32) or dilute sulfuric acid solutions (7) and higher than those reported when using FeCl3, (10) steam explosion, (23) or steam explosion combined with dilute phosphoric acid. (6)

Figure 1

3.3 EH and Overall Sugar Yields at Different Pretreatment Conditions

The effects of pretreatment operational conditions on WIS enzymatic digestibility are shown in Table 5, expressed as EH yields referred to the glucose present in the pretreated material. Table 5 also shows glucose recovery by EH referred to the total potential glucose in the raw material (extracted OTP) and the overall sugar yields taking into account the sugar content in the liquid fraction from pretreatment (Table 2).

Table 5

The influence of the pretreatment temperature on the enzymatic susceptibility of WIS is clear. EH yields rise as the pretreatment temperature did, reaching values around 85% at 210 °C. This fact is widely proven in numerous works. (3, 23) However, the phosphoric acid concentration had a limited effect in the range assayed (only at 170 °C did the acid concentration show significant differences between the acid concentrations assayed). As an example, Figure 2 shows the evolution of the glucose concentration in the EH performed with WIS obtained at 170 °C and different acid levels.

Figure 2

The effect of pretreatment in terms of improvement of cellulose susceptibility for all of the conditions assayed in this work when comparing results to those obtained from the EH of the control (extracted OTP not submitted to pretreatment) is noticeable. In this case, only 1.8 g/L glucose was obtained, probably resulting from the residual extractive fraction (Table 1). Maximal glucose concentrations are obtained at 72 h, although the hydrolysis rate is greater in the first 24 h.

Despite the beneficial effect of the pretreatment temperature on the enzymatic digestibility of pretreated materials, when a glucose recovery based on the glucose content in the raw material is determined, the best results (around 67%) were obtained at 190 °C (Table 5), because of the significant cellulose solubilization and glucose degradation detected during the pretreatment at 210 °C (Figure 1A). In addition, because the main goal of this work is to produce ethanol from both cellulose and hemicellulose fractions, an overall sugar yield has been determined to select more appropriate pretreatment conditions, taking into account also the recovery of sugars in the prehydrolysates. As seen in Table 5, rising the pretreatment temperature results in a decrease in overall sugar yields, because of hemicellulosic sugar degradation during pretreatment (Figure 1B). A very small enhancement of overall sugar yields was detected only at 170 °C when the H3PO4 concentration increased from 0.5 to 1.5%, whereas the influence on the generation of inhibitory compounds was significant (Table 4). This is the reason why the pretreatment conditions selected for the subsequent fermentation experiments were 170 °C and 0.5% (w/v) phosphoric acid concentration.

In comparison to a previous work with the same raw material and the use of phosphoric acid and a catalyst in steam explosion pretreatment, (6) the selected conditions provided a WIS less susceptible to enzymatic attack but a better sugar recovery in the prehydrolysates. Furthermore, sugar concentrations in the liquid fraction were remarkably higher.

3.4 Fermentation of Selected Prehydrolysates Obtained with and without the Water Extraction Step

The process configuration used in this work included a water extraction step on OTP biomass (section 2.1). This operation has been proven in previous works (6, 23) as an advantageous option for the improvement of the EH yield of the cellulose fraction and to avoid condensation lignin reactions occurring when pretreatments were performed with the original raw material, which has a high content of extractive fraction. (4, 7) In this work, the effect of this extraction step on the fermentabiliy of prehydrolysates was tested in the selected pretreatment conditions, 170 °C and 0.5% (w/v) H3PO4. Tables 6 and 7 show the sugar and inhibitor compositions of prehydrolysates obtained with and without the extraction step, respectively, and also after the acid posthydrolysis needed to break down the oligomeric sugars. In comparison to sugar composition (Table 6), the main differences were found in glucose and xylose contents. In the case of no extraction step, the free glucose present in the prehydrolysates was almost triple and the total glucose more than double that obtained from pretreatment of water-extracted OTP. The reason is the presence of a significant amount of glucose in the extractive fraction of original raw material (Table 1). In the case of xylose, the trend was the contrary: more xylose is found in the experiment performed with the water extraction step before pretreatment, because of a better solubilization of the hemicellulose fraction when the extractive fraction was removed. The EH tests (data not show) also confirm the positive effect of the extraction step to increase the digestibility of cellulose (59 versus 27%) that has been reported in previous works. (6, 23)

Table 6

Table7

Concerning inhibitory compounds, it can be clearly stated from data in Table 7 that there were more degradation compounds in the case that OTP was pretreated without a previous extraction step, except for acetic acid and furfural, which were slightly lower. HMF from glucose degradation (29) reached 1.3 g/L (versus 0.2 g/L with the extraction step), and formic acid, which could be formed from HMF, (33) reached 4.4 g/L (versus 1.3 g/L with the extraction step); in both cases, this fact could be explained by the higher presence of glucose when the OTP is pretreated without a previous extraction, as mentioned above. This glucose, mainly from the extractive fraction (Table 1), could be easily solubilized and then degraded during the pretreatment. The presence of the extractive fraction during the pretreatment could also explain the differences in total phenolic compounds, whose concentration was more than doubled when no extraction step was performed. The posthydrolysis step required to release all sugars available in monomeric form, before fermentation, also had some effects on inhibitory compounds. As seen in Table 7, the acetic acid concentration increased significantly after posthydrolysis, suggesting a high level of the degree of substitution of oligomers by acetyl groups. (3) Furan contents slightly increased by posthydrolysis, probably because of some sugar degradation occurring in this step. (34)

The fermentation of the selected prehydrolysates after the posthydrolysis step was assayed with *E. coli* MS04. No growth was detected with these undetoxified liquors (data not shown), indicating the inhibitory effect exerted by toxic compounds present in prehydrolysates. Therefore, a detoxification process, overliming, was applied to both prehydrolysates (from pretreatment at 170 °C and 0.5% H_3PO_4 with and without a previous extraction step). Overliming has been widely reported in the literature for different lignocellulosic materials, (26) and it has demonstrated its efficiency to improve the fermentability of OTP prehydrolysates with the yeasts *P. stipitis* and *P. tannophilus*. (8, 35) As seen in Tables 6 and 7, the sugar loss during the detoxification step was limited and the amount of some inhibitors as furans and phenolic compounds was also reduced. However, no decrease in the acetic acid was detected, whereas the formic acid concentration showed a small reduction. In comparison of these results to other detoxification methods applied to OTP prehydrolysates, (6) an alkaline treatment similar to overliming but using NaOH instead of $Ca(OH)_2$ resulted in lower reduction of the amount of inhibitory compounds compared to overliming. Nonetheless, in a previous work, (6) the treatment with ion-exchange resins led to an almost complete elimination of inhibitory compounds, although, in this case, toxic compound concentrations were lower than in the present work.

When fermentation experiments were performed with the detoxified prehydrolysates, a good microbial performance was obtained in the case of liquid fractions issued from extracted OTP pretreated at 170 °C and 0.5% (w/v) H_3PO_4 (Figure 3), whereas the prehydrolysates obtained in the same pretreatment conditions but using original OTP as raw material (without an extraction step) showed poor fermentability (data not shown), with neither growth nor ethanol production after 72 h of fermentation. Therefore, the water extraction step of OTP improved the fermentability of the prehydrolysates obtained in the further pretreatment, because of the absence of the extractive fraction in this step, which causes a minor presence of both phenolic compounds and degradation products coming from the glucose present in extractives. This fact, jointly with the reported improvement in sugar recoveries, (23) confirms that including an extraction step is an interesting choice for an overall process of bioethanol production from OTP.

Figure 3

Figure 3 shows that *E. coli* MS04 was able to efficiently convert to ethanol all sugars present in the prehydrolysates resulting from water-extracted OTP pretreated at 170 °C and 0.5% phosphoric acid concentration. Glucose and arabinose were completely consumed before 48 h, whereas the rest of the sugars present in the prehydrolysates were almost exhausted at 72 h of fermentation, with a maximum ethanol concentration of 23 g/L and an ethanol yield of 0.46 g of ethanol/g of sugar consumed. These results compare favorably to the results available about pentose and hexose mixture fermentation of the same raw material (water-extracted OTP) with *S. stipitis*, (6) where a maximum of 8 g/L ethanol was obtained with a yield of 0.37 g of ethanol/g of sugar consumed and no total consumption of all sugars, because *S. stipitis* was not able to assimilate arabinose. In other works, OTP (without water extraction) pentose-containing detoxified prehydrolysates were fermented with the yeasts *P. tannophilus* and *P. stipitis* (8, 9, 17, 35) but lower yields and ethanol concentration were obtained, a maximum of 14 g/L in the best case, (8) after fermentation with *P. stipitis* of prehydrolysates obtained with dilute acid pretreatment at 180 °C and 1% (w/v) sulfuric acid concentration and a solids loading of 20% in the pretreatment step, instead of 30% employed in this work.

3.5 Overall Process Material Balance

The findings obtained in this work can be summarized in Figure 4. The flow diagram proposed included a water extraction of original tree pruning to remove most of the extractive fraction that has been demonstrated favorable to improve the overall sugar recovery of the process. (23) Besides that, this work has proven that the removal of the extractives also has a beneficial effect on prehydrolysate fermentability. In addition, in the context of a biorefinery process to valorize the OTP residue, it can be taking into account that the liquid obtained from the water extraction step contains potential high-value compounds, such as phenolic compounds with antioxidant capacity, mannitol, or oligosaccharides.

Figure 4

Concerning pretreatment, the conditions selected in this work (170 °C and 0.5% H_3PO_4) allowed for a good overall sugar recovery with similar results to that obtained in previous works with the same raw material and different acid catalysts but with lower temperature and chemical requirements, which is a more sustainable and lower energy demanding process. The higher solids concentration employed in this work allowed for a higher sugar concentration in the liquid fraction to obtain higher ethanol concentrations in the fermentation step. As a first time with OTP, a modified microbial strain of *E. coli* was used for the fermentation of the mixture of pentose and hexose sugars derived from pretreatment, with promising results as an almost total consumption of all of the sugars in 72 h and a yield of 0.46 g of ethanol/g of sugar consumed. With the aim to obtain an overall ethanol yield of ethanol of the whole process, the fermentation of the enzymatic hydrolysates obtained from the solid fraction rich in cellulose was made with *E. coli*. Therefore, considering the two steps of fermentation from both liquid and solid fractions obtained from pretreatment, a total ethanol yield of 13.2 g of ethanol/100 g of original OTP was obtained (16.3 g of ethanol/100 g of extracted OTP). This result is similar to the result reported by Negro et al. (6) (16 g of ethanol/100 g of extracted OTP) using a different batch of the same raw material and steam explosion as a pretreatment. The final solid residue contains some cellulose and a high proportion of lignin that could make it an energetic source for the whole process. As a conclusion, the integration of ethanol production from sugars, other valuable chemicals from the water extraction step, and energy generation from the rich lignin final residue could improve the global economy of a possible biorefinery based on OTP as raw material. As far as ethanol production is concerned, the results obtained in the present work represent a starting point, which requires further research. Next efforts will focus on process optimization, including the fermentation of the whole slurry with ethanologenic *E. coli*.

Acknowledgment

The authors thank support from Ministerio de Economía y Competitividad (Project ref ENE 2011-29112-C02-02, including FEDER funds) and Junta de Andalucía (Project AGR-6103). Juan Miguel Romero-García gratefully acknowledges his schorlarship to Junta de Andalucía.

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Figure caption

Figure 1. Mass sugar recovery, expressed as a percentage of the sugar content in the raw material, in solids (dotted bars) and liquid (hatched bars) fractions issued from pretreatment with H_3PO_4 at different acid concentrations (%, w/v) and temperatures (°C): (A) glucose mass recovery and (B) hemicellulosic sugar mass recovery.

Figure 2. Time course of the glucose concentration obtained by EH of WIS resulting from pretreatment at 170 °C and different phosphoric acid concentrations (%, w/v): 0.5% (blue diamonds), 1% (red squares), and 1.5% (green triangles). The control with un-pretreated raw material (purple crosses) (extracted OTP) is also represented.

Figure 3. Time course during *E. coli* MS04 fermentation of detoxified prehydrolysates obtained from extracted OTP pretreated at 170 °C and 0.5% H_3PO_4: glucose (blue diamonds), XGM (sum of xylose, galactose, and mannose) (red squares), arabinose (green triangles), and ethanol (purple crosses) concentrations.

Figure 4. Material balance flow scheme of the overall process for ethanol production from OTP after water extraction and dilute acid pretreatment with 0.5% phosphoric acid at 170 °C.

Figure 1

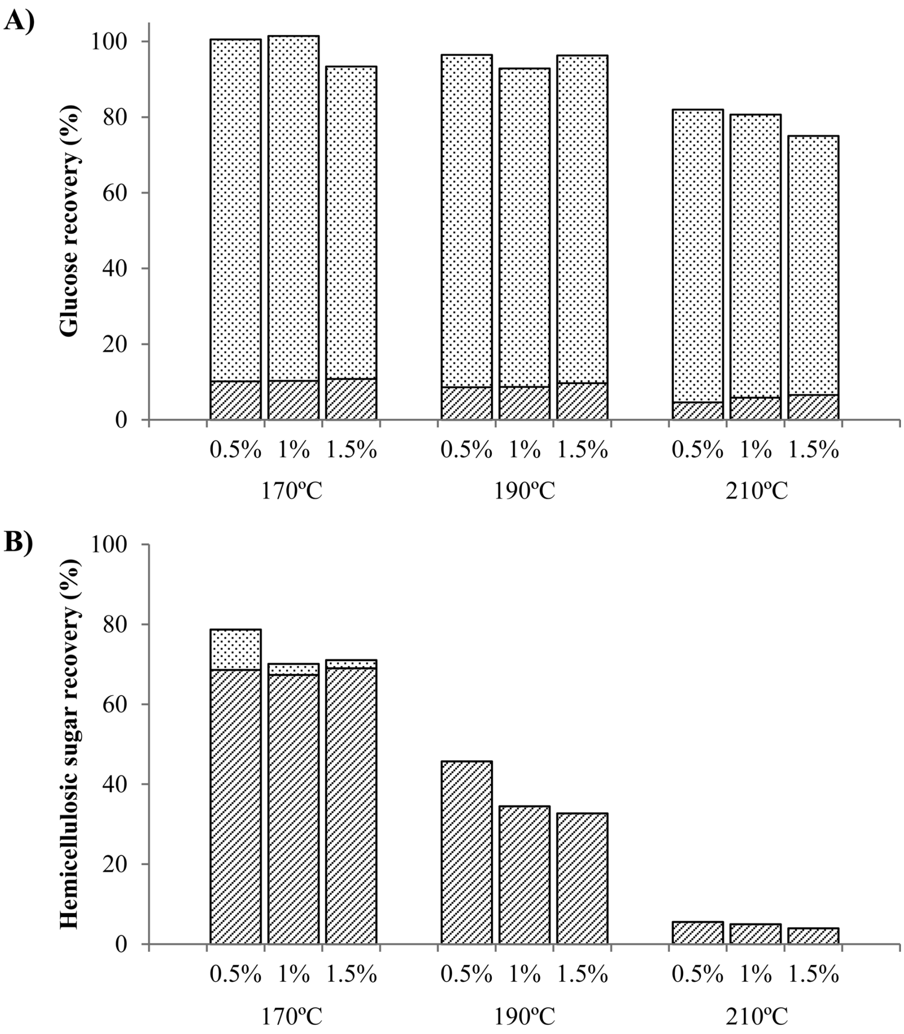
A)
Glucose recovery (%)
0
20
40
60
80
100
0.5%
1%
1.5%
0.5%
1%
1.5%
0.5%
1%
1.5%
170ºC
190ºC
210ºC
B)
Hemicellulosic sugar recovery (%)
0
20
40
60
80
100
0.5%
1%
1.5%
0.5%
1%
1.5%
0.5%
1%
1.5%
170ºC
190ºC
210ºC

Figure 2

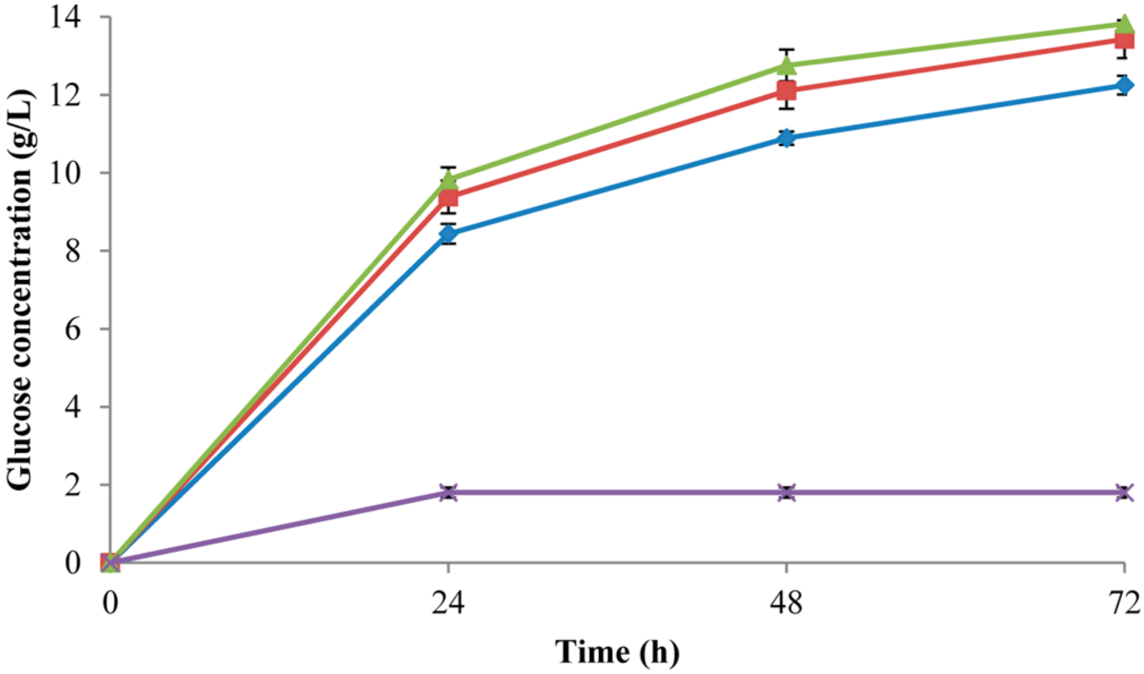

Figure 3

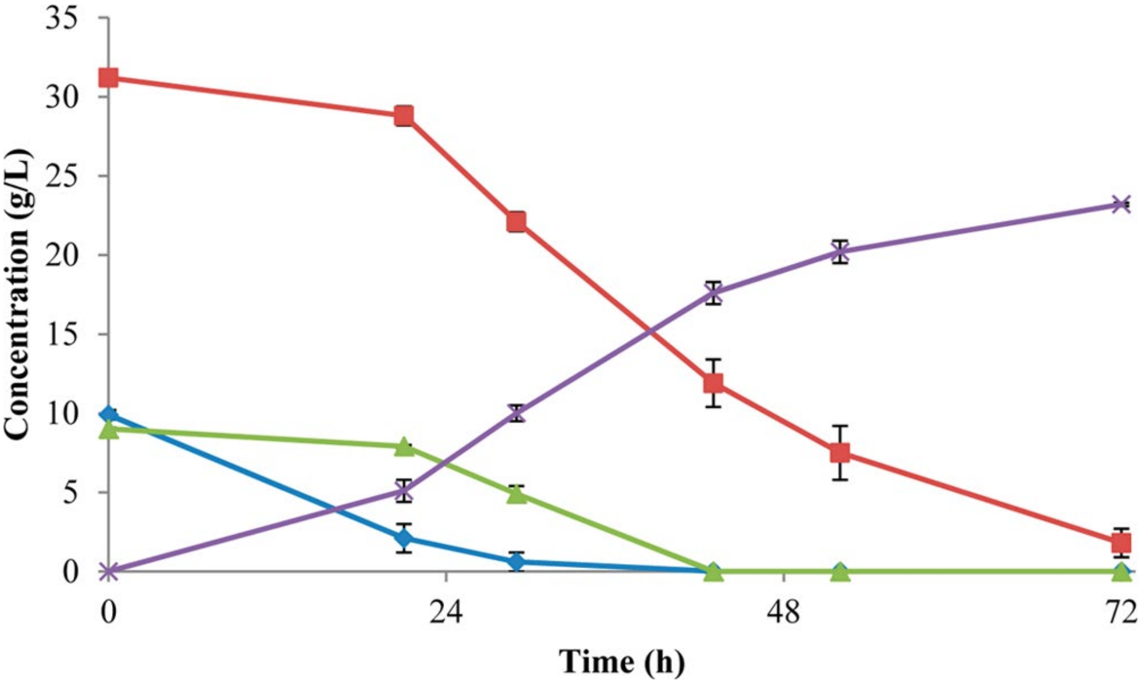

Figure 4

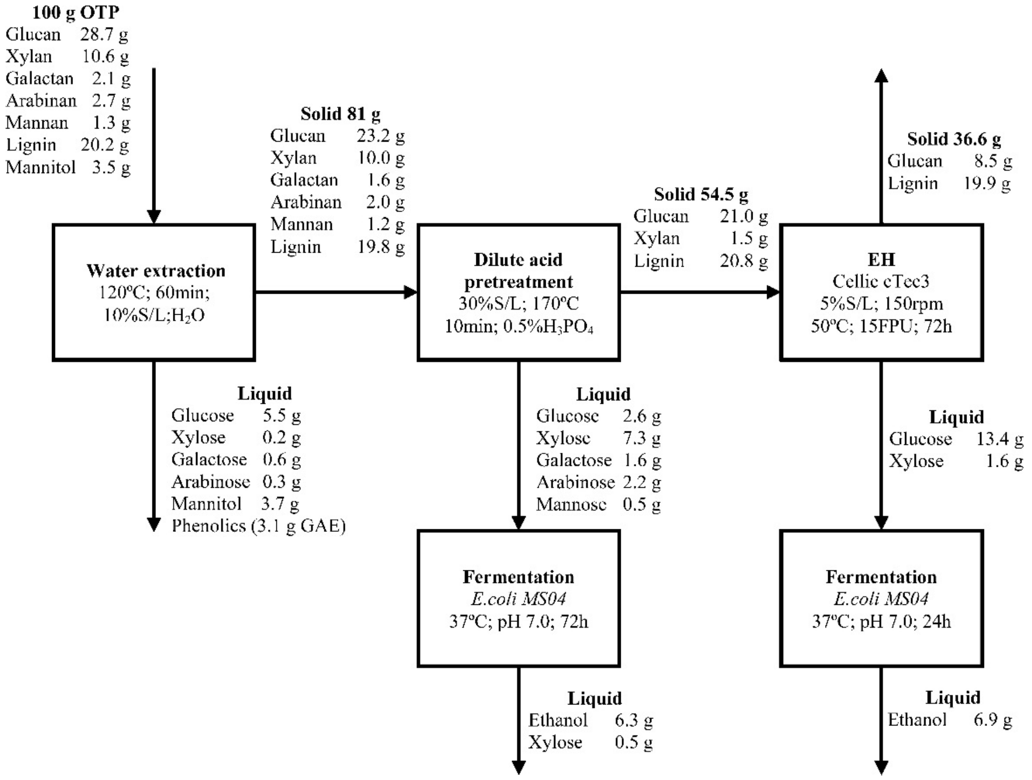
100 g OTP
Glucan 28.7 g
Xylan 10.6 g
Galactan 2.1 g
Arabinan 2.7 g
Mannan 1.3 g
Lignin 20.2 g
Mannitol 3.5 g
Water extraction
120°C; 60min;
10%S/L;H2O
Solid 81 g
Glucan 23.2 g
Xylan 10.0 g
Galactan 1.6 g
Arabinan 2.0 g
Mannan 1.2 g
Lignin 19.8 g
Liquid
Glucose 5.5 g
Xylose 0.2 g
Galactose 0.6 g
Arabinose 0.3 g
Mannitol 3.7 g
Phenolics (3.1 g GAE)
Dilute acid pretreatment
30%S/L; 170°C
10min; 0.5%H3PO4
Solid 54.5 g
Glucan 21.0 g
Xylan 1.5 g
Lignin 20.8 g
Liquid
Glucose 2.6 g
Xylose 7.3 g
Galactose 1.6 g
Arabinose 2.2 g
Mannose 0.5 g
Fermentation
E.coli MS04
37°C; pH 7.0; 72h
Liquid
Ethanol 6.3 g
Xylose 0.5 g
EH
Cellic cTec3
5%S/L; 150rpm
50°C; 15FPU; 72h
Solid 36.6 g
Glucan 8.5 g
Lignin 19.9 g
Liquid
Glucose 13.4 g
Xylose 1.6 g
Fermentation
E.coli MS04
37°C; pH 7.0; 24h
Liquid
Ethanol 6.9 g

Table 1

Table 1. Composition of OTP before and after the Aqueous Extraction Stage

composition (%,dry matter)	OTP	extracted OTP[a]
cellulose (as glucose)	26.0 ± 0.3	30.9 ± 0.3
hemicellulosic sugars	17.4 ± 0.2	19.9 ± 0.3
xylose	11.7 ± 0.1	13.9 ± 0.2
galactose	1.7 ± 0.1	1.9 ± 0.1
arabinose	2.7 ± 0.2	2.4 ± 0.1
mannose	1.4 ± 0.0	1.6 ± 0.0
acid-insoluble lignin	17.5 ± 0.1	21.4 ± 0.2
acid-soluble lignin	2.6 ± 0.0	3.0 ± 0.1
extractives	25.6 ± 0.3	8.8 ± 0.7
glucose in extractives	5.9 ± 0.1	1.0 ± 0.2
ash	2.8 ± 0.2	3.4 ± 0.1

[a] In an autoclave at 120 °C for 60 min.

Table 2

Table 2. Total Gravimetric Recovery (TGR) and Composition (%, Dry Matter) of WIS Obtained after Different Pretreatment Conditions

temperature (°C)	**phosphoric acid concentration (%, w/v)**	**TGR**	**glucan**	**xylan**	**acid-insoluble lignin**	**ash**
170	0.5	67.3	38.5	2.7	38.3	3.9
	1.0	64.2	40.8	0.8	41.4	4.2
	1.5	64.0	37.1	0.6	41.7	4.6
190	0.5	62.6	40.2	Nd[a]	44.7	5.0
	1.0	60.5	40.0	Nd	47.0	4.5
	1.5	60.6	41.0	Nd	47.1	4.3
210	0.5	61.5	36.1	Nd	49.5	4.9
	1.0	60.7	35.5	Nd	51.7	5.0
	1.5	58.3	33.8	Nd	53.8	4.4

[a] Nd = not detected.

Table 3

Table 3. Sugar Composition (g/L) of Liquid Fractions Issued from Pretreatment and Percentage of Oligomeric Sugars

			sugar composition[a]				
temperature (°C)	**phosphoric acid concentration (%, w/v)**	**oligomeric fraction (%)[b]**	**glucose**	**xylose**	**galactose**	**arabinose**	**mannose**
170	0.5	53.4	9.7	27.0	6.0	8.0	1.7
	1.0	24.8	9.8	26.7	6.1	7.1	1.9
	1.5	28.2	10.3	27.5	6.2	7.4	2.0
190	0.5	13.1	8.2	16.8	4.9	4.7	2.1
	1.0	2.3	8.3	11.6	4.3	3.9	1.8
	1.5	2.5	9.3	10.7	4.4	3.8	1.6
210	0.5	19.1	4.4	1.0	1.3	0.6	0.6
	1.0	6.9	5.6	0.8	1.3	0.6	0.4
	1.5	6.8	6.3	0.6	1.1	0.4	0.4

[a] After a mild posthydrolysis step [4% (w/v) sulfuric acid at 120 °C for 30 min]. [b] Obtained from subtracting the sugars released after the posthydrolysis step and the sugars present as monomers in the liquid fraction resulting from pretreatment at different conditions.

Table 4

Table 4. pH and Inhibitor Composition (g/L) of Liquid Fractions Issued from Pretreatment

temperature (°C)	phosphoric acid concentration (%, w/v)	pH	formic acid	acetic acid	HMF	furfural	total phenolic compounds[a]
170	0.5	3.2	1.9	3.0	0.4	1.3	3.5
	1.0	2.8	2.2	5.2	0.6	2.9	
	1.5	2.4	2.9	5.8	0.6	3.1	4.9
190	0.5	3.5	2.4	8.2	1.6	7.1	4.5
	1.0	2.9	2.2	9.8	2.4	10.0	3.8
	1.5	2.5	2.4	10.1	2.7	10.6	5.2
210	0.5	3.4	3.0	11.1	4.7	11.2	5.1
	1.0	3.1	3.0	11.1	5.7	12.4	5.4
	1.5	3.0	3.0	11.1	6.2	12.1	5.8

[a]Measured as gallic acid equivalent.

Table 5

Table 5. Sugar Yields (%) Obtained after Pretreatment and EH of Pretreated Materials (WIS)

temperature (°C)	**phosphoric acid concentration (%, w/v)**	**EH yield**[a]	**glucose recovery by EH**[b]	**overall sugar yield**[c]
170	0.5	59.4	53.6	65.7
	1.0	61.4	55.9	66.7
	1.5	69.4	57.3	68.4
190	0.5	77.0	67.6	64.0
	1.0	78.8	66.3	59.0
	1.5	77.9	67.5	59.6
210	0.5	84.0	65.0	44.3
	1.0	85.1	63.7	44.0
	1.5	85.8	58.7	41.1

[a] Percentage of glucose released from EH referred to the glucose content in WIS. [b] Percentage of glucose released from EH referred to the glucose content in extracted OTP. [c] Sum of the glucose released from EH and total sugar content in prehydrolysates, expressed as a percentage of the total sugar content in the extracted OTP.

Table 6

Table 6. Sugar Composition (g/L) in Initial Prehydrolysates, after Posthydrolysis, and after Detoxification by Overliming of OTP Pretreated with 0.5% H_3PO_4 at 170 °C[a]

	glucose		**xylose**		**galactose**		**arabinose**		**mannose**	
	WE	**NE**	**WE**	**NE**	**WE**	**NE**	**WE**	**NE**	**WE**	**NE**
prehydrolysates	4.8	13.9	7.2	3.2	3.0	3.1	9.1	9.2	0.4	
after acid posthydrolysis	10.1	20.5	26.3	19.2	5.4	5.4	8.8	8.6	2.0	1.1
after detoxification	9.6	20.7	25.0	19.2	5.2	5.4	8.5	8.6	2.0	1.3

[a] WE columns stand for water-extracted material, and NE columns stand for pretreated material without water extraction.

Table 7

Table 7. Inhibitor Composition (g/L) in Initial Prehydrolysates, after Posthydrolysis, and after Detoxification by Overliming of OTP Pretreated with 0.5% H_3PO_4 at 170 °C[a]

	formic acid		**acetic acid**		**HMF**		**furfural**		**total phenolic compounds**[b]	
	WE	**NE**	**WE**	**NE**	**WE**	**NE**	**WE**	**NE**	**WE**	**NE**
prehydrolysates	1.3	4.4	2.5	2.3	0.1	1.3	1.1	0.8	3.3	7.4
after acid posthydrolysis	1.1	3.5	7.5	6.3	0.2	1.7	1.4	1.0	3.5	6.9
after detoxification	0.7	3.4	7.6	7.0	0.1	0.6	0.5	0.4	2.2	3.4

[a] WE columns stand for water-extracted material, and NE columns stand for pretreated material without water extraction. [b] Measured as gallic acid equivalent weight.