ALKALINE TWIN-SCREW EXTRUSION FRACTIONATION OF OLIVE-TREE PRUNING BIOMASS

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ABSTRACT

The present study investigates and optimizes a one-step alkaline-extrusion pretreatment process using olive tree pruning as feedstock. In this work, a range of pretreatment conditions (temperature, screw speed and alkaline catalyst to dry matter ratio) were evaluated according to different parameters: composition of pretreated substrates, glucose and xylose recovery, degradation products generation, enzymatic hydrolysis yield and overall sugars yield. Results show that enzymatic digestibility is remarkably improved by extrusion although not significant variations are found on the chemical compositions of extruded material produced at different conditions. The maximum glucose sugar production value, after pretreatment and enzymatic hydrolysis, was close to 21 g/ 100 g raw material, which corresponds to about 69 % of the theoretical production yield.

Keywords: Bio-refineries; extrusion; agricultural waste; pretreatment, lignocellulose, enzymatic hydrolysis.

1. INTRODUCTION

Lignocellulosic biomass has been receiving major research attention during the last three decades due to its important potential for conversion to sugars and fuels (Balat, 2011). Its role in the diversification of the current bioethanol production based on starchy or sugar-based biomass appears to be a key factor to boost implementation of lignocellulosic biomass conversion to ethanol into the current fuel market. The production of fuel ethanol from agricultural or other lignocellulosic residues may be advantageous because of the local availability of the raw material, usually at reduced prices. Olive tree pruning (OTP) biomass is a highly available renewable agricultural residue in the Mediterranean countries with no industrial applications. Olive tree pruning is a periodical culture operation performed every two years after fruit harvest by means of which less productive branches are cut off and trees are regenerated, the main objective being to improve production. Currently, olive trees are cultivated in more than forty countries, and the total dedicated surface is about 10.4 million ha (Faostat, 2013). In Mediterranean areas, the residual biomass from olive pruning reaches an average 1.31 t ha^{-1} in annual, and 3.02 t ha^{-1} in biennial, pruning (Velázquez-Martín et al., 2011). Other studies state the residue yield ranging from 1 to 5 and from 4 to 11 t ha^{-1}, respectively, for the Spanish and the Italian orchard (Sánchez et al., 2002; Spinelli and Picchi 2010). A typical OTP lot includes leaves (around 25% by weight), thin branches (around 50% by weight), and thick branches or wood (25% by weight), although the proportions may vary depending on culture conditions, tree age, production and local pruning practice. This biomass constitutes an important energy and chemicals source that, till date, is not being used commercially. This residue contains variable amounts of carbohydrates as well as phenolic and terpenic compounds, etc., which makes it an interesting source for bio-refinery products (Romero-García et al., 2014).

The composition of OTP biomass permits to develop a multiproduct industry that takes advantage of the various components in biomass and their intermediates, therefore maximizing the value derived from the biomass feedstock.

As an alternative, olive tree pruning residues may be used as raw material for ethanol production. Due to the recalcitrant nature of the lignocellulose, a pretreatment step is required for increasing fermentable sugars in the hydrolysis step. It is necessary to choose pretreatment conditions that produce highly digestible solid material resulting in high sugar yields from enzymatic hydrolysis and at the same time, prevent the degradation of soluble sugars, so maximizing overall sugar yield. Many pretreatment methods have been evaluated for ethanol production (Alvira et *al.,* 2010). Particularly for OTP biomass dilute acid (Cara et al., 2008), liquid hot water (Cara et al., 2007), un-catalysed steam explosion (Ballesteros et al, 2011), phosphoric acid-catalysed steam explosion (Negro et al., 2014) and salts such as $FeCl_3$ (López-Linares et al., 2013) have been tested. All these pretreatments have in common the obtaining of a pretreated material in which soluble fraction is mainly composed of the hemicellulose sugars, while cellulose and lignin remain in insoluble solid fraction.

Regarding pretreatment, extrusion process is a novel and promising physical method for biomass fractionation. Twin-screw extruders are a specialized category of continuous processing equipment that is especially suited for aggressive mixing under reactive conditions. They contain synchronous, parallel axis shafts wit intermeshing screw elements that can be configured to impose very high compression and shear forces on materials (Scott et al., 2011). In extrusion pretreatment, the material is subjected to heating, mixing and shearing, resulting in physical and chemical modifications during the passage through the extruder (Karunanity and Muthukumarappan, 2010). Screw speed and barrel temperature are believed to cause important effect in the disruption of the lignocellulose structure caused by extrusion, which results in defibrillation and shortening of the fibres, and, in the end, increased accessibility of carbohydrates to enzymatic attack (Karunanithy and Muthukumarappan, 2010). The different extrusion parameters must be taken into account to achieve the highest efficiency in the process. Recent extrusion studies showed a significant improvement on sugar recovery from corn stover (Liu et al., 2013), switchgrass (Karunanithy and Muthukumarappan, 2010), *Miscanthus* (Kang et al., 2013), prairie cord grass (Karunanithy and Muthukumarappan, 2010), pine wood (Karunanithy et al., 2012), and barley straw (Duque et al., 2013) through enzymatic hydrolysis. This improvement is attributed to the reduction in cellulose crystallinity, increase in surface area, pore size and volume (Karunanithy and Muthukumarappan, 2013), and the delignification effect during dissolution-regeneration steps (Um *et al*., 2013).

On the other hand, alkali treatment is reported to break hydrolysable linkages in lignin and glycosidic bonds of carbohydrates (Carvalheiro et al., 2008). As a result, it produces swelling of the fibers leading to increase in internal surface area, reduction in the degree of polymerization and crystallinity, and disruption of the lignin structure. Moreover, alkaline saponification of acetyl and uronic ester bonds also occurs, improving the enzymatic digestibility of pretreated material (Chen et al., 2013).

The high potential of OTP biomass as raw material for the production of fuels and chemicals makes the search of new and efficient fractionation technologies a matter of interest. The present study focuses on the extrusion of OTP biomass in a one-step alkaline-extrusion process, in order to obtain a biomass fractionation. To our best knowledge, there is no literature on alkali-extrusion of olive tree pruning residues. In this work, a range of pretreatment conditions [temperature (70, 90, 110 ºC), screw speed (70 and 140 rpm) and alkaline catalyst to dry matter ratio (5 and 10 g NaOH/100 g DM biomass) were evaluated according to different parameters: composition of pretreated substrates, glucose and xylose recovery, degradation products generation, enzymatic hydrolysis yield and overall sugar yield.

2. METHODOLOGY

2.1 Olive tree pruning

OTP was collected after fruit-harvesting, air-dried at room temperature to equilibrium moisture content of about 10%, and milled using a hammer mill to a particle size smaller than 4 mm. Fraction 1-4 mm was utilized in this work, while fraction less than 1 mm was discharged.

2.2 Pretreatment

A twin-screw extruder) consisting in six modules (Clextral Processing Platform Evolum® 25 A110, Clextral, France), was used in this study. OTP biomass was fed into the first module through a volumetric screw feeder KMV KT20 (K-tron), which has a flow capacity up to 16 Kg/h for OTP milled at 4 mm. Biomass was feed at 0.6 kg/h to provide a continuous and constant feeding flow. The screw profile, which diagram is depicted in Figure 1, has been previously described by Duque et al. (2013). Briefly, the screws were configured to have a constantly decreasing pitch in module 1 and 2, a zone with neutral kneading blocks in module 3 and reverse screws in module 4. In module 5 a filtration step was set up in order to separate liquid from solid fraction (filtrate and extrudate, respectively) after extrusion. Right after that module, a reverse screw is used in module 6. In order to add the catalyst (NaOH solution at 10-20% w/v, flow rate 0.3 L/h) and water (flow rate 6 L/h) to the process, two metering pumps connected to the extruder were used.

Operating conditions were set to achieve moderate values of NaOH/DM ratio, 5 and 10 % (w/w), barrel temperature (70, 90 and 110 ºC) and screw speed of 70 and 150 rpm. These conditions were chosen based on previous studies carried out in our laboratory with other herbaceous residues, such as barley straw (Duque et al., 2013). Pretreatment runs were performed in triplicate.

After extrusion, OTP extruded material was recovered and washed thoroughly with slightly acidic water (pH around 4), until neutral pH. The filtrate was collected and total soluble solids, sugars, aliphatic acids, furans and phenols content was determined. A portion of washed extruded solid (WES) was dried at 40ºC and analyzed for carbohydrates and lignin composition, as described below.

[Insert Figure 1 here]

2.3 Enzymatic hydrolysis

The washed water-insoluble residue of pretreated OTP was enzymatically hydrolyzed by the novel enzyme preparations Cellic CTec 2 and Cellic HTec 2. The enzymes where kindly provided by Novozymes A/S (Denmark). Cellic CTec 2 is a cellulase preparation, which in addition shows high beta-glucosidase activity. Cellic HTec 2 is a hemicellulase preparation with endoxylanase activity. All the enzymatic hydrolysis assays were performed in 100-mL Erlenmeyer flasks in triplicates using WES as substrate. Enzyme preparation used was a mixture of Cellic Ctec2:Cellic Htec 2 (in a proportion 3:1 in volume) and was added in a dosage of 15 FPU of cellulose/g substrate. The assays were run in 50 mM sodium citrate buffer (pH 5) at 50 ºC, 150 rpm and 5% (w/v) dry WES load. At 72 h, samples were withdrawn, centrifuged at 9300 *g* for 10 min and the supernatants were analysed for sugars concentration by HPLC, as described below in analytical methods. Additionally, blanks of the enzyme mixtures were analyzed by HPLC to subtract the sugar content present in the enzyme preparations used. Enzymatic hydrolysis yields (EH) were determined considering the glucose/xylose produced during enzymatic hydrolysis, which is referred to the potential glucose/xylose (calculated based on the glucan/xylan content in the WES) and is reported as percentage. Average values of the three replicates were presented.

2.4 Analytical Methods

The composition of raw material and WES obtained after pretreatment were determined according to National Renewable Energy Laboratory (NREL) analytical methods for biomass (Sluiter et *al.*, 2011).

Sugar content in filtrate and EH media was quantified by high performance liquid chromatography (HPLC) using a Waters 2695 liquid chromatograph with refractive index detector. A CARBOSep CHO-682 LEAD column (Transgenomic, Omaha, NE) operating at 75 ºC with Milli-Q water (Millipore) as mobile-phase (0.5 mL /min) was used.

Phenolic compounds were analysed by HPLC (Agilent, Waldbronn, Germany) employing an Aminex HPX-87H column (Bio-Rad Labs, Hercules, CA) at 65 ºC. The mobil phase was 89% (5 mM H_2SO_4) and 11% acetonitrile at flow rate of 0.7 mL/min. A 1050A Photodiode-Array detector (Agilent, Walsbronn, Germany) was employed for detection. Total phenols were also quantified according to a slightly modification version of Folin-Ciocalteau as described Moreno et al. (2013).

Formic and acetic acid were quantified by HPLC (Waters, Milfors, MA) using a 410 Water refractive index detector. An Aminex HPX-87H (Bio-Rad Labs, Hercules, CA) column maintained at 65 ºC and mobile phase of 5 mM H_2SO_4 at flow rate of 0.6 mL/min were employed.

Total starch content was measured using the Total Starch Assay Kit (Megazyme, Ireland).

3. RESULTS

3.1 Composition of raw material

Table 1 shows olive tree pruning biomass composition. OTP has 22.3% of cellulose and 17.4% hemicellulose (oven dry weight). Total lignin content accounts for 17.8%. Acetyl groups represent about 2.3% of raw material and total ash accounts for 4.1 %. It is worth noticing that this lignocellulosic residue has an extractive content of 24.5%, which includes 6.2% of glucose (as oligosaccharides, probably starchyose and raffinose). Other sugars are present in the water extract in trace amounts. The proportion of the extractive fraction is greater than that reported for other agricultural residues like barley straw. In previous reports on OTP, extractive contents ranged from 23.3% (Ballesteros et al., 2011) to 31.4% (Cara et al., 2008), and the variability was attributed mainly to the heterogeneity of the residue (variable proportions of small branches and leaves). The high proportion of extractives could be related to a higher content of leaves in the raw material.

[Insert Table 1 here]

3.2. Extrusion pretreatment in combination with alkali

Results of WES and filtrate composition after the different extrusion experiments are depicted in Tables 2 and 3, respectively. The alkali-extrusion pretreatment resulted in a cellulose and hemicellulose enriched-solid (Table 2), compared to raw material. Glucan in WES (values ranging from 31.0 to 38.8 %), is increased by 1.3 to 1.6 fold in relation to the content in raw material. Hemicellulose content in WES ranges from 21.2 to 26.5%., while AIL was 24.4-27.3%. Hemicellulose was mostly composed of xylan (70%) and arabinan (16 %).

Regarding total solid recovery values, in most cases it was close to 100% (data not shown).The recovery of glucan is in the range 92-100 % in the solid fraction, while xylan recovery in solid fraction varies from 92 to 99%. It is interesting to attain high values of hemicellulose recovery in the pretreated solid to enhance the total fermentable sugars production through enzymatic hydrolysis of xylan using specific enzymes.

[Insert Table 2 here]

In the water-soluble fraction generated from pretreatment (filtrate) (Table 3), sugars were present in considerable proportions as oligomers, so that a post-hydrolysis step was performed to determine the total amount of sugars. The sugar production ranged from 7.2 to 9.5 g/100 g raw material. It is worth noting that glucose is the most abundant sugar in the liquid fraction at any condition. Considering that non-structural derived glucose was present at high proportion in the aqueous extract fraction of raw material, it is likely that the most part of this component was transferred to liquid fraction after one-step alkaline-extrusion process. The second major sugar found in filtrates was mannitol; sugar production of this sugar ranged from 1.65 to 3.5 g/100 g raw material. This component, with interesting applications in the food and pharmaceutical industries, is also present in olive tree leaves (Ghoreishi and Shahrestania, 2009). Mannitol is used as an excipient in pharmacy, and as anticaking and free-flow agent, lubricant, stabiliser and thickener, and low calorie sweetener, in the food industry.

[Insert Table 3 here]

Regarding other products in filtrates, acetic acid was detected in all pretreatment conditions. Acetic acid production is due to the action of soda on acetyl groups release from hemicelluloses. In fact, when pretreatment was done with water instead of soda, acetic acid was not found in the filtration liquid (data not shown).

Totals phenols were also determined in the filtrate, and values ranged from 1.7 to 3.3 g/100 g raw material. By HPLC analysis of monomeric phenols, cumaric acid and ferulic acid were detected (about 30 mg/100 g raw material, about 27 mg felulic acid /100 g raw material), and in less proportion, hydroxybenzoic acid (10 mg/100 g raw material). During alkaline pretreatment, the lignin macromolecule is dissolved and degraded into small fractions. It has been reported that the reaction involves the cleavage of phenolic alfa-O-4 linkages, cleavage of non-phenolic beta-O-4 linkages, and removal of residual lignin fractions, either by cleavage of C-C linkages or carbohydrate degradation, releasing lignin-carbohydrate fractions that are mainly oxidized into aliphatic carboxylic acids (Sun et al., 2002). As expected, due to low operation temperatures and basic conditions, neither furfural nor 5-hydromethyl furfural were detected.

3.3 Enzymatic saccharification

The effect of temperature, alkali concentration and screw speed on the enzymatic digestibility of the solid fraction obtained after extrusion pretreatment was evaluated and results are shown in Table 2. EH yield depends on the barrel temperature and in general, EH yield increases as the temperature rises. The untreated raw material displayed maximum EH yield about 8 % after 72 h enzymatic hydrolysis in tests performed in parallel to pretreated substrates and 19 % EH yield when extrusion pretreatment was undertaken with water instead of alkali. The alkali extruded samples exhibited a higher enzymatic digestibility, yielding up to 65%. The comparison of EH yield values from water-extruded and alkaline- extruded OTP demonstrates the positive effect of alkaline addition during extrusion on enzymatic hydrolysis of extruded OTP. The addition of 5 g NaOH/100 g DM allows increasing EH yield by 1.7 fold, while 10 g NAOH/g DM results in 3.4 fold increase in experiments at 110ºC. The EH yield increased with NaOH loading and barrel temperature, but screw speed effect was not significant ($p<0.05$) by ANOVA analysis.

On the other hand, xylan conversion yield rises as alkaline concentration increases in all temperatures tested, attaining values close to 70% of theoretical in WES at 110ºC and 10 NaOH/100 g DM and 150 rpm. In untreated material, yield was 3.3%. It means that the digestibility of xylan is enhanced by one-step alkaline extrusion pretreatment due to deconstruction of lignocellulose structure and facilitating of the xylanase enzymes action. Similar results in xylan hydrolysis were reported on barley straw using the same equipment, where the hydrolysis yield for xylan resulted in 71% of theoretical (Duque et al., 2013).

In order to optimize the overall process yield, both carbohydrate recovery in the solid residue after pre-treatment step, and hydrolysis yield in the enzymatic step must be taken into account. This parameter is an important indicator of the potential amount of sugars that could be used for ethanol or other by-products production. Overall sugar yields were evaluated and results are shown in Table 2. At the best conditions (150 rpm, 110ºC, 10 NaOH g/100 g DM) an overall yield of 21.01 g glucose /100 g olive tree pruning and 9.54 g xylose/100 g olive tree pruning was obtained. A 68.7% of total glucose is available after one-step alkaline extrusion pretreatment and enzymatic hydrolysis yield. The maximum sugars recovery recorded in this study was comparable to those obtained in pine were a maximum of 66.1% of sugars was obtained. However these experiments were carried out at significantly higher temperature of 180ºC without alkaline treatment (Kuranunithy and Muthukumarappan, 2012).

The comparison of the effectiveness of one-step alkaline extrusion process with other pre-treatments performed on OTP resulted in an improvement in overall sugar yield. Table 4 show results obtained for different pretreatments on OTP biomass in relation to glucose overall yield and sugars overall yield. Results are also expressed as percentage of theoretical, due to the different composition of raw materials. Maximum overall sugar recovery yield achieved in the extrusion pretreatment of OTP is highest than those obtained using different pre-treatments such as liquid hot water and steam explosion (both un-catalysed and acid-catalysed) pretreatments. Though overall sugars yield values obtained were slightly lower than results from diluted acid pretreatment, one of the main advantages of extrusion fractionation process over other thermo-chemical methods is that the process can be carried out at lower temperature, preventing the formation of inhibitory compounds coming from the degradation of hemicellulos/lignin.

[Insert Table 4 here]

CONCLUSIONS

Results in this work show that one-step alkaline –extrusion process is a suitable method to fractionate OTP resulting in high sugars recovery values. Fractionation followed by enzymatic saccharification leads to a glucose yield equivalent to 69% of potential glucose present in raw material. Regarding the effect of process parameters studied on EH yield, it is demonstrated by ANOVA analysis that NaOH loading and barrel temperature positively affect sugars release by EH ($p<0.05$), but screw speed effect was not significant. This result together with the huge amount of this residue yearly generated, its low cost and lack of other alternatives of use, makes this process an attractive option for its upgrading. Nevertheless, research on the improvement of sugar yield using extrusion process must be continued to optimize the use all sugars present in this biomass.

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