

Article

Biorefinery of the Olive Tree—Production of Sugars from Enzymatic Hydrolysis of Olive Stone Pretreated by Alkaline Extrusion

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Abstract: This work addresses for the first time the study of olive stone (OS) biomass pretreatment by reactive extrusion technology using NaOH as the chemical agent. It is considered as a first step in the biological conversion process of the carbohydrates contained in the material into bio-based products. OS is a sub-product of the olive oil extraction process that could be used in a context of a multi-feedstock and multi-product biorefinery encompassing all residues generated around the olive oil production sector. OS biomass is pretreated in a twin-screw extruder at varying temperatures—100, 125 and 150 °C and NaOH/biomass ratios of 5% and 15% (dry weight basis), in order to estimate the effectiveness of the process to favour the release of sugars by enzymatic hydrolysis. The results show that alkaline extrusion is effective in increasing the sugar release from OS biomass compared to the raw material, being necessary to apply conditions of 15% NaOH/biomass ratio and 125 °C to attain the best carbohydrate conversion rates of 55.5% for cellulose and 57.7% for xylan in relation to the maximum theoretical achievable. Under these optimal conditions, 31.57 g of total sugars are obtained from 100 g of raw OS.

Keywords: lignocellulosic biomass; extrusion; pretreatment; olive oil industry; sub-product; fermentable sugars

1. Introduction

The world faces a series of global challenges (climate change, food and energy security, depletion of natural resources, etc.) that require turning upside down the way we produce and consume as a society. A sustainable and circular bioeconomy, in which renewable biological resources replace fossil fuels as precursors for materials, chemicals and energy, could be the solution to these problems [1]. Lignocellulosic biomass has been postulated as one key resource in a bioeconomy scenario due to its abundance, widespread availability and relatively low cost [2].

Lignocellulosic biomass can either come from woody or herbaceous energy crops or be generated as a residue in agriculture, forest and industrial sectors. Particularly, the agro-industrial sector produces a series of by-products and wastes that are considered as potential feedstocks for the production of different bio-based products, hence reinforcing the development of a sustainable bioeconomy. This approach has significant advantages, since these biomass wastes are usually abundant and cheap, have a negative environmental impact and they would otherwise require a disposal system that can this way be avoided. Their revalorization through the conversion to bio-products can solve some of

the problems associated to the handling of biomass wastes, while contributing to improve the social and economic development of agricultural areas.

Among the main agro-industries of the Mediterranean countries, the olive oil production sector stands out in Spain. In fact, it is the first country worldwide in terms of production and exportation. In Spain, more than 3 million tons of olives are processed every year for virgin olive oil production, in close to 1800 operating olive mills [3]. In addition to the main product, this industry generates a series of lignocellulosic by-products that constitute an abundant biomass source. These biomasses hold a high potential for the production of bio-based products, considering their widespread availability and production in Spain. The main residues and by-products produced in this industry are olive tree pruning (OTP), derived from the pruning operation in olive tree crop; extracted dry olive pomace (EOP), produced in olive oil pomace extraction industries; and olive leaves (OL) and stones (OS), produced at different steps of the olive oil extraction process in olive mills [4]. Several studies have already demonstrated the considerable potential of using OTP as a feedstock in a multi-product biorefinery, with focus on bioethanol as the main product among other valuable compounds such as xylitol and antioxidants [5–7]. Furthermore, EOP and OL have also been evaluated as valuable sources of antioxidants, biofuels and bioenergy in a process strategy that would be applied as a multi-feedstock biorefinery built around the olive oil sector [8,9]. One of the main advantages of using these materials within this concept of advanced biorefinery is that they are all generated in a very specific area, which may facilitate the transport and logistic operations by providing a constant and sufficient feedstock supply according to its availability in a given moment [10].

OS are separated from the olive fruit either in the olive mill, the olive-oil pomace industry or in both, depending on the local process operations. According to Ruiz et al. [11], OS mean proportion of the whole olive weight varies between 8% and 15%. If the production of olives in Spain of 6,496,972 tons in 2019, provided by the Ministry of Agriculture [12] is taken into account, an estimated OS production ranging from 520,000 to 975,000 tons could be estimated for that year. According to the Spanish Biomass Association AVEBIOM (<https://www.avebiom.org/>), around 77% of the olive stone produced is commercialized and the remaining portion is available to be used as solid fuel in olive mills and related industries due to its elevated higher heating value, close to 4000 kcal/kg [13]. However, this is not a fixed scenario and variations can be encountered depending on the energy market conditions and environmental requirements for OS use as solid fuel. This changing situation may result in a surplus stock with serious challenges for their commercialization, which motivates the search of a new OS management alternative. The lignocellulosic nature of this biomass material makes it a promising candidate for sugar production based on its composition of up to 50% of structural carbohydrates, cellulose and hemicellulose, on a dry weight basis (dwb). Sugars could be subsequently transformed by different biological conversion routes into a series of high-added value compounds such as ethanol, xylitol, lactic acid and so forth, in an integrated process within a multi-feedstock (along with OTP, OL and EOP biomasses) and multi-product biorefinery [11].

The conversion of lignocellulosic biomass into bioenergy and bio-based chemicals and materials in biorefineries holds great potential. However, in order to be economically viable, lignocellulosic biorefineries need to make a complete use of all biomass components. Lignocellulosic biomass is mainly composed of cellulose, hemicellulose and lignin, along with minor amounts of other compounds. Cellulose is the major constituent of lignocellulosic biomass and it is composed of cellobiose chains tightly arranged in crystalline and amorphous regions. Hemicellulose is the second most abundant polymer, with a heterogeneous composition and structure that interlinks with the cellulose fibres. Lignin is a complex polymer composed of phenylpropanoid units which acts as glue between the cellulose and hemicellulose chains and confers strength and hydrophobicity to the fibres. Together, the three components form a compact structure very recalcitrant to degradation. The relative amount of each of these components and their exact composition varies depending on the type of biomass.

In order to fully break down this highly packed structure and make a complete use of all components in the lignocellulosic biomass, an adequate selection of the pretreatment and primary refining steps

has to be done, depending on the type and quality of the feedstock used [14]. In the particular case of the residues generated around the olive oil industry, several hydrothermal and chemical pretreatments such as steam explosion, liquid hot water or dilute acid have been tested as fractionation methods, with different results depending on the pretreatment type and conditions [8,15,16]. These types of pretreatment generate various liquid streams containing easily solubilized sugars and other soluble compounds. The recovery of these compounds is essential to improve the efficiency of the overall process, an aspect of utmost importance to enhance the economic viability of the biorefinery. However, hydrothermal and acid pretreatments have the disadvantage of requiring high temperatures (190–220 °C) in order to achieve an effective disruption of the fibres. The harsh conditions used in these processes can lead to degradation of sugars and other soluble compounds, resulting in several inhibition products, such as 5-hydroxymethylfurfural (HMF) or furfural, which may affect the subsequent biological steps. As an alternative pretreatment method, twin-screw extrusion is revealed as a technology that can effectively break down lignocellulosic biomass into its components at mild temperatures and low chemical loadings, avoiding the production of inhibitory by-products [17].

Extrusion has been described as a versatile pretreatment due to its capacity to adapt to different process configurations and feedstocks [18–20]. Extrusion alone is a mechanical process that provides high shear, fast heat transfer and thorough mixing [20] and the possibility to combine it with other pretreatments adds interest to the process application [21,22]. Chemical reagents and biocatalysts can also be added during extrusion [17,23–25], resulting in a mixture of physical, thermal, chemical and/or biological effects that enhance the disruption of the lignocellulosic matrix. Among the different process possibilities, extrusion combined with an alkaline chemical agent (most notably NaOH) has been proven to be successful operating at moderate temperatures and pressures and causing no or very low sugar degradation compared to other chemical pretreatments [19,26]. This high adaptability confers extrusion an enormous potential for pretreating a variety of biomasses. Although most of the scientific literature related to the extrusion of lignocellulosic biomass is focused on herbaceous materials, which are easier to pretreat, there are some studies on woody biomasses or other complex residues [27,28]. In the context of the olive oil industry by-products, Negro et al. [28] studied the alkaline extrusion of OTP and optimized some key process variables such as temperature, screw speed and soda to dry matter ratio, demonstrating the feasibility of the technology to fractionate OTP biomass and obtain high sugars recovery values.

The aim of this work is to focus on another by-product of the olive oil industry, olive stone (OS) and its potential to produce sugars by means of alkaline extrusion pretreatment. To the best of the authors' knowledge, there is no previous evidence on alkaline extrusion applied to OS biomass. The effect of temperature and NaOH/OS dry matter in extrusion was evaluated by measuring the recovery of main components and the enzymatic digestibility of pretreated OS. Regarding the different process strategies that can be followed in extrusion technology, a previous paper by the authors [29] described an integrated process consisting in alkaline extrusion followed by neutralization inside the extruder. This way, the pretreated substrate is ready to be incubated with enzymes and the incorporation of an external washing step is not required. This is the basis for the first approach tested in this work to pretreat OS. Additionally, some other operational modifications were needed to adapt this biomass to the extrusion technology.

2. Materials and Methods

2.1. Biomass

Olive stones (OS) were purchased from a company located in Jaén (Andalusia, Spain). A bulk sample with particle size between 3.35 mm and 1 mm and 8% moisture, was used throughout the work. OS biomass was kept as received until use in the laboratory experiments.

2.2. Alkaline Extrusion

OS biomass was extruded in a co-rotating twin-screw extruder Evolum[®]25 A110 (Cletral, France). The extrusion machine has six modules of 100 mm length each, equipped with temperature control. Biomass was fed to the extruder with a volumetric feeder KMV KT20 (Ktron AG, Niederlenz, Switzerland).

The basic configuration for the extrusion of OS material was adapted from Duque et al. [29] and is represented in Figure 1. The extrusion strategy consisted in a reactive extrusion using a solution of NaOH as alkaline agent and a subsequent step of neutralization of the alkali-treated biomass by pumping a solution of H₂SO₄. The pretreated solid material (onwards referred to as extrudate) had a pH at the exit of the machine of around 5, which means that the extrudate could be incubated with the cellulolytic enzymes with no need of post processing operations.

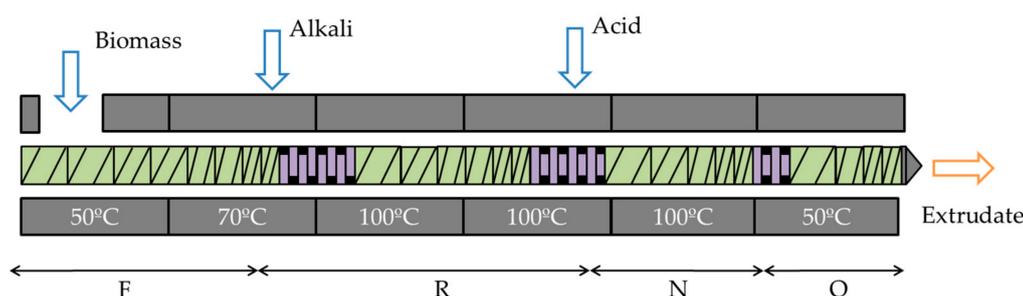


Figure 1. Functional zones, screw profile, temperature profile and material inputs and outputs used for the extrusion of olive stone. Green—transport screws; Purple—mixing screws. F: feeding zone; R: reaction zone; N: neutralization zone; O: output zone.

The extruder is divided in four functional zones—feeding (F), reaction (R), neutralization (N) and output (O). The feeding zone covers from the entrance of the biomass to the point of introduction of the alkali; in this zone, the material is pre-heated to 70 °C and conveyed to the next zone. The reaction zone is the space between the introduction of the alkali and the point where the acid solution is pumped; there the biomass and alkali are thoroughly mixed and heated and reaction takes place. The R zone is closed by a reverse screw that prevents the backflow of acid from the neutralization zone to the reaction zone. Neutralization happens in a short space where the alkaline biomass is mixed with the acid solution, getting optimal pH values for the subsequent enzymatic hydrolysis. On-site neutralization inside the extruder reduces the need for further steps between pretreatment and hydrolysis, optimizing the process in a continuous way. However, the feasibility of this step is determined by the pretreatment conditions and the capability of the extrusion process to increase the liquid-to-solid ratio [27]. Finally, the material is cooled down to 50 °C and exits the machine through the output zone.

The operational strategy comprehended introduction of biomass in barrel #1, addition of alkali solution in barrel #3, heating of the mixture and neutralization by pumping a H₂SO₄ solution at the end of barrel #4. The output of the pretreatment was a mixture of liquid and solid biomass (extrudate) coming out of barrel #6. The alkali loadings (NaOH/DM, dry biomass matter) tested were 5% and 15%, the temperatures being 100 °C for 5% NaOH/DM and 100, 125 and 150 °C for 15% NaOH/DM. Experiments are respectively referred to as 5–100, 15–100, 15–125 and 15–150. In order to avoid acid corrosion and/or liquid-to-solid (L/S) ratios too high, on-site neutralization was only carried out for an alkali loading of 5% using a 5% (*w/v*) H₂SO₄ solution. Extrudates treated at 15% NaOH/DM were neutralized by diluting a sample in acidulated water until optimal pH.

In order to process and characterize the extrudates, samples of 100 g were centrifuged and filtered, obtaining separately a solid and a liquid fraction. The solid fraction was analysed for its composition in carbohydrates, lignin and acetyl groups, according to the characterization methodology described in the following section. The liquid fraction was analysed for its content in sugars and acetic acid to assess the effect the pretreatment has on the solubilization of carbohydrates.

2.3. Material Characterization

OS raw biomass and all extrudates produced were analysed according to the methodology described by Slutier et al. [30]. The biomass was first submitted to a two-step extraction process with water and ethanol, consecutively, for extractives quantification. The amount of main biomass components, that is, cellulose, hemicellulose and lignin, was quantified by submitting the extractives-free biomass sample to a two-step acid hydrolysis with sulphuric acid. Soluble sugars and acetic acid in the liquid hydrolysate were analysed by high performance liquid chromatography (HPLC) as described below. The remaining non-hydrolysed biomass is the acid-insoluble lignin, which was determined by weight.

2.4. Enzymatic Hydrolysis

Enzymatic hydrolysis was carried out in triplicates in Erlenmeyer flasks by adding 15 Filter Paper Units (FPU)/g dry extrudate of commercial cellulolytic cocktail Cellic[®] CTec2 (kindly provided by Novozymes A/S, Bagsværd, Denmark) in a 0.05 M sodium citrate buffer solution of pH 5, along with 5% sodium azide (NaN₃) in order to avoid contamination. The flasks were incubated in an orbital shaker at 5% (*w/w*) solids consistency, 150 rpm and 50 °C for 72 h. At the end of the experiment, samples were withdrawn and analysed for its sugar content as described below.

Efficiency of EH was evaluated by calculating glucan conversion (GC) and xylan conversion (XC) according to Equations (1) and (2) below. These formulas consider sugars liberated in the process and correct the amount of glucose with the value of its content in the enzyme.

$$GC (\%) = \frac{(C_{\text{glu}} - C_{\text{glu,enz}}) \cdot V_{\text{EH}}}{m \cdot \% \text{Cel} \cdot \frac{180}{162}} \cdot 100 \quad (1)$$

$$XC (\%) = \frac{C_{\text{xyl}} \cdot V_{\text{EH}}}{m \cdot \% \text{Xyl} \cdot \frac{150}{132}} \cdot 100, \quad (2)$$

where, C_{glu} and C_{xyl} are the final concentration of glucose and xylose at a certain time; $C_{\text{glu,enz}}$ is the glucose concentration in the enzymatic cocktail; m is the dry olive stone weight; V_{EH} is the volume of the media, and $\% \text{Cel}$ and $\% \text{Xyl}$ are the cellulose and xylan content in the extrudate respectively, each followed by their stoichiometric conversion factors to the monomers.

Moreover, the sugar production was expressed as the amount of glucose, xylose or the sum of both, liberated during the process, divided by 100 g of dry raw biomass. This calculation takes into account the mass balance of the pretreatment, in terms of extrudate outlet flow/biomass inlet flow ratio.

2.5. Analytical Methods

Monomeric sugars were analysed by high-performance liquid chromatography (HPLC) in a Waters 2695 liquid chromatograph with refractive index detector. A CARBOsep CHO-782 LEAD column (Transgenomic, Omaha, NE, USA) was used, operating at 70 °C with Milli-Q water (Millipore, Burlington, MA, USA) as mobile-phase (0.5 mL/min). Total sugars were also quantified chromatographically after mild acid hydrolysis (3% *v/v* H₂SO₄, 120 °C and 30 min).

Acetic acid was analysed by HPLC in a Waters 2414 liquid chromatograph with refractive index detector. An ionic exclusion column Aminex HPX-87H (Bio-Rad Labs, Hercules, CA, USA) was operated at 65 °C with sulphuric acid 0.05 M as mobile-phase (0.6 mL/min).

2.6. Statistical Analysis

Experimental results were statistically tested using the software Statgraphics XVII Centurion[™] (Statpoint Technologies, Inc., Warrenton, VA, USA).

3. Results and Discussion

3.1. Characterization of OS Biomass

As highlighted in the introduction, the type of biomass used as feedstock greatly affects the performance of the pretreatment. Composition is, therefore, essential information to take into account when designing the pretreatment process. In the present work, the composition of OS biomass was determined as explained in the methodology section. The results are presented in Table 1.

Table 1. Composition in % of dry weight basis (dwb) of raw OS biomass analysed.

Component	Composition (%)
Cellulose	20.8 ± 0.2
Hemicellulose	25.9 ± 0.1
Xylan	23.4 ± 0.1
Galactan	1.2 ± 0.0
Arabinan	1.2 ± 0.0
Mannan	0.1 ± 0.0
Acetyl groups	5.9 ± 0.1
Acid insoluble lignin	33.8 ± 0.6
Acid soluble lignin	1.7 ± 0.1
Ash	0.6 ± 0.0
Extractives	6.4 ± 0.5
Others	5.0

OS is an industrial residue which shows relatively high hemicellulose content (~26% dwb) and low cellulose content (~21% dwb), in comparison to other lignocellulosic biomasses, such as hardwoods or herbaceous type biomass, in which cellulose may constitute up to 1.4 fold the value of hemicelluloses, according to the data provided by Tayyab, et al. [31]. Hemicelluloses are mostly composed by xylan, accounting for up to 23.4% of raw OS and thus showing a high potential for xylose extraction. A significant content in acetyl groups was also found, which indicates highly acetylated xylan structure. Regarding lignin, the value found denoted a relatively high lignified biomass, since a large number of lignocellulosic biomasses show lignin content values below 30% dwb [31,32]. However, other biomasses as hazelnut shell or Japanese cedar also exhibit high lignin contents between 33%–40% dwb [31].

The methodology followed for the compositional analysis of the biomass includes a previous extraction step that removes all the non-structural compounds that could interfere with the analysis and that could be valorised upon extraction. In other olive related-materials such as olive tree pruning, olive leaves or extracted olive oil pomace, extractives content may represent up to 48% of dry weight biomass [3] but in OS biomass, the amount of extractives is substantially lower, comprising only 6.4% dwb. Extractives in the aforementioned materials have been shown to contain significant amounts of antioxidant bioactive compounds [33,34] that make the removal and recovery of this fraction important. The lack of a substantial extractives amount in OS biomass highlights the importance of the carbohydrates in its revalorization process.

3.2. Alkaline Extrusion Pretreatment of OS Biomass

3.2.1. Extrusion Configuration and Performance

The configuration tested in this work for OS extrusion was based on a previous research carried out by the authors using other lignocellulosic materials such as barley straw [17,29]. The work concluded that the addition of a neutralization step after the alkali pretreatment in the extrusion run provided a significant advantage by integrating thermo-mechanic-chemical pretreatment of the biomass and by providing the conditioning operations of the material produced in a single machine. The criterion followed in the neutralization was to reach a pH around 5–6 in the extrudate estimating the amount of acid necessary for neutralization outside the extruder in the same NaOH conditions used in the

alkaline extrusion of OS. This estimation led to a suitable selection of the H_2SO_4 concentration in order to maintain the L/S ratio inside the neutralization zone within reasonable values.

The first experimental trials in OS biomass were performed according to the conditions demonstrated to be effective for barley straw—5% (*w/w*) NaOH/OS dry matter ratio and 100 °C temperature in the reaction zone modules (3 to 5, Figure 1) [29]. In this experiment, neutralization with a solution of 5% (*w/v*) H_2SO_4 was employed, according to the process scheme shown in Figure 1. The outcome of this experiment in terms of efficacy to improve the enzymatic digestibility of OS biomass showed a rather poor result (see Section 3.3) and so, more severe operation conditions were conceived for further experiments. A previous work by the authors performed on eucalyptus biomass (with a similar high lignin content as OS) had indicated the suitability of increasing NaOH/DM ratio over 10% to get significant results [27] and thus, a ratio of 15% NaOH/DM was selected for the following set of experiments. Temperatures over 100 °C, namely 125 and 150 °C, were assayed with the aim of increasing the severity of the pretreatment conditions. However, the use of these high soda (NaOH) loadings made neutralization unfeasible due to the high sulphuric acid concentrations required for maintaining admissible L/S ratios within the extruder, which would cause severe corrosion problems. Consequently, the extrudates produced under these conditions were neutralized outside the extruder as previously explained in Section 2.2.

A summary of the operation conditions for all alkaline extrusion trials of OS (screw speed, temperature, amount of alkali by weight of feedstock and liquid to solid ratio in the reaction zone) is shown in Table 2 below. The alkali flow and the concentration of the NaOH solution were varied accordingly to the ratio alkali/biomass tested.

Table 2. Operation inputs and outputs in the alkaline extrusion of olive stone (OS) biomass.

	5–100	15–100	15–125	15–150
Inputs				
S_s (rpm)	150	120	150	150
T (°C)	100	100	125	150
Q_B (kg/h)	1.3	2.0	1.0	1.0
Q_{NaOH} (kg/h)	0.6	1.8	0.92	0.92
c_{NaOH} (% <i>w/v</i>)	10	15	15	15
R_{NaOH} (% <i>w/w</i>)	5	15	15	15
L/S_1	0.5	1	1	1
$Q_{H_2SO_4}$ (kg/h)	1.5	0	0	0
$c_{H_2SO_4}$ (% <i>w/v</i>)	5	-	-	-
$R_{H_2SO_4}$ (% <i>w/w</i>)	6.2	0	0	0
L/S_2	1.8	1	1	1
Outputs				
SME (Wh/kg)	134.8	67.7	226.8	226.8
Q_E (kg/h)	1.5	2.2	1.3	1.1
DM_E (%)	56.0	59.0	83.1	86.5

S_s : screw speed (rpm); T: temperature of the modules in the reaction zone (°C); Q_B : biomass feed rate (kg/h); Q_{NaOH} , $Q_{H_2SO_4}$: alkali or acid flow rate (kg/h); c_{NaOH} , $c_{H_2SO_4}$: concentration of the alkali or acid solution (% *w/v*); R_{NaOH} , $R_{H_2SO_4}$: ratio of alkali or acid to dry biomass (% *w/w*); L/S_1 : liquid-to-solid ratio in the reaction (1) or neutralization (2) zones; SME: specific mechanical energy (Wh/kg dry biomass inlet); Q_E : extrudate flow rate (kg/h); DM_E : extrudate dry matter content (%).

Screw speed was maintained constant at 150 rpm for all experiments except for 15–100, carried out at 120 rpm. Initially, the experiments with higher soda loading were planned to work at a higher biomass flow rate of 2 kg/h and slightly lower screw speed of 120 rpm. However, unforeseen difficulties in the processing showed up when the temperature was increased from 100 °C to 125 °C due to water loss that led to blockage of the biomass inside the extruder. In order to alleviate this clogging effect, the input biomass flow was lowered to 1 kg/h and the screw speed was increased to 150 rpm. Carrying out the process at a lower biomass flowrate resulted in a higher specific mechanical energy (SME), increasing from roughly 70 Wh per kg of raw OS to 227 Wh/kg. SME is a parameter that estimates the mechanical energy that is required in an extrusion machine to generate a certain weight

of extruded biomass. Therefore, the output of pretreated material in a particular extrusion run highly influences the SME value [18].

The increase of temperature had an important effect on the humidity of the extrudate produced. Working at 100 °C produced a pretreated biomass with less than 60% dry matter (56% for 5–100, 59% for 15–100); however, at higher temperatures, the loss of water inside the extruder was considerably higher and the extrudates produced showed considerably lower moisture contents—18.9% when working at 125 °C and 13.5% when working at 150 °C. This also resulted in an increased SME value, as the decreased water content meant a lower lubrication inside the extruder and thus a higher amount of energy was required for the processing.

3.2.2. Characterization of Extrudates and Liquid Fractions

A deeper insight into the effects of alkaline extrusion in the pretreated biomass was obtained by the analysis of the output products—the extrudate and the liquid fraction obtained during the characterization process. The composition of extrudates regarding main components is presented in Table 3, while the results of liquid fraction characterization are depicted in Table 4.

Table 3. Extrudates composition in % dry weight basis (dwb).

Component	5–100	15–100	15–125	15–150
Cellulose (%dwb)	18.3 ± 2.8	18.3 ± 0.4	18.1 ± 0.3	17.8 ± 0.3
Hemicellulose (%dwb)	22.4 ± 0.4	21.1 ± 0.4	19.5 ± 1.3	22.4 ± 0.6
Xylan (%dwb)	20.8 ± 0.0	19.2 ± 0.3	18.9 ± 0.2	20.6 ± 0.5
Lignin (%dwb)	39.0 ± 0.2	41.9 ± 0.3	34.3 ± 0.2	36.5 ± 0.3
Acetyl groups (%dwb)	3.2 ± 0.05	0.6 ± 0.02	1.9 ± 0.02	2.1 ± 0.1

Table 4. Liquid fraction composition in terms of insoluble solids (IS) per weight of filtrate, sugar content (in both monomeric and oligomeric form) per weight of dry matter inlet (dmi) and acetic acid per dry weight matter inlet.

Component	5–100	15–100	15–125	15–150
IS (%dwb)	n.d. ²	0.50	9.80	11.83
Glucose (%dmi)	0.09	0.03	0.07	0.10
Xylose (%dmi)	0.62	1.35	1.89	1.79
Other ¹ (%dmi)	0.35	0.18	0.40	0.46
Acetic acid (%dmi)	0.20	1.24	3.39	4.50

¹ Includes arabinose, galactose and mannose. ² no data.

Table 3 shows the composition of the extrudates obtained in the different experiments. Cellulose seems to be unaffected by both temperature and soda loading, slightly decreasing with increasing temperature but differences between them are not statistically significant for a 95% confidence level. Results of hemicellulose and xylan content seem to indicate a downward trend as NaOH ratio increases from 5 to 15% and temperature raises at 15% ratio. However, this effect is not so evident when temperature increases from 125 to 150 °C, suggesting that 125 °C would be the threshold value from which no effect on hemicellulose solubilisation is obtained. The increased solubilisation of xylose as temperature raises is consistent with the higher values of xylose recovered in the liquid fraction as the severity increases, as shown in Table 4. The variation of lignin composition does not follow a clear tendency, showing a slightly increased value when increasing the soda loading at 100 °C and then diminishing as the temperature increases to 125 °C and over.

The characterization of the liquid fractions presented in Table 4 includes the determination of the amount of insoluble solids (IS) in the liquid. The percentage of IS corresponds to the portion of insoluble solids or fines in raw material that is released to the soluble fraction of extrudate during the extrusion run. A higher percentage of those fine particles found as temperature increased is a clear evidence of the breaking up effect of the OS biomass structure during extrusion.

Table 4 shows the percentage of sugars that were found in the filtrate related to the sugars determined in the raw biomass. In general, solubilization of sugars was low, with only the values of xylose for 15% NaOH/OS exceeding 1%, meaning that the extrudate virtually contains all the carbohydrates initially present in the biomass. If the pretreatment effective in avoiding any sugar loss, then all sugars would be available in one single stream. The low amounts of solubilized sugars serve as a preliminary confirmation of the effectiveness of alkaline extrusion for OS as a pretreatment that retains most of carbohydrates in the extrudate for further enzymatic hydrolysis step.

Alkaline pretreatment with NaOH is known, among other effects, to hydrolyse the acetyl groups present in the hemicellulose, rendering them more accessible to enzymes [35]. The content of acetic acid found in the filtrates gives an idea of the extent of the deacetylation. Higher acetic acid content in the extrudates is found for the case with lower soda loading, while the opposite trend is seen in the liquid fractions. This suggests a higher deacetylation at higher soda loadings, which is expected to enhance enzymatic digestibility.

3.3. Evaluation of the Enzymatic Digestibility of the Extrudates

The potential of sugar release from the extrudates produced was tested by standard enzymatic hydrolysis tests, as explained in Section 2.4. Results in terms of sugars concentration and conversion of cellulose and xylan out of the theoretical maximum attainable are shown below in Figure 2.

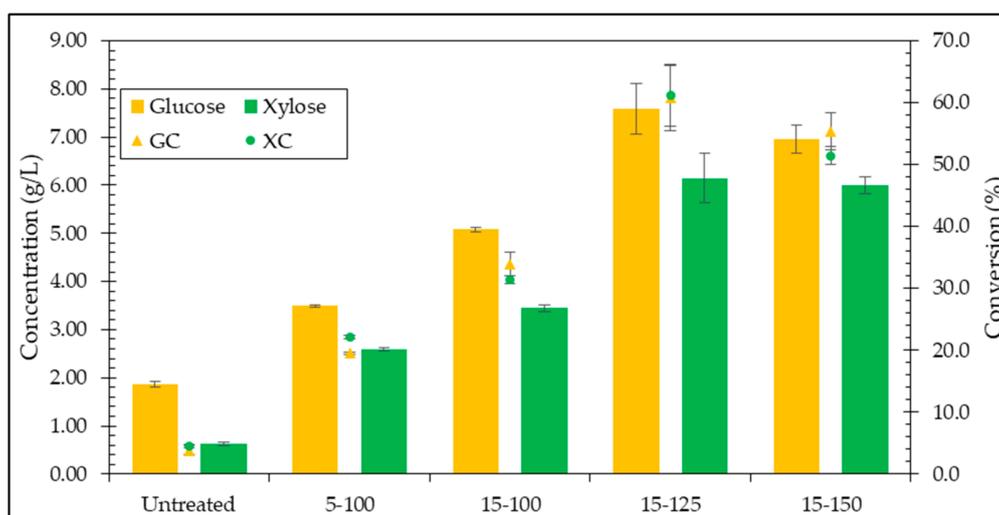


Figure 2. Glucan and xylan conversions (GC, XC) in % of theoretical (spare marks, right) and glucose and xylose concentration in the media in g/L (plain bars, left), after enzymatic hydrolysis of raw OS and OS extrudates.

In all cases, the pretreatment of alkaline extrusion was effective in increasing the sugar release from the lignocellulosic biomass by EH. Extruding OS with a soda loading of 5% NaOH/OS resulted in an increase close to 200% on glucose concentration, more than a 400% increase in xylose concentration, while the conversion of both sugars were increased up to 6 times and 5 times respectively, compared to the untreated biomass. The effect of soda loading was also confirmed—at a constant temperature of 100 °C, increasing the soda loading from 5% to 15% provided a higher sugar production in terms of both concentration and conversion, for glucose (3.50 g/L and 19.5% vs. 5.08 g/L and 33.9%) and xylose (2.58 g/L and 22.1% vs. 3.44 g/L and 31.4%). The differences between the results at 5 and 15% NaOH/OS were statistically significant for a confidence level of 95%. The 15–100 experiment showed a better enzymatic digestibility, proving that the higher soda loading caused the deconstruction of the complex polymer, allowing for a better action of the enzymatic cocktail and thus resulting in increased sugar liberation [36].

The effect of temperature on EH was analysed by statistically comparing the results of the different experiments carried out at a constant soda loading of 15% NaOH/OS. Working at temperatures of 125 °C or above resulted in higher sugars production compared to 100 °C (p -value < 0.05). The best results were obtained for the case of 15–125: 7.58 g/L and 60.8% glucose concentration and conversion yield respectively and 6.15 g/L and 61.2% xylose concentration and conversion. The increase of 25 °C up to 150 °C resulted in a slight decrease of sugar production, that could be caused by the partial degradation of carbohydrates that occur due to the action of alkali at high temperatures [35]. However, the statistical analysis of the results seen in Figure 2 for 15–125 and 15–150 revealed that there was no significant difference between these two experiments. Nevertheless, the ANOVA test confirmed the significance of the improvement in sugars production and conversion of both 15–125 and 15–150 in regard to 15–100.

Considering the extrudate outlet flow/biomass inlet flow ratio, Table 5 shows the amount of sugars produced in g per 100 g of raw OS pretreated for every experiment studied. The results show a similar trend to that obtained from Figure 2—there was a significant increase in sugars production ($p < 0.05$) when increasing the soda loading from 5% to 15% (from 13.51 to 19.61 g/100 g), which was further increased when raising the extrusion temperature to 125 °C or higher. The best production result was indeed obtained for 15–125: 31.57 g/100 g (59% of the carbohydrates present in the raw material), proving the positive effect of temperature in the release of sugars up to 125 °C (the effect of T was significant for a confidence level of 95%). The effect of a further increase beyond 125 °C to 150 °C was not conclusive with the available data (differences not significant for 95% confidence level) and further study is required in this sense.

Table 5. Overall glucose, xylose and total sugars production per 100 g of raw olive stone.

Experiment	Glucose Production (g/100 g Raw OS)	Xylose Production (g/100 g Raw OS)	Total Sugars (g/100 g Raw OS)
5–100	7.76 ± 0.07	5.75 ± 0.12	13.51 ± 0.19
15–100	11.69 ± 0.16	7.92 ± 0.22	19.61 ± 0.38
15–125	17.43 ± 1.07	14.14 ± 1.62	31.57 ± 3.35
15–150	15.97 ± 0.95	13.80 ± 0.55	29.77 ± 1.50

OS has attracted increasing interest over the last years as a potential feedstock in a lignocellulosic biorefinery. Most of the studies carried out focused on different pretreatment methods for an effective breakdown of its structure, being the present work the first developed with alkaline extrusion. Saleh et al. [37] worked with OS pretreated by means of dilute sulphuric acid hydrolysis for the production of xylitol and ethanol, achieving a recovery of 89.7% of the total xylose available and an enzymatic conversion yield of 56% of cellulose to glucose, in line to the glucan conversion result hereby obtained. Cuevas et al. [38] treated olive stones with liquid hot water, dilute sulphuric acid and organosolv processes, achieving high conversion and production yields in certain experiments. Although they obtained high individual production yields in the prehydrolysate (21 kg xylose/100 kg dry OS using dilute sulfuric acid at 200 °C and 11 kg glucose/100 kg dry OS at 230 °C), they were faced with the dilemma of maximizing either the xylose or the glucose recovery in the liquid. The pretreated solid was submitted to simultaneous saccharification and fermentation and a maximum of 9.2 kg/100 kg dry OS was obtained from the pretreated solid resulting from dilute sulfuric acid. A more recent study by the same authors [39] optimized the pretreatment of olive stone by dilute acid at high temperatures ranging from 172 to 228 °C. They achieved a maximum glucose production of 19.8 kg per 100 kg of raw biomass (later fermented yielding 9.7 kg ethanol/100 kg dry OS) along with 20 kg of xylose in the acid prehydrolysate. The combined recovery of glucose from the pretreated solid and xylose in the prehydrolysate was higher than the maximum value obtained in the present study; however, regarding the glucose, it must be taken into account the high enzyme amount used by Cuevas et al. [39] in the enzymatic hydrolysis of the pretreated solid (40 FPU/g solid compared to 15 FPU/g dry extrudate here). Furthermore, the higher xylose recovery can be explained because acid pretreatments are especially good at solubilizing hemicellulose. In contrast, as shown in this work, by using alkaline extrusion all the sugars were obtained in a single stream, which may be advantageous depending on

the final use of the fermentable sugars. Padilla-Rascón et al. [40] carried out a sequential acid-steam explosion pretreatment process for enhancing the production of sugars from olive stone. The best conditions studied resulted in an overall production of 15.9 kg of glucose and 23.1 kg of xylose per 100 kg of raw OS, an overall yield of 83.2% of the total carbohydrates present in the biomass. It is important to note that, although the xylose produced was higher compared to the present work, the amount of glucose recovered by enzymatic hydrolysis after the sequential pretreatment was comparable to that obtained by alkaline extrusion (14.7 kg glucose/100 kg dry OS versus 17.43 kg glucose/100 kg dry OS). This means that alkaline pretreatment is really effective increasing the enzymatic digestibility of OS. Moreover, the work from Padilla-Rascón et al. [40] confirmed that, while a single pretreatment process can already be effective in promoting sugars liberation, this can be further enhanced by applying a sequential process to the biomass. In the present case, the xylose production could be boosted by a sequential treatment to reach the values obtained in the papers previously cited [38–40]. For instance, alkaline extrusion could work as a second step following a preliminary step of steam explosion, a combination that has already been proven successful with other biomasses such as barley straw [21].

4. Conclusions

The pretreatment of olive stone biomass by alkaline extrusion has been proven to significantly improve the accessibility of cellulolytic enzymes to carbohydrates, while at the same time preventing the degradation of sugars during the process. Extrusion causes disruption of the OS structure that can be supported by the notable deacetylation and changes in the particle size occurred, resulting in an enhanced enzymatic digestibility of the biomass as confirmed both in terms of final sugar concentration and conversion in regard to the untreated biomass.

In relation to extrusion process conditions, the results of process performance and pretreatment effectiveness demonstrate that OS biomass is a rather recalcitrant biomass in comparison to other biomasses submitted to such a process technology. Thus, high soda/dry matter ratio (15% dwb) and temperatures of 125 °C must be used to attain glucan and xylan conversion rates around 60% of the maximum theoretical and a maximum sugar production of 31.6 g total sugars/100 g raw OS, representing a total production yield of 59% out of the maximum achievable from the raw material. However, the highest figures attained for sugars conversion suggest that there is still room for improvement. This could be achieved by further optimizing process parameters or by carrying out a combined sequential process combining alkaline extrusion with other pretreatment techniques. Previous works with other biomasses suggest the suitability of combining hydrothermal techniques with extrusion in order to further enhance the enzymatic digestibility, which is yet to be studied for olive stone.

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