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Ethanol production from olive stones using different process strategies

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ABSTRACT

In this work, olive stone is used as a lignocellulosic raw material for ethanol production. In order to optimise the ethanol production yield, three different process strategies are studied considering the different streams produced in a sequential pretreatment of olive stone with dilute sulfuric acid/steam explosion (SE), which has been previously tested and demonstrated to be an effective fractionation strategy for olive stone biomass. Strategy 1 features fermentation with *E. coli* SL100 of the mixture of the detoxified prehydrolysate from the dilute sulfuric acid stage and the enzymatic hydrolysate of WIS and detoxified SE liquid fractions. Strategy 2 consists of fermentation with *E. coli* SL100 separately from the prehydrolysate and the enzymatic hydrolysate of WIS and detoxified SE liquid fractions. Strategy 3 considers fermentation with *E. coli* SL100 of the prehydrolysate from the acid stage and presaccharification and simultaneous saccharification and fermentation with *S. cerevisiae* "Ethanol Red" of WIS and detoxified SE liquid fractions. Strategies 2 and 3 reach a similar ethanol production of ~162 kg/t, which is the highest ethanol yield reported so far from olive stones. The latter strategy uses two different microorganisms that allow an ethanol concentration close to 30 g/L.

1. Introduction

The olive tree is a vital crop in Mediterranean countries. In particular, Spain is the largest olive oil producer worldwide, with ~2.3 Mha cultivated. Olive stone (OS) is an interesting and abundant by-product from this industry, with a production of ~600,000 tons per year in Spain [1]. OS is separated by mechanical means in oil mills, in the extraction industry or both, presenting a logistic advantage due to its centralised location in these facilities. Today, most OS is commercialised for energy applications (~80%) and the rest is used in olive mills and related industries for heat generation [2]. However, because this situation is strongly influenced by the conditions of the environmental requirements and energy market for use of OS as solid fuel, an excess stock of this by-product may occur. Therefore, there is an ongoing search for new sustainable applications of OS in order to promote a sustainable circular economy.

OS is a lignocellulosic biomass primarily composed of cellulose (21%), hemicellulose (26%), mainly xylan, and lignin (36%) [3], in addition to other components, such as proteins and extractives. This complex composition makes it an interest feedstock for obtaining biopower, biofuels and/or high value-added bio-based products in the context of biorefineries [4]. Since OS has a chemical composition rich in carbohydrates, its use as a raw material for the production of bioethanol can be interesting with a suitable bioconversion process [5,6]. The production of biofuel such as bioethanol with lignocellulosic raw materials that do not compete with food is a key element for the sustainability of biofuels, thus avoiding the controversy food vs fuel in relation to the feedstocks use [7]. Besides its use as an alternative fuel to gasoline, bioethanol has excellent potential to be converted into chemical products, having been catalogued as a chemical building block for biorefineries [8].

The bioconversion of lignocellulosic materials into biofuels and other value-added compounds requires a pretreatment to modify

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their complex and recalcitrant lignocellulosic structure, followed by enzymatic hydrolysis and fermentation steps [9]. The aim of the pretreatment is to alter the structure of lignocellosic biomass and to ease the release of sugars from carbohydrates in the subsequent enzymatic hydrolysis. Released sugars will be next converted into fuels or chemicals. In particular, in the case of OS, several pretreatment methods, such as liquid hot water, steam explosion (SE), dilute acid or extrusion, have already been tested [3,5,10,11]. Through hydrothermal/acid pretreatments, a high solubilisation of the hemicellulosic fraction is achieved and a solid rich in cellulose and lignin suitable for enzymatic hydrolysis obtained. However, some inhibiting compounds, which can affect cell growth in the fermentation stage, are also released and produced during the pretreatment stage [12]. The most common are carboxylic acids (acetic and formic acids), furan aldehydes, furfural and 5-hydroxymethylfurfural (HMF) and lignin degradation products (phenolic compounds) [13]. Although it is very common to perform fermentation without a prior detoxification, in some cases it is necessary to treat the acid hydrolysates with detoxification methods in order to reduce the presence of these inhibitory compounds for their use in the fermentation step. Common detoxification methods include over-liming, ion-exchange resins, liquid-liquid extraction, activated charcoal and organic solvents [14].

To optimise the ethanol yield and maximise the use of sugars contained in lignocellulosic pretreated materials, different strategies can be used. Enzymatic hydrolysis and fermentation can be carried out separately in order to perform each process under optimal conditions. As well, these steps can take place simultaneously in a process called presaccharification and simultaneous saccharification and fermentation (PSSF), avoiding the end-product inhibition of enzymes due to the presence of a high sugar concentration [9,15]. Several strategies employing different process configuration have been studied using various raw materials such as olive tree pruning [16,17], olive pomace [18] and barley straw [19]. In addition, due to the high concentration of hemicellulosic sugars (mainly xylose) in this type of biomass, there is significant interest in fermenting these hemicellulosic sugars to increase the ethanol yield, thus improving the economic profitability of the process. This requires the use of microorganisms capable of fermenting pentoses in any process strategy. In nature, there are microorganisms capable of fermenting pentoses to ethanol, such as *Pachysolen tannophilus, Scheffersomyces (Pichia) stipitis* and *Candida shehatae*, although fermentation yields are usually low, they are also more sensitive to inhibitors and high ethanol concentrations and they need microaerophilic conditions [20]. Research has been carried out for the last 30 years to genetically engineer microorganisms capable of co-fermenting both glucose and pentoses from hemicellulose by genetically improving microorganisms that have the ability to ferment both pentoses and hexoses, such as *Escherichia coli*, *Klebsiella oxytoca* [21,22] or genes involved in pentose metabolism can be introduced into hexose-fermenting microorganisms, such as *Saccharomyces cerevisiae* or *Zymomonas mobilis* [21,23,24].

In a previous study, OS was subjected to a sequential fractionation process based on acid pretreatment and SE, followed by enzymatic hydrolysis. The pretreatment conditions were previously optimised for both stages: 128 °C, 10.5 g acid/100 g OS at 33% solids for the first acid stage and 195 °C for 5 min in the case of SE [3]. Following this process strategy, in this work, the liquid fraction obtained after the first stage and liquid and solid fractions after SE are used as substrates for ethanol production.

The objective is to integrate all sugar-containing streams in a bioconversion process of OS biomass to ethanol to maximise the final production yield. To this end, different strategies for enzymatic hydrolysis and fermentation are evaluated. Ethanologenic *E. coli*, one of the most widely used and studied bacteria in this field, is used to co-ferment the sugars (pentoses and hexoses) present. Specifically, strain *E. coli* SL100 is used [25]. Several strategies are also used by combining the use of *E. coli* and *S. cerevisiae* to find the process scheme that results in the highest ethanol yield.

2. Material and methods

2.1. Feedstock

The OS composition in dry weight is 20.9% cellulose, 26.0% hemicelluloses (with xylose accounting for more than 80% of the sugars), 35.6% lignin, 6.3% extractives, 0.6% ash and 5.9% acetyl groups, with a particle size in the interval 1–3.35 mm and a moisture of 8% [3].

2.2. Process strategies

Fig. 1 shows the three strategies to be studied in this work for the production of ethanol from OS. Prior to the fermentation strategies, the OS was subjected to a sequential pretreatment, which consist of an acid pretreatment followed by steam explosion. The first stage consists of acid pretreatment which uses sulfuric acid as catalyst. Through this pretreatment a solid fraction and a liquid fraction (prehydrolysate) are obtained. Afterwards, the solid fraction is subjected an SE pretreatment to obtain two fractions: a liquid fraction (SE liquid) and a water insoluble solid (WIS) fraction. The latter was washed with water (twice its weight) to remove residual acid. The sequential pretreatment conditions were selected according to an optimisation study described in a previous work [3]. These fractions were used as substrates for ethanol production according to the following strategies:

- Strategy 1: the mix of washed WIS and SE liquid detoxified with activated carbon (AC) was used as a substrate for enzymatic hydrolysis. Afterwards, the whole enzymatic hydrolysate (E-hydrolysate) obtained was mixed with the prehydrolysate, which was previously detoxified with AC and fermented with *E. coli*. This strategy seeks to integrate all the streams in a single fermentation. In this strategy, two different tests containing 30 and 40 wt% E-hydrolysate were carried out.
- Strategy 2: the mixture of WIS and SE liquid detoxified with AC was enzymatically hydrolysed. The resulting whole E-hydrolysate and prehydrolysate were then fermented separately with *E. coli* in contrast to strategy 1.
- Strategy 3: the mixture of WIS and SE liquid detoxified with the ion-exchange resin (IER) was subjected to a PSSF with *S. cerevisiae*. In contrast, the prehydrolysate fraction detoxified with AC was fermented using *E. coli*.

All the processes involved in the different strategies are described in the following sections.

2.2.1. Sequential pretreatment

The OS was subjected to a two-step pretreatment, a first step with sulfuric acid in a laboratory autoclave and a second one by SE. The first acid pretreatment was carried out at 128 °C with a solid/liquid ratio of 33% (w/v) and 10.5 g H_2SO_4/100 g OS for 60 min. The resulting slurry after acid pretreatment was filtered to obtain a liquid (prehydrolysate) and a solid fraction. The latter was subjected to a second pretreatment using a SE unit at 195 °C for 5 min. A detailed description of SE pilot plant and procedure used is included in Negro et al. [26]. These pretreatment conditions were selected according to an optimisation study described in a previous work [3]. Completed the second pretreatment step, the SE slurry

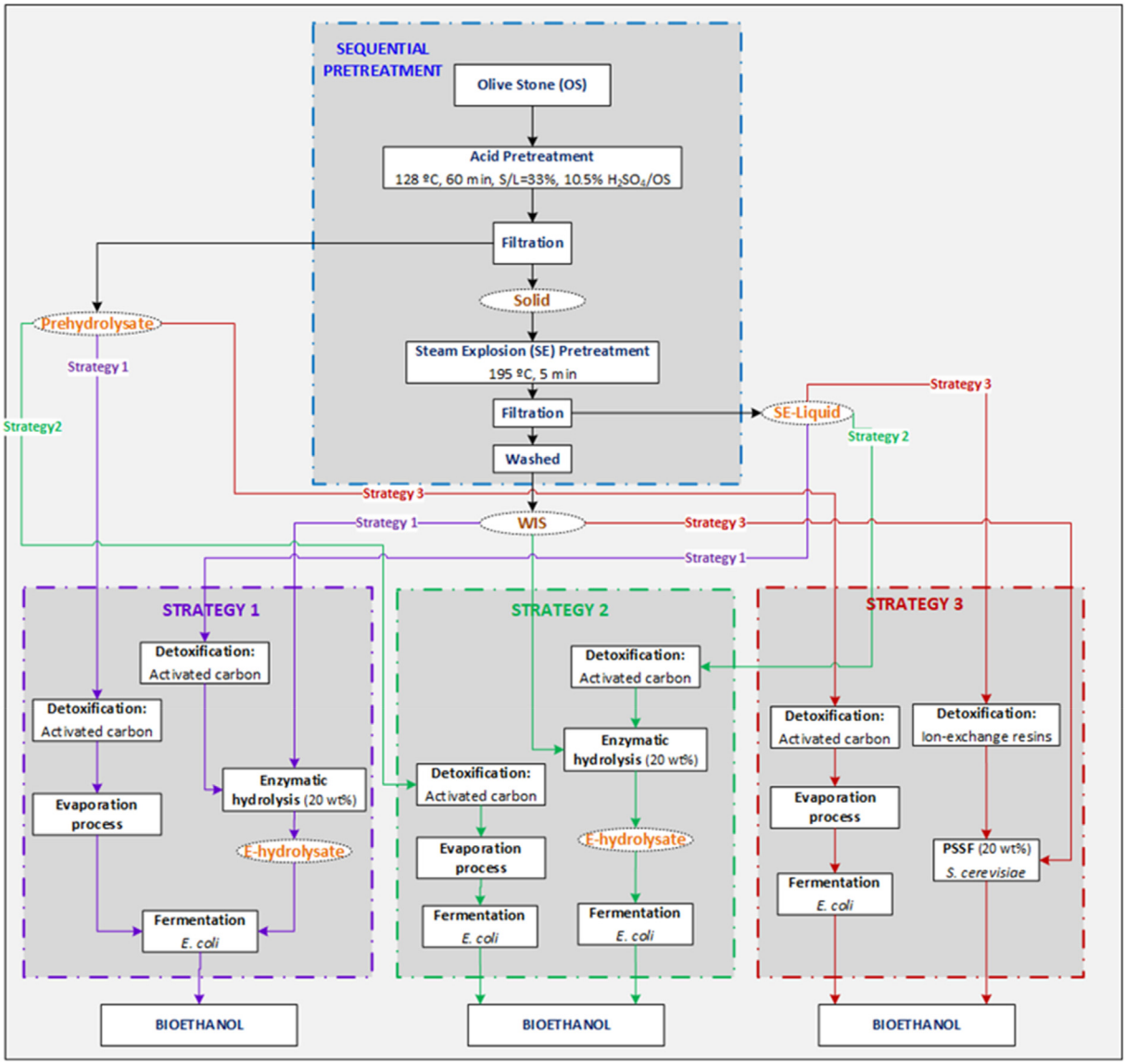

Fig. 1. Bioethanol production strategies from OS.

was vacuum filtered to obtain the solid (WIS) and liquid fractions (SE liquid). Then, the WIS fraction was washed with water to remove residual acid and soluble toxic compounds. The amount of water used was twice the weight of the WIS fraction. The prehydrolysate, SE liquid and WIS were used as substrates in the different strategies previously described.

2.2.2. Prehydrolysate and SE liquid detoxification

In this study, three detoxification methods were used: AC, IER and evaporation. In strategies 1 and 2, the prehydrolysate and SE liquid were detoxified by the AC method, as described by Fernandes-Klajn et al. [9]. In addition, in all strategies the prehydrolysate fraction is also subjected to an evaporation process after AC detoxification. This evaporation was carried out in an ventilated oven at 45 °C after adjusting the pH to 3, reducing the mass around half after 24 h and subsequently adjusting the mass to the initial one with distilled water and the pH to that of fermentation. In strategy 3, the SE liquid was detoxified by the IER, as described by Ref. [6], while AC was used for the prehydrolysate.

2.2.3. Enzymatic hydrolysis (EH)

The experiments were carried out in 100 mL Erlenmeyer flasks with 25 g (11.1 g WIS (55 wt% moisture) and 13.9 g of detoxified liquor) (pH 4.8) to reach 20 wt% WIS load at 50 °C for 72 h. Different enzyme doses (30 and 40 FPU/g of dry substrate) of the commercial enzyme solution Cellic® CTec2 (SAE0020, Sigma-Aldrich, Co) were tested. The FPU activity and protein content of the enzymatic solution are 200 FPU/ml enzyme and 260 mg/mL, respectively After EH completion, the glucose content was analysed by high performance liquid chromatography (HPLC), as described below in Section 2.3.

2.2.4. PSSF

For SSF experiments, 250 mL Erlenmeyer flasks with 100 g of 0.05 M sodium citrate buffer (pH 4.8) at 15 and 20 wt% WIS load were used. A presaccharification step of WIS from SE pretreatment was performed at 50 °C for 4 h at 150 rpm using different enzyme doses (15, 30 and 40 FPU/g of dry substrate) of Cellic® CTec2 (Novozymes A/S, Bagsværd, Denmark). After presaccharification, the temperature was reduced to 35 °C and the flasks were

inoculated with 1 g/L of *S. cerevisiae* ("Ethanol Red", kindly provided by Fermentis, France). Inoculum was grown overnight (at 35 °C and 150 rpm on a rotatory shaker) in a basal medium containing (in g/L): yeast extract (2), NH_4Cl (1), KH_2PO_4 (1), $MgSO_4$–$7H_2O$ (0.3) and glucose (30). The preculture was then centrifuged at 10,000 g for 10 min. The supernatant was discarded and the cells were washed with a saline solution and then diluted to obtain an inoculum level of 1 g/L. Samples were withdrawn after 20, 24, 48 and 72 h and analysed for ethanol and glucose. All tests were carried out in triplicate.

2.2.5. Fermentation with ethanologenic E. coli

Ethanologenic *E. coli* SL100 strain was used to co-ferment pentoses and hexoses [25]. *E. coli* SL100 (kindly provided by Dr. Ingram from the University of Florida, USA) was maintained in 40% glycerol tubes at −80 °C and transferred before inoculation to an AM1 culture medium [27].

The preparation of the inoculum and experimental conditions were described in Ref. [28] and the fermenters used in Ref. [29]. Fermentation salts of the AM1 culture medium, sodium metabisulfite (1.5 mM) to reduce the toxicity [30], and nutrient of Luria-Bertani (10 g/L tryptone, 5 g/L sodium chloride and 5 g/L yeast extract) were added to the fermentation media (prehydrolysate, E-hidrolysate or mix). The fermentation was carried out at pH 6.5, 37 °C, and 350 rpm with a little magnetic stir bar. Samples were taken periodically to determine ethanol production and sugar consumption. Duplicate experiments were performed.

2.3. Analytical methods

Pretreatment liquors and fermentation liquids were analysed by HPLC to determine the content of sugars (glucose, xylose, galactose, arabinose and mannose) and other compounds such as ethanol, acetic acid furfural, HMF and formic acid. The conditions and equipment used were described by Padilla- Rascón et al. [3].

3. Results and discussion

3.1. Prehydrolysate and SE liquid conditioning

Table 1 shows the composition of the prehydrolysate and SE liquid fractions before and after detoxification. As commented in the introduction, the presence of compounds, such as furfural, HMF, acetic acid, formic acid and levulinic acid, can inhibit fermenting microorganisms or greatly reduce their productivity. Therefore their reduction or elimination is a fundamental step in this kind of processes. As can be seen in Table 1, acetic acid and furfural were the main inhibitor compounds found in the prehydrolysate fraction at concentrations of 14.9 and 2.5 g/L, respectively. Considering that xylose is the main sugar present in the prehydrolysate (>90%), it is necessary to use a microorganism with the ability to ferment this sugar. In this work, *E. coli* SL100 was selected, since it presented good yields in similar studies in which prehydrolysates obtained from other olive industry-derived biomasses, such as olive tree pruning [9] or exhausted olive pomace [31], were used. This microorganism also showed good yields (>80%) with other biomasses, such as brewers' spent grain [28], *Eucalyptus benthamii* [32], sweet sorghum bagasse and sugarcane bagasse [25]. These studies highlighted the need to detoxify those prehydrolysates with furfural concentrations higher than 1 g/L in order to avoid inhibition of the microorganism.

Considering the above, AC was selected as the detoxification method for the prehydrolysate, since it is capable of eliminating furfural, as well as not significantly affecting the concentration of sugars [28]. As can be seen in Table 1, the reduction in the concentration of furfural in the prehydrolysate detoxified with AC is close to 90% and the variation in the concentration of sugars is practically negligible. Referring to acetic acid, the reduction was only 5%, leaving its concentration above 14 g/L. This concentration is still very high but in principle it should not be an impediment for the selected microorganism (*E. coli* ethanologenic), since it has a high tolerance to this compound and could also use it as a carbon source [33]. The presence of a high concentration of acetic acid and a low concentration of furan compounds could have a non-negative synergy, not reducing ethanol production according to Martinez et al. [34], working with an E. coli predecessor to the strain used in this work.

Regarding the SE liquid, a high concentration of furfural (close to 2 g/L) was also found. The acetic acid concentration was also high but much less than in the case of the prehydrolysate, not even reaching half (6.8 g/L). This acetic acid concentration, in combination with the high concentration of furfural, can negatively affect both the enzymes responsible for the hydrolysis of cellulose contained in the WIS and the microorganisms responsible for fermenting the sugars produced in EH (*E. coli* SL100) or during PSSF (*S. cerevisiae*, "Ethanol Red").

The SE liquid was detoxified with AC and IER. By detoxifying the SE liquid with AC, it is possible to eliminate 90% of the furfural as in the prehydrolysate and also more than 90% of the HMF (Table 1). Despite the fact that in the SE liquid, the concentration of acetic acid is much lower than in the prehydrolysate, the elimination was about 5% of initial content. However, by using the IER, a much higher removal of acetic acid is achieved, higher than 95%, in addition to totally eliminating furfural and HMF. Acetic acid has been reported as one of the main inhibitors for *S. cerevisiae*. Therefore, when the SE liquid was used for fermentation with that yeast, an IER was employed in the detoxification step, since it has been proven to remove this compound [6]. The detoxification process using the IER achieved the total elimination of toxic compounds without almost affecting the sugar concentrations. Negro et al. [6] observed similar results, with the use of anion exchange resin to detoxify liquid fractions coming from steam exploded olive

Table 1
Composition of prehydrolysate and SE liquid (g/L) before and after detoxification (error <5%).

	Prehydrolysate	Prehydrolysate detoxified with AC	SE-liquid	SE-liquid detoxified with AC	SE-liquid detoxified with IER
	g/L	g/L	g/L	g/L	g/L
Glucose	1.3	1.3	19.2	18.9	18.1
Xylose + Galactose	65.3	65.3	29.9	30.5	29.7
Arabinose	4.5	4.3	1.5	1.6	1.0
Formic acid	0.8	0.9	1.3	1.2	0.1
Acetic acid	14.9	14.2	6.8	6.6	0.3
Levulinic acid	0.0	0.0	0.0	0.0	Nd
HMF	0.1	0.0	0.5	0.1	Nd
Furfural	2.5	0.3	2.0	0.1	Nd

tree pruning resulted in total removal of toxic compounds and only small reduction in sugars was observed. Martínez-Patiño et al. [12] obtained very similar results using AC and IER to detoxify two liquors obtained from olive tree pruning with similar concentrations regarding acetic acid (5.2–11.4 g/L), furfural (1.8–3.5 g/L) and HMF (0.3–0.5 g/L) to prehydrolysate and SE liquid.

3.2. Fermentation strategies

In a bioconversion process of lignocellulosic biomass to ethanol, it is important to reach ethanol concentrations that permit an efficient distillation, with a minimum of 4 wt% having been benchmarked [35]. To reach this ethanol concentration in the bioconversion process, a high solid content over 15 wt% solids in the substrate has to be used, which may imply difficulties in mixing and fluidification of the process media. In this work, three different strategies have been carried out at 20 wt% solids in order to achieve ethanol concentrations suitable for distillation.

3.2.1. Strategy 1

In this strategy the enzymatic hydrolysis of the SE slurry (WIS and SE liquid detoxified with AC) with a high solid load of 20 wt% (20 g WIS + 80 SE liquid detoxified with AC) has been performed. Other authors have carried out the enzymatic hydrolysis of slurries from, for example, brewers' spent grain [28] or rapeseed straw [36], but with lower solid loads (less than 10%) and a subsequent concentration stage of the hydrolysates has been required to achieve high sugar concentrations. Godoy et al. [37] Oliva et al. [17] have found higher concentrations of sugars without the need for a concentration step using solid loads higher than 15% [17,37].

For this first stage, two enzymatic loads have been studied, 30 and 40 FPU/g of dry substrate. These relatively high dosages were used only for comparative purpose since previous assays using lower solid loading (5 wt%) and enzyme doses (data not shown) indicated the convenience of increasing the amount of enzyme to perform tests at high solid loading of 20 wt%

During the first 24 h of hydrolysis, ~45 g/L of glucose are produced, with the difference between the different enzymatic loads being very small. After 72 h of hydrolysis, the increase in glucose concentration did not reach 7% compared to 24 h (data not shown). Considering these results, the lowest enzymatic load of 30 FPU/g of dry substrate and a time of 24 h was selected.

In the second stage of this strategy, *E. coli* was used to ferment the E-hydrolysate together with the AC-detoxified prehydrolysate. The mixture (E-hydrolysate + AC-detoxified prehydrolysate) was made in two proportions of E-hydrolysate, 30 and 40 wt% of E-hydrolysate, to analyse the effect of solids in the E-hydrolysate and the influence of different glucose-xylose ratios on final ethanol production. The total concentration of sugars is above 80 g/L in both cases, with xylose as the major sugar (Fig. 2a and b). In the mixture of 30 wt% E-hydrolysate, xylose concentration was ~55 g/L and glucose concentrations of ~20 g/L, while in the mixture of 40 wt% of E-hydrolysate, these concentrations are similar; 50 g/L and 25 g/L for xylose and glucose, respectively. The mixtures had been supplemented as described in Section 2.2.5 and adjusted to pH 6.5 before being inoculated with the microorganism.

Contrary to what was expected, the microorganism was not able to ferment these mixtures despite the fact that the concentration of furfural is very low (<0.3 g/L). However, the presence of 11 g/L of acetic acid in the medium could affect the metabolism of *E. coli*. Similar behaviour was observed by Martínez-Patiño et al. [12] when fermenting an olive pruning hydrolysate detoxified with CA using *E. coli* MM160 (predecessor of *E. coli* SL100), whose acetic acid concentration was higher than 11 g/L. Since *E. coli* SL100 was used in previous works to successfully ferment an AC detoxified liquor

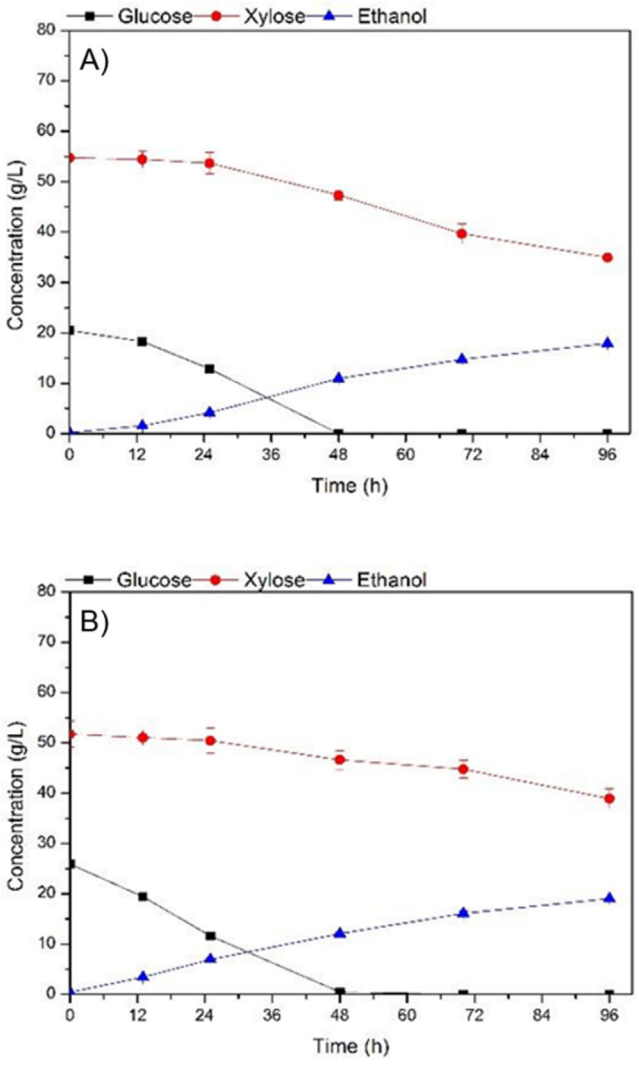

Fig. 2. Fermentation with *E. coli* SL100 of the prehydrolysate detoxified with CA and E-hydrolysate mixtures: a) 30 wt% E-hydrolysate; b) 40 wt% E-hydrolysate.

produced from another olive biomass, exhausted olive pomace, with an acetic acid concentration of 5.4 g/L [31], an evaporation process to reduce the concentration of acetic acid below 5 g/L was carried out. After this step, no sugar losses were detected, only the reduction of the acetic acid concentration was observed. The mixtures were then inoculated with *E. coli* and the microorganism began to consume sugars and produce ethanol.

The results of fermentation with *E. coli* SL100 of the prehydrolysate detoxified with AC and E-hydrolysate mixtures, at 30 wt% E-hydrolysate and 40 wt% E-hydrolysate are shown in Fig. 2a and b, respectively. The evolution of fermentation in both cases was quite similar, with glucose being preferentially consumed until it disappears completely after 48 h and afterwards the consumption of xylose is accelerated. After 96 h, the consumption of xylose in both cases is less than 40% of the initial concentration. As can be seen, ethanol concentration below 20 g/L was obtained in both cases, corresponding to an ethanol yield lower than 50% of the theoretical yield (0.51 g/g). There is a difference in the ethanol yields with respect to consumed sugars, being 74% and 84% with respect to the theoretical for the mixture of 30 and 40 wt%, respectively. This difference is due to the fact that in the 40 wt% case, there was a higher concentration of glucose that is transformed more efficiently

into ethanol and preferably by *E. coli* [38]. Ethanol production yields with respect to consumed sugar of ~80% are similar to those obtained from hydrolysates of brewers' spent grain [28] with the same microorganism, those obtained from rapeseed straw hydrolysates with *Scheffersomyces stipitis* [36] and those achieved from Napier grass with *S. cerevisiae* TISTR 5339 [39].

3.2.2. Strategy 2

In this case, as in Strategy 1, after detoxification an enzymatic hydrolysis stage of SE slurry (WIS and SE liquid detoxified with AC) at a high solid load of 20 wt% WIS (30 FPU/g of dry substrate and 24 h) was carried out. In this strategy, E-hydrolysate and the prehydrolysate were fermented by separate to avoid the poor utilisation of xylose by *E. coli* in the presence of high glucose concentrations, at least in the fermentation of the prehydrolysate.

Fig. 3 shows the results of the fermentation with *E. coli* SL100 of the E-hydrolysate and prehydrolysate fractions. The sugar concentration of the E-hydrolysate fraction was close to 100 g/L, composed mainly of glucose (67 g/L) and xylose (27 g/L). The high initial concentrations of sugars are desirable to obtain a high concentration of ethanol. E-hydrolysate was prepared according to Section 2.2.5 and adjusted to pH 6.5 with KOH before inoculating the microorganism. As can be seen in Fig. 3a, the microorganism began to consume sugars from the beginning. The consumption of glucose is much faster than that of xylose, being totally consumed before 68 h. It can also be seen that the consumption of xylose is faster when the glucose concentration is higher, resulting in that after 48 h where the glucose concentration is lower than that of xylose, xylose practically stops being consumed. After 96 h of fermentation, more than 60% of the initial xylose concentration remained in the medium. A high ethanol concentration of more than 36 g/L is reached at 96 h (0.37 g/Lh), being almost the same at 70 h, which would improve volumetric productivity. This ethanol concentration corresponds to 73% of the theoretical ethanol yield. If only the sugars consumed in the fermentation are considered, this yield increases considerably to ~90% of the theoretical one. The microorganism had a good performance considering the concentration of acetic acid in the medium was close to 5 g/L. In this strategy, evaporation is not necessary to reduce the acetic acid amount since the microorganism is capable of fermenting the E-hydrolysate. Rojas-Chamorro et al. [28] used the same microorganism to ferment a brewers' spent grain hydrolysate (~57 g/l glucose, ~32 g/l xylose, 0 g/l furfural, 1.3 g/l acetic acid) but more than 100 h were needed to reach 36 g/L of ethanol and this hydrolysate was more favourable containing less than 1.5 g/L of acetic acid.

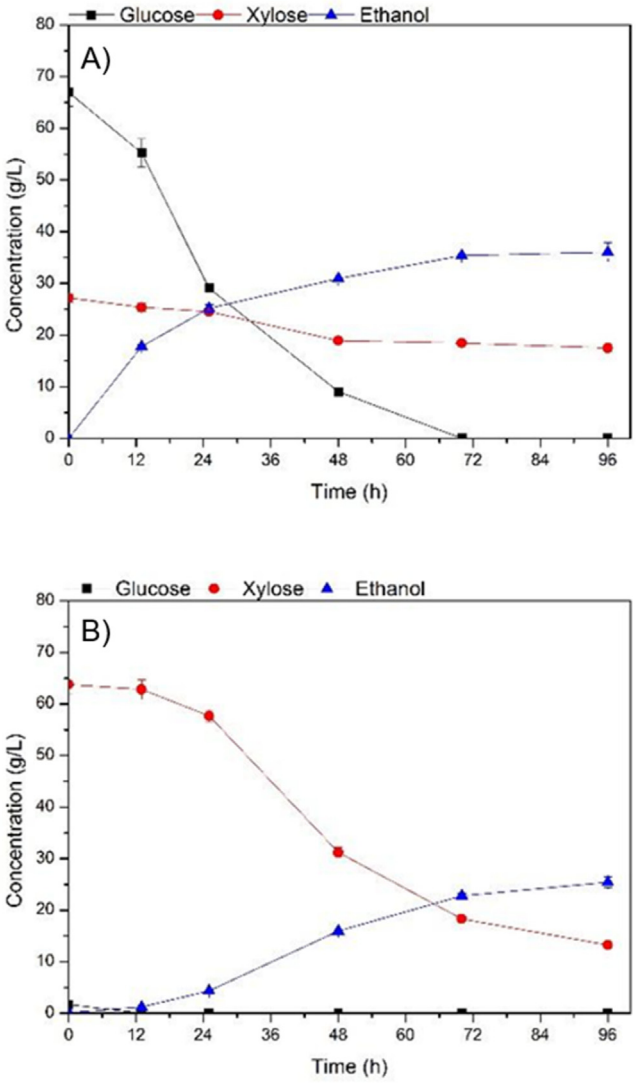

Fig. 3. Fermentation with *E. coli* SL100: a) E-hydrolysate; b) prehydrolysate detoxified with AC.

In contrast, the detoxified prehydrolysate with AC was fermented with the same microorganism (Fig. 3b). As can be seen in Table 1, the concentration of xylose stands out as the major sugar and also the high concentration of acetic acid greater than 14 g/L. With this composition, *E. coli* SL100 was not capable of fermenting the prehydrolysate, as occurred in Strategy 1. *E. coli* MM160 (the predecessor of E. coli SL100) fermented a similar prehydrolysate from OS with a concentration of acetic acid of 20 g/L, although the volumetric productivity was very low [29]. In this work, the prehydrolysate detoxified with AC was evaporated at a low temperature to reduce the concentration of acetic acid, leaving a concentration lower than 1.5 g/L without affecting the rest of the components. Fig. 3b shows how *E. coli* is already capable of fermenting the prehydrolysate, although during the first 12 h it has an adaptation stage. After 96 h of fermentation, an ethanol concentration higher than 25 g/L, was reached, which represents a yield of 72% compared to the theoretical one. Romero-García et al. [29] obtained a similar ethanol concentration and yield using a prehydrolysate of OS (~5 g/l glucose, ~63 g/l xylose, 0.1 g/l furfural, 20.2 g/l acetic acid) and another strain of *E. coli* but needed a much longer time (216 h). Martínez-Patiño et al. [40] required more than 120 h to reach a concentration of 25 g/L (71% yield) using the same strain to ferment a prehydrolysate from olive tree pruning (~16 g/l glucose, ~40 g/l xylose, 0.4 g/l furfural, 7.5 g/l acetic acid).

If the sugars consumed are considered, the ethanol yield is higher than 88% compared to the theoretical one, since at 96 h, ~13 g/L of xylose remained without consuming. From a prehydrolysate of exhausted olive pomace (~5 g/l glucose, ~27 g/l xylose, 0.5 g/l furfural, 11.2 g/l acetic acid) fermented with the same microorganism, a yield of ~90% was obtained, but the ethanol concentration reached was less than 15 g/L [31]. In the case of the prehydrolysate from corn stover, which presents higher sugars content available for fermentation (~24 g/l glucose, ~67 g/l xylose), 38 g/L are reached using *Zymomonas mobilis* 8b, corresponding to a yield of 80% [41].

Higher ethanol concentration was achieved in Strategy 2 compared to strategy 1, which may be related to the better adaptation of the microorganism to the composition of the prehydrolysate and E-hydrolysate separately.

3.2.3. Strategy 3

In this strategy, the prehydrolysate detoxified with AC was fermented with *E. coli*, as previously described in strategy 2. Furthermore, a PSSF process at a 20 wt% WIS fraction using *S. cerevisiae* as fermenting microorganism was carried out, with the objective of maximising both enzymatic hydrolysis and fermentation yields using WIS fraction as a substrate. In a second version of this process scheme, the SE liquid, previously detoxified with IER, is mixed with WIS to undergo the PSSF process, aimed at integrating all fractions from SE pretreatment in a single transformation process. It is proposed as an alternative use of the SE liquid mixed with EH media in previous strategies 1 and 2, which has shown certain limitations. In the PSSF using only WIS, a 4 h presaccharification step at 50 °C was performed in order to reduce viscosity previous to inoculation. Different enzymes loading (15, 30 and 40 FPU/g of substrate) were used, based on previous work carried out in OS biomass [10].

The results of ethanol production by a PSSF process with a 4-h presaccharification step using different enzymes loadings are shown in Fig. 4. Only at the beginning of the fermentation process glucose presence was observed. At this time, sugar concentrations were 35, 50 and 58 g/L for 15, 30 and 40 FPU, respectively (data not shown). As can be seen, a similar fermentation pattern was found in all cases, reaching the highest ethanol concentration at 72 h (21.5, 24.6 and 30 g/L for 15, 30 and 40 FPU/g of substrate, respectively). However, almost 90% final ethanol concentration was achieved at 48 h of fermentation. The PSSF yields were in the range of 44–60% of the theoretical yield, assuming that all the glucose contained in the pretreated material was available for the microorganism. The maximum ethanol concentration was close to 30 g/L.

In previous studies, using OS as a substrate [5,10], SE was used as pretreatment in order to increase the enzymatic accessibility of the pretreated material and obtain fermentable sugars. Ballesteros et al. [10], using SE-pretreated OS in a SSF process, reached 59% of the theoretical yield when using a 10% substrate consistency and a final ethanol concentration of ~18 g/L. In that work, at a 20 wt% substrate loading, a strong inhibition of ethanol fermentation was observed and authors stated this fact was due to the formation on inhibitory compounds during the SE pretreatment. In the present work, the WIS was rinsed with abundant water in order to remove inhibitors. Therefore, washing the WIS resulted in more efficient fermentation and no inhibition when using this relative high substrate concentration was observed. In scaling up the process, the use of water in the washing step should be reduced to the minimum possible. The wash water stream could be integrated into the process and used in the pre-adaptation of microorganisms, *S. cerevisiae* or *E. coli*.

In contrast, as stated above, since the SE liquid contained ~19 g/L of glucose, this fraction could be employed as fermentation broth together with WIS in order to increase the final ethanol concentration. As expected, the presence of inhibitory compounds (Table 1) caused a total inhibition of the microorganism and no ethanol production was observed in a first approach (data not shown). Thus, this fraction was subjected to a detoxification step with IER, as previously described in Section 2.2.2 and the mixture of WIS and SE-liquid detoxified with IER were subjected to a PSSF with *S. cerevisiae* at a 20 wt% substrate consistency using two enzyme loadings (30 and 40 FPU/g of substrate). In this case, a 4-h presaccharification step at 50 °C was also performed. Fig. 5 shows the ethanol production reached through this second version of strategy 3. In this case, at the beginning of fermentation glucose concentrations were 61 and 70 g/L for 30 and 40 FPU, respectively (data not shown).

As can be seen, both versions of the PSSF process followed a similar pattern and maximum ethanol concentration was reached after 48 h from the inoculation. The highest ethanol concentration was ~36 g/L (~65% of theoretical yield) when using a 20 wt% substrate consistency and a 40 FPU enzyme loading. This concentration corresponds to 4.5 %v, close to the concentration required to reach benchmark for an efficient distillation before mentioned. When the amount of enzyme was reduced (30 FPU/g), the ethanol concentration decreased to 30.5 g/L. These results show that the supplementation of WIS with SE liquid after detoxification with IER, resulted in an increase in final ethanol concentration in the range 6–8 g/L when compared to PSSF only with WIS, without decreasing the process yield.

3.3. Strategy comparison

According to the ethanol concentration reached, strategies 2 and 3 were selected as the best schemes. A simplified process diagram and the mass balance for both strategies are shown in Fig. 6. In

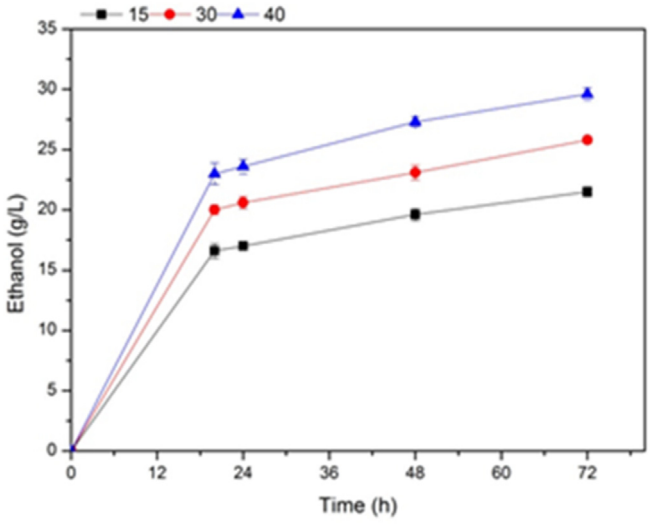

Fig. 4. Ethanol production in a PSSF process at 20 wt% WIS using different enzyme loadings (15, 30 and 40 FPU/g of dry WIS).

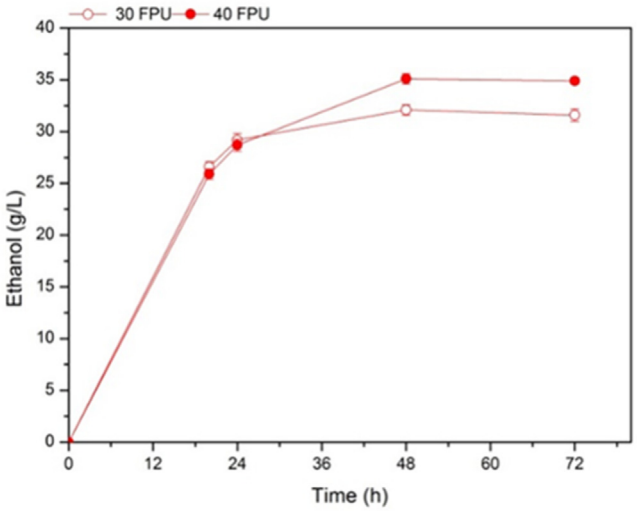

Fig. 5. Ethanol production at 20 wt% WIS consistency supplemented with the SE liquid detoxified with IER.

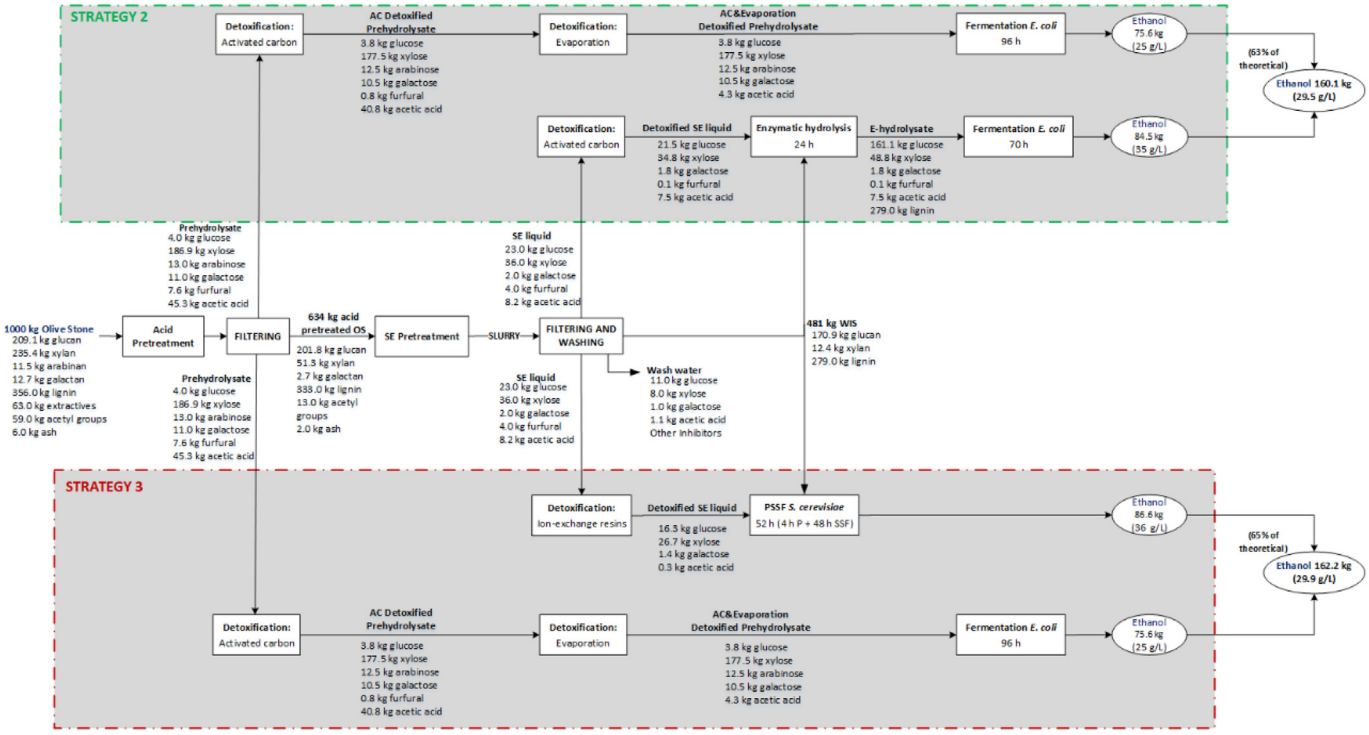

Fig. 6. Simplified diagram and mass balance for ethanol production in strategies 2 and 3.

strategies 2 and 3, the prehydrolysate obtained in the first acid treatment stage is detoxified with AC and subsequently fermented with *E. coli* SL100. After 96 h of fermentation, an ethanol concentration higher than 25 g/L (72% yield compared to the theoretical of dissolved sugars) is reached, which means a production of 75.6 kg of ethanol per ton of OS.

In strategy 2, an enzymatic hydrolysis of the SE slurry (WIS and SE liquid detoxified with AC, 20 wt% WIS, 30 FPU/g of substrate) is carried out for 24 h to obtain an E-hydrolysate with a high concentration of sugars. This enzymatic hydrolysate is fermented with *E. coli* and an ethanol concentration of more than 35 g/L is reached (73% yield with respect to the theoretical of dissolved sugars), after 70 h of fermentation, which corresponds to 85.3 kg of ethanol per ton of OS. In contrast, in strategy 3, a PSSF of SE-slurry (WIS and SE liquid detoxified with IER, 20 wt% WIS, 40 FPU/g of substrate) is performed with a presaccharification time of 4 h. After 48 h of PSSF, an ethanol concentration greater than 36 g/L was reached, representing an amount of 86.8 kg of ethanol/t OS.

Overall, strategy 2 yielded 160.8 kg of ethanol/t OS are obtained, while strategy 3 resulted in 162.3 kg of ethanol/t OS. Both strategies required 96 h to ferment the prehydrolysate. However, the bioconversion of sugars content in streams from SE pretreatment required different times in each strategy. Thus, strategy 2 needed 94 h (24 h of enzymatic hydrolysis and 70 h for the subsequent fermentation of the hydrolysate with *E. coli*), while in Strategy 3 the time was noticeably reduced to 52 h of PSSF (4 h of presaccharification and 48 h of SSF) (Fig. 6).

As can be seen in Fig. 6, both strategies reached an overall ethanol yield of approximately 65%. These results may be due to not only to the relatively low EH yield of OS, but also in the failure of *E. coli* to ferment all xylose present in the fermentation media (Fig. 3) and the sugars losses in detoxification steps mainly related to IER technology (Strategy 3). So, although ethanol concentrations close to those required to reach benchmark for an efficient distillation were achieved, both ethanol concentration and yield should be increased in order to improve the economic viability of the process. In this context, the use of recombinant yeast could be taken into account in a further research.

3.4. Ethanol from olive-derived biomass

In order to compare the ethanol production yields attained in this work with other references in the literature of wastes and by-products generated in the olive oil production sector, Table 2 shows a compilation of some examples of ethanol production from different olive-derived biomass. Olive tree pruning, olive pomace, exhausted olive pomace and OS have been used as raw materials for ethanol production using different pretreatments and different fermenting microorganisms. Among the pretreatments used, examples of one stage processes as dilute sulfuric acid, steam explosion or ethanol-based organosolv, can be found, as well as of two stage-pretreatments, such as alkaline extraction/dilute sulfuric acid, dilute sulfuric acid/alkaline peroxide or dilute sulfuric acid/steam explosion, used in this work. Among the fermenting microorganisms, we mainly find different strains of *S. cerevisiae* (Ethanol Red, F12, ATCC 24860 or IR2-9a) and different strains of ethanologenic *E. coli* (SL100, MM160 or FBR5). In this work, the fermentation strategies applied to OS have implied the utilisation of more than one fermenting microorganism in the same process, as is the case of strategy 3 in which *E. coli* SL100 and *S. cerevisiae* "Ethanol Red" were used.

Regarding the different biomasses, olive tree pruning is the olive-derived biomass most studied for the production of ethanol, since it is the one that is produced in the highest annual quantity and does not present any application for the most part [42]. From this biomass, ethanol productions of around 170 kg/t have been achieved [9]. Olive pomace and exhausted olive pomace have been little studied as raw material for ethanol production and the yields obtained are low, just over 70 kg/t [31].

OS is increasingly gaining interest as a raw material for ethanol

Table 2
Ethanol production from different olive-derived biomasses.

Raw Material	Pretreatment	Hydrolysates detoxification	Microorganism	Cofermentation	Yield (kg ethanol/t dry raw material)	Reference
Olive tree pruning	Alkaline extraction/Dilute sulfuric acid	Yes (Ammonium hydroxide)	*E. coli* SL100	Yes	169.9	[9]
Olive tree pruning	Dilute sulfuric acid/Alkaline peroxide	Yes (Overliming)	*E. coli* MM160/*S. cerevisiae* "Ethanol Red"	Yes	150	[40]
Olive tree pruning	Dilute sulfuric acid	Yes (Overliming)	*E. coli* MM160	Yes	144	[12]
Olive tree pruning	Steam explosion	Yes (IER)	*S. cerevisiae* F12	Yes	142	[17]
Olive pomace	Dilute sulfuric acid	Yes (Overliming)	*E. coli* FBR5	Yes	60	[45]
Olive pomace	Dilute sulfuric acid	Yes (AC)	*S. cervisiae* ATCC 24860	Yes	30	[46]
Exhausted Olive Pomace	Dilute sulfuric acid	Yes (Overliming) Yes (AC)	*E coli* SL100	No (Pentoses)	72.5	[31]
Olive stone	Steam explosion	No	*Kluyveromyces marxianus*	No (Hexoses)	78	[10]
Olive stone	Dilute sulfuric acid	Yes (vacuum distillation)	*P. tannophilus* ATCC 32691	Yes	122	[44]
Olive stone	Ethanol-based organosolv (with sulfuric acid)	No	*S. cerevisiae* IR2-9a	No (Hexoses)	131	[43]
Olive stone	Dilute sulfuric acid/Steam explosion	Yes (AC)	*E coli* SL100	Yes	160.8	This work (Strategy 2)
Olive stone	Dilute sulfuric acid/Steam explosion	Yes (AC/IER)	*E. coli* SL100/*S. cerevisiae* "Ethanol Red"	Yes	162.3	This work (Strategy 3)

production. It has logistic and process advantages compared to other olive-derived biomass, since it is located in industries, has a small size and low humidity. Ballesteros et al. [10], using a SSF configuration of the solid resulting from steam explosion pretreatment, reached an ethanol yield of 78 kg ethanol/t OS just from glucose, with *Kluyveromyces marxianus*. Cuevas et al. [43], using the same configuration, reported an ethanol yield of 131 kg of ethanol/t OS from organosolv pretreated OS using the yeast *S. cerevisiae*. Ethanol production from OS hydrolysates, containing pentoses and hexoses, using *P. tannophilus* was reported, with an overall yield of 122 kg of ethanol/t OS [44]. In this work, the highest ethanol yield from OS reported so far has been obtained, with a value of 162.3 kg/t OS.

4. Conclusions

The process schemes applied to OS biomass in this work result in a successful bioconversion of carbohydrates contained in OS to ethanol, considering all streams generated in a sequential dilute acid/steam explosion pretreatment and an enzymatic hydrolysis step. The results of this study confirm that a previous detoxification step is required for the fermentation with *E. coli* of both xylose-rich liquid streams generated in the sequential pretreatment. The best strategies evaluated in this work (strategies 2 and 3), in terms of ethanol production, involved the fermentation of the detoxified acid liquor separately and a second step of hydrolysis and fermentation of the pretreated solid. The strategy that involves a PSSF with *S. cerevisiae* (strategy 3), in spite of fermenting only C6 sugars, was proved to be more efficient in terms of whole yield (hydrolysis and fermentation), reaching a similar ethanol yield than in strategy 2, using *E. coli* in all process stages. The process scheme that includes a PSSF resulted in the highest ethanol production reported so far from OS (162.3 kg/t), which proves the effectiveness of the process strategies applied in this work to improve the ethanol production yield. Although ethanol concentrations close to benchmark value were achieved, both ethanol concentration and yield should be increased in order to improve the economic viability of the process. The use of recombinant yeast could be an option to reach this goal in further research.

CRediT authorship contribution statement

J.M. Romero-García: Investigation, Conceptualization, Writing – original draft. **A. Susmozas:** Investigation, Writing – original draft. **C. Padilla-Rascón:** Investigation, Writing – original draft. **P. Manzanares:** Funding acquisition, Project administration, Writing – review & editing. **E. Castro:** Project administration. **J.M. Oliva:** Conceptualization, Writing – original draft. **I. Romero:** Funding acquisition, Project administration, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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