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Bioethanol production from extracted olive pomace: dilute acid hydrolysis

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Abstract: Residues from olive oil industry such as Extracted Olive Pomace (EOP) are potential substrates for bioethanol production. In this work, enzymatic hydrolysis of EOP pretreated by dilute acid hydrolysis (DAH) was assessed, and the enzymatic hydrolysis and bioconversion were carried out both by separate hydrolysis and fermentation (SHF) and pre-saccharification followed by simultaneous saccharification and fermentation (PSSF). DAH led to a significant removal hemicellulose, but the subsequent enzymatic treatments showed that the resulting residue was still partially recalcitrant to cellulase hydrolysis. Size reduction and further treatment of EOP-DAH with an alkaline solution were also tested. Alkaline post-treatment allowed a decrease in lignin content, but had little effect on enzymatic saccharification comparing to size reduction. Hence fermentation study was performed with ground EOP-DAH. The PSSF process showed a relatively higher bioethanol fermentation yield (0.46 gg^{1}) when compared to the SHF process.

Keywords: Alkaline treatment; Biomass pretreatment; Enzymatic hydrolysis; High solid loading; Sequential Hydrolysis and Fermentation (SHF), Simultaneous Saccharification and Fermentation (SSF).

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1 Introduction

Sugar sources that cannot be used within the food chain are considered to be the most appropriate feedstocks for bioethanol production [1], since they do not interfere with food supply and prices. Lignocellulosic materials such as agricultural or forestry residues and by-products, together with agro-food industrial residues are typical examples of such sources. All these materials are widespread and are usually cheap, as they do not have many (if any) industrial applications. Agro-food industrial residues have further advantages, as they are available in high quantities in the industrial sites, thus expediting the logistic aspects of their upgrade.

The olive oil industry is such a case, as it annually produces large volumes of residues throughout the Mediterranean basin [2] and other regions (e.g. Chile) where olive trees are grown. Specifically, the agrarian region of Alentejo with 177, 288 ha of olive grove orchards is the major olive oil producing region in Portugal [3,4] yielding about 40, 000 ton of olive oil in 2012 [5]. The majority of virgin olive oil extraction facilities in this region use the state-of-the-art [4] two-phase extraction system. This process yields the wet olive pomace a high moisture by-product composed by olive skin, pulp and stones and aqueous soluble materials, that totalizes over 250,000 ton/year [3,6,7]. This by-product, as well as olive cake and olive pomace derived from the traditional or the three-phase systems (Figure 1), still contain economical relevant amounts of oil, that are further processed to extract olive pomace oil, in chemical extraction facilities. During this process, the olive pomace is first pitted (2 mm sieve) to remove big pieces of olive stones, dried in rotary heat dryers which both the wet pomace and hot combustion gases are introduced at high temperatures (400 to 800°C) [8], cooled-down, pelletized, and only then extracted using a commercial food-grade organic solvents (commonly hexane). Due to this industrial processing, the final residue obtained, here called Extracted Olive Pomace (EOP), is quite different from the lignocellulosic materials

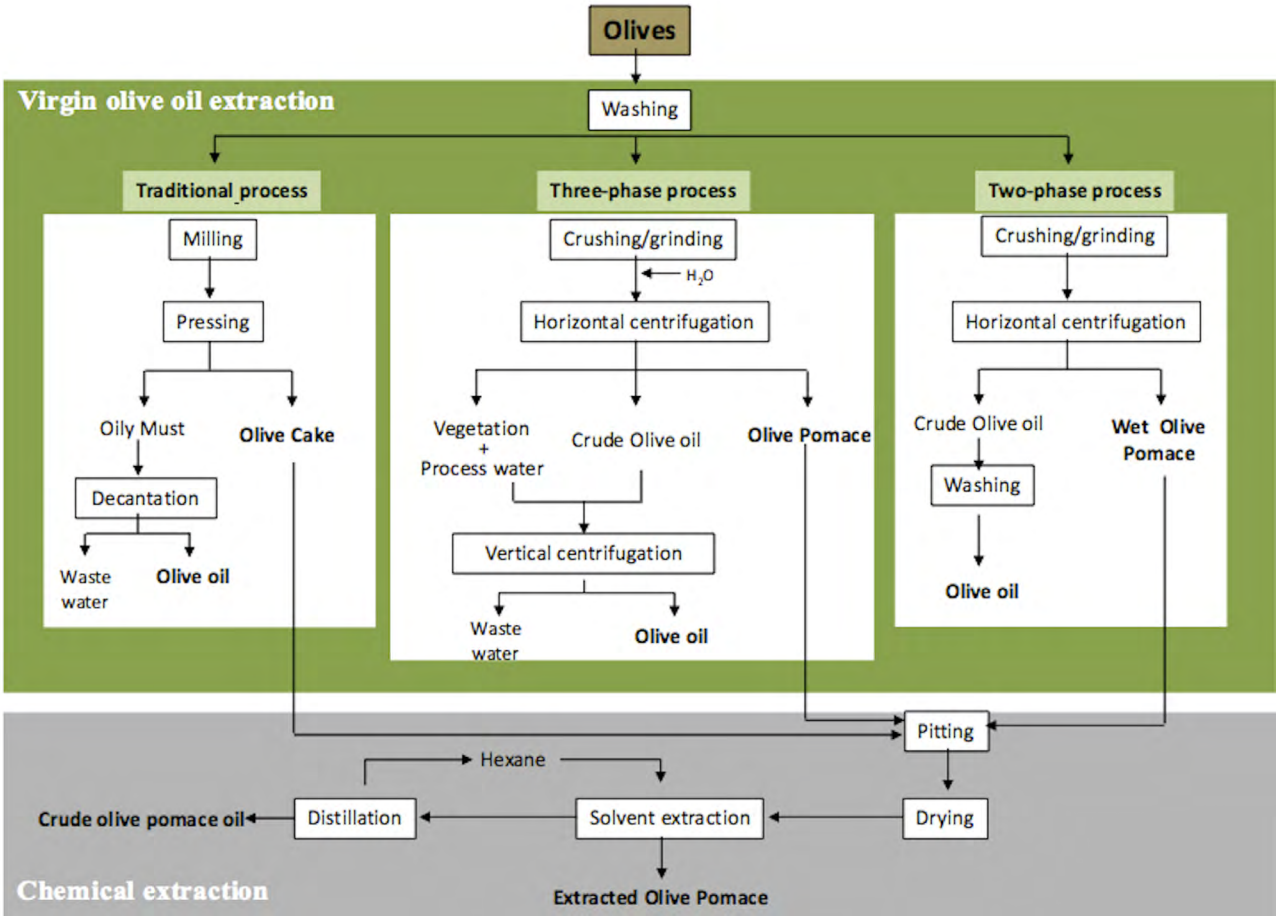

Figure 1: Different methods of olive oil and olive pomace oil extraction and their products and by-products.

usually reported in literature in the same context which include olive cake from the traditional process, olive pomace from the three phase process [9,10], wet olive pomace from two-phase processes [11–14], or olives stones from food olives preparation [11,15], and specially different from olive tree pruning (branches and leaves), which are agricultural residues [16,17].

Currently, EOP is partially used in olive pomace extraction facilities as a low cost energy source (solid biofuel for direct combustion) [8], and the remaining material is sold to other biomass fuel users. Therefore, there is an obvious need of a more value-adding upgrade strategy. To the best of our knowledge, this work evaluates for the first time the use of EOP for bioethanol production.

Ethanol production from lignocellulosic materials is not a trivial task, since structural sugars must be made accessible to fermentation this. In fact, due to the different composition of lignocellulosic materials and recalcitrance to cellulolytic enzymes action [18], the optimization of pre-treatment parameters for each different feedstock is a key issue for increasing sugar conversion efficiency during the lignocellulosic biomass-to-ethanol processes [19]. Among pretreatments currently available, dilute acid hydrolysis (DAH) is still one of the most studied and widely used due to its low operational costs, regardless of its potential drawbacks, such as equipment corrosion issues and the need of a final neutralization step [20] Nevertheless, the main advantages are its high efficiency on the conversion of hemicelluloses into monosaccharides and also its positive impact on cellulose digestibility of the solid residues obtained [21], even though these two traits may not be fully coincident.

This work presents a first approach of bioethanol production from EOP after pretreatment by dilute acid hydrolysis (DAH). The effects of subsequent biomass treatments, namely size reduction and alkali treatment on cellulose enzymatic digestibility were also assessed. In order to improve the process, several fermentation strategies were also tested, namely both separate hydrolysis and fermentation (SHF) and pre-saccharification followed by simultaneous saccharification and fermentation (PSSF).

2 Methods

2.1 Raw Material

Extracted Olive Pomace (EOP) was supplied by UCASUL – União de Cooperativas UCRL (Alvito, Portugal). In order to avoid fine particles and pellets with uneven sizes and heterogeneous composition the material was sieved to obtain the fraction between 3.55 and 1 mm, corresponding to approximately 62% of the raw material. This fraction is mainly composed of extracted olive stone fragments (around 85-90%) and olive pomace (10%).

2.2 Biomass pretreatment

In Figure 2 is depicted a schematic representation of the experimental work, both for the biomass pretreatment and fermentation stages.

2.2.1 Dilute acid hydrolysis

Dilute acid hydrolysis pretreatment was carried out under previously optimized conditions [22]. Briefly, 3.5% (w/w) H_2SO_4, was added to the EOP at a liquid-to-solid ratio (LSR) of 3 (g acid solution/g EOP dry weight basis), at 130°C in an autoclave for 130 min. After the reaction time had elapsed, the autoclave was rapidly cooled down then, the liquid and solid phases were separated by pressing using a hydraulic press (up to 200 bar). The solid phase was thoroughly washed with deionized water until reaching pH 4.8 (hereinafter EOP-DAH) and dried at 40°C or frozen until further analysis or enzymatic hydrolysis, respectively.

2.2.2 Alkaline treatment

Washed EOP-DAH, was treated with sodium hydroxide as previously reported [23]. Briefly, 50 mL of 2% (w/v) aqueous sodium hydroxide solution was added to 10 g

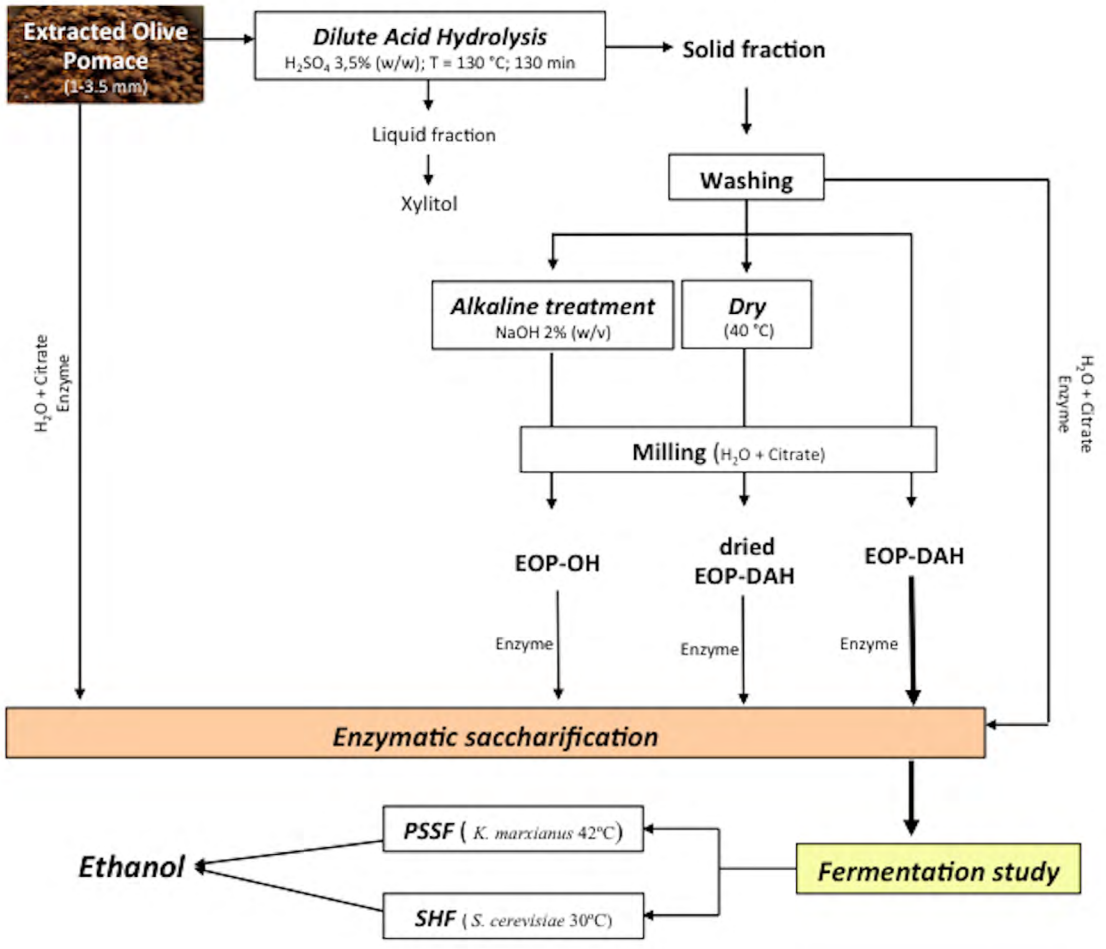

Figure 2: Schematic representation of biomass pretreatment, enzymatic hydrolysis and fermentation process.

(dry weight) of EOP-DAH, and allowed to react for 15 min at room temperature, with occasional agitation with a rod. Then, the obtained residue, (hereinafter EOP-OH), was separated by filtration, thoroughly washed with hot deionized water, until the pH of the washing water reached ca. 4.8. The remaining solid was dried at 40ºC or frozen for later analysis and enzymatic hydrolysis, respectively.

2.3 Enzymatic hydrolysis

EOP and EOP-DAH samples (5% solid loading) were mixed with cellulase (72 FPUmL^{-1}, 15 β-glucosidase activity (CBU) mL^{-1}) 45 FPUg^{-1} dry substrate in a total volume of 10 mL containing 50 mM sodium citrate buffer (pH 4.8) and 1% sodium azide. Assays were carried out at 50°C, at 130 rpm for 72 h and stopped by boiling the reaction mixtures for 5 min. Substrates and enzyme controls were performed simultaneously.

Each material: EOP-DAH, dried EOP-DAH and EOP-OH, was mixed with citrate buffer (5% solid loading) and then grounded with a household hand blender. Ten mL of each mixture were collected and treated with 45 FPU of cellulase per g dry substrate in a closed 50 mL Erlenmeyer. Assays were carried out as described above. All assays were done in duplicate. Saccharification yields are expressed in percentage as the ratio between obtained glucose and potential glucose in the substrate. For grounded material saccharification yield was normalized to the saccharification yield of unground EOP-DAH.

2.4 Fermentations studies

In order to increase glucose concentration for fermentation and consequently final ethanol concentration, fermentation studies were carried out with 15% of solids. Since β-glucosidase activity measured in the Celluclast 1.5L enzyme suspension was very low (11 CBUmL^{-1}), an additional enzyme with β-glucosidase activity (Novozyme 188) was also added to avoid cellobiose accumulation and to improve saccharification.

2.4.1 Separate Hydrolysis and Fermentation

EOP-DAH (15% w/w) was mixed with citrate buffer and then ground with an household hand blender, pH was adjusted to 4.8 and the mixture sterilized in autoclave for 15 min at 121°C. All assays were done with total volume of 100 mL in a closed 250 mL Erlenmeyer flasks, caped with a bubble trap and the fermentation was performed under semi-anaerobic conditions. Filter sterilized Celluclast 1.5L (75 FPUmL^{-1}, 20 FPUg^{-1} dry biomass) and Novozyme 188 (639 CBUmL^{-1}, 14 CBUg^{-1} dry biomass) were aseptically added, and saccharification was conducted at 50°C, 130 rpm for 72 h. Saccharification data were corrected by substrate and enzymes blanks.

An active culture of *Saccharomyces cerevisiae* NCYC 1119, from the United Kingdom National Collection of Yeast Cultures, was obtained by growing the yeast in YPD medium containing 10 gL^{-1} yeast extract, 20 gL^{-1} peptone, and 50 gL^{-1} glucose, at 30°C, 130 rpm for 15 h.

After the saccharification step, sterile peptone and yeast extract were added to each flask, to obtain a final concentration similar to that of YPD medium. A 5 mL *S. cerevisiae* inoculum (0.090 g dry weight) was added and the fermentation was carried out under the same conditions as for inocula preparation. Samples were taken over time, centrifuged at 13000 *g*, 5°C for 10 min and the supernatant filtered with 0.45 μm filters for HPLC analysis of cellobiose, glucose, xylose and ethanol.

Fermentation yield was calculated as the ratio between the obtained ethanol concentration and the maximum glucose concentration in the fermentation medium plus the one formed during the process. All tests were performed, at least, in duplicate.

2.4.2 Pre-saccharification followed by Simultaneous Saccharification and Fermentation

PSSF assays were prepared in the same conditions as for SHF, with a pre-saccharification of 15 h at 42°C, 130 rpm, to assure the presence of glucose in the beginning of the fermentation process. Before fermentation step a sample was taken for sugar analysis, for saccharification control.

A thermotolerant yeast, *Kluyveromyces marxianus* PYCC 3884 obtained from the Portuguese Yeast Culture Collection was used in the SSF experiments. Active cultures of *K. marxianus* were prepared in a similar way as for *S. cerevisiae*, except for the incubation that was done at 42°C. Fermentation was also carried out at 42°C in a similar way as in SHF, with an 5 mL *K. marxianus* inoculum (0.017 g dry weight). All Fermentation assays were done in duplicate.

2.5 Chemical analysis

EOP, EOP-DAH and EOP-OH were ground with a knife mill to a particle size smaller than 0.5 mm and characterized

according to a procedure based on NREL protocols [24]. Briefly, the samples were incubated with 72% (w/w) H_2SO_4 for 1 h with occasional shaking, followed by dilution with water to obtain a final 4% (w/w) H_2SO_4 concentration and hydrolysis at 120°C for 1 h in an autoclave. The acid insoluble residue was considered as Klason lignin, after correction for ash (determined by igniting the contents at 575°C for 5 h).

Monosaccharides, cellobiose, acetic acid and ethanol were analyzed by HPLC (Agilent 1100 series, Waldbronn, Germany) using an Aminex HPX-87H column (BioRad) with a refractive index detector, at 50°C and 0.5 $mLmin^{-1}$ flow of 5 mM H_2SO_4. An AminexHPX-87P column was also used to further refine the analysis of xylose, that is known to co-elute with galactose, mannose and fructose, but as xylose is quantitatively the most significant sugar present that data is presented based only on the HPX-87H data. Concentrations were calculated using a calibration curve with external standard solutions.

3 Results and discussion

3.1 Raw material and treated materials chemical composition

The upgrading potential of a given material in the biorefinery framework is a function of many factors [25], among which the chemical composition plays a major role. Table1 summarizes the chemical characterization of the raw material used in this work (EOP), after dilute acid hydrolysis (EOP-DAH) and after sequential processing by of dilute acid hydrolysis and alkali treatment (EOP-OH).

EOP has a quite similar composition to what is described for olive stones or olive stone fragments in some literature references [11,15], and a lower glucan content and higher lignin content than others [22,26]. These differences may be explained by the natural biological variability of the olives and variations in plant biosynthesis depending on the geografic location and the climatic and soil conditions as well as by the differences in the olive oil /olive pomace oil extraction process.

On the other hand, EOP has a higher content of carbohydrates and Klason lignin than olive pulp/pomace [11–14], which confirms that the residue used in this work has a substantially different composition than the common reported in literature for materials coming from the primary oil extraction [11–14].

Regarding the treated material composition, the dilute acid hydrolysis pretreatment was very selective for hemicellulose (Table 1), whose removal is essential to increase the efficiency of the subsequent enzymatic treatments for the depolymerization of cellulose. Furthermore, the liquid fraction obtained with this treatment is rich in pentoses, as shown before [27]. The solid residue is richer in Klason lignin and in glucan, which is an advantageous outcome for ethanol production. Similar results were found with equivalent material treated by autohydrolysis [15,28]. Nevertheless, EOP-DAH shows a higher content of hemicellulose, than olive pomace after autohydrolysis [12]. When compared to other lignocellulosic materials pretreated with dilute acid hydrolysis, it behaves quite similarly to corn stover [29], cardoon [21] and sugar cane tops [30].

Mild alkaline post-treatment was also applied in order to remove lignin and other alkali-extractable compounds from the residue. As a consequence, EOP-OH showed a slightly lower percentage of Klason lignin than EOP-DAH and a further percentage increase in glucan content, which is an advantage for the purpose of bioethanol production.

Table 1. Chemical composition of raw material (EOP) and of pretreated materials (EOP-DAH and EOP-OH).

	EOP	EOP-DAH	EOP-OH
Solid Yield (%)	-	63.6	89.3
Glucan	22.87 ± 0.19	27.83 ± 1.64 (17.70)[a]	37.68 ± 2.05 (21.40)[a]
Hemicellulose	23.34	5.75 (3.66)	3.99 (2.21)
Xylan	15.60 ± 0.16	5.75 ± 0.20 (3.66)	3.89 ± 0.48 (2.21)
Arabinan	1.05 ± 0.11	nd	nd
Acetyl groups	6.64 ± 0.12	nd	0.10 ±0.02
Klason lignin	33.90 ± 0.63	56.49 ± 0.22 (35.99)	53.60 ± 3.25 (30.44)
Ash	4.35 ± 0.15	ND (-)	ND (-)
Others (by difference)	15.45	9.93	4.58

nd-not detected

ND - not determined

EOP- Extracted olive residue; EOP-DAH- EOP after dilute acid hydrolysis; EOP-OH – EOP after DAH followed by alkaline treatment

[a] - Data are expressed in parentheses as a percentage based on dry weight of EOP

Similar results were obtained, with alkaline extraction postreatment of olive stones [26] , rockrose and cardoon stems [31] [32], all previously treated by steam explosion.

3.2 Enzymatic hydrolysis process

Enzymatic cellulose hydrolysis is still one of the major factors influencing the economics of bioethanol production[33] and thus it requires a special attention.

The untreated EOP presented no saccharification (data not shown) conversely to what is described for extracted olive cake treated with cellulase 15 $FPUg^{-1}$ substrate (6 gL^{-1}) [34] and untreated olive pulp from two phase oil extraction systems where saccharification can reach values in the range of 23 to 45% [11,14,35] . This result is an additional evidence that this material is considerably different from the primary oil extraction pomaces described in the literature. It resembles stone fragments from olive processing reported by Fernández-Bolaños *et al.* [23] that obtained less than 5% of saccharification yield for ball milled material. In our case there was no size reduction of the material and that might explain the absence of saccharification.

Saccharification yields obtained for enzymatic hydrolysis of EOP-DAH (5% solids loading) were 8.9±0.8 and 3.7±0.2% for glucose and xylose, respectively. Although xylan was also saccharified by the enzyme complex used, this result will not be discussed in this work, since the majority of yeasts used for fermentation processes like *S. cerevisiae* only use hexose sugars as substrate. The obtained yield for cellulose conversion was lower than what was previously described for steam exploded stone fragments (between 20-85%) [23], but similar to the reported by Cuevas *et al.* (lower than 10%) with total pomace slurry [15].

In order to further study possible limitations to enzymatic hydrolysis, assays with ground EOP-DAH, EOP-OH and dried EOP-DAH, were carried out as indicated in section 2.3. The normalized saccharification yield to unground EOP-DAH ratios were: 2.51, 2.64 and 0.51 for ground EOP-DAH, EOP-OH and dried EOP-DAH, respectively. Drying the EOP-DAH reduced to half the enzymatic accessibility to cellulose, despite the size reduction. Conversely, for ground EOP-DAH and EOP-OH saccharification yield increased at least 2.5 times when compared to unground EOP-DAH (8.9±0.8%). Size reduction was more effective on increasing enzyme accessibility towards cellulose than the alkali treatment, since the increment from ground EOP-DAH to grounded EOP-OH was only in 0.13 time in comparison to 2.5 times. Enzymatic saccharification obtained was similar to that reported for olive stones (23.3%) [15]. The obtained results where equivalent to studies done by Rodríguez et al [35] with olive pomace treated with steam explosion and posterior alkaline post-treatment. For post treatment in equivalent conditions to ours (5%) no increment on saccharification was observed. Only with 25% of NaOH an increment from 60 to 80% on saccharification yield was observed.

Since EOP-OH preparation implies one more step in the process, and taking in account that practically the same yield was obtained with ground EOP-DAH, fermentation studies were carried out using the latter.

3.3 Fermentation studies

Fermentation profiles obtained for SHF and SSF of EOP-DAH are represented in Fig. 3.

Glucose concentration in the beginning of SHF (8.8 gL^{-1}) was higher than the value obtained during enzymatic hydrolysis study (1.4 gL^{-1}). The saccharification step had a yield of 22.4%, similar to that obtained in the enzymatic assay with the ground EOP-DAH. In fact this assay showed a high sugar content, despite the different enzyme load, addition of β–glucosidase and lower cellulase content, probably due on one hand to the high solid loading in the fermentation assay (15%) when compared to enzymatic hydrolysis assay (5%) and, on the other, to the effect of size reduction of EOP-DAH with the hand blender, as mentioned before. The initial glucose concentrations for the SHF fermentation experiments are similar to data reported in literature for olive pomace [13] and pulp [14] in equivalent liquid-to-solid rate. Glucose concentration remained almost constant until 14 h of fermentation with practically no production of ethanol, probably because during that period the cell culture was in lag phase using energetic resources to adaptation and to grow. The large lag phase observed in the beginning of the fermentation can be partially explained by the relatively high solid loading, although some inhibitory effect of possible solubilized phenols during the saccharification process may also affect the yeast growth. The concentration of cellobiose (Cel) during SHF was not measurable (data not shown), indicating that the enzymatic reaction conditions used allowed a complete conversion of the glucose dimer. After 14h glucose content decreased to values around 4 gL^{-1} with a corresponding increasing in ethanol concentration, that reached the maximum concentration of 3.5 gL^{-1} at 48 h, with a fermentation efficiency of 14.8% and an ethanol productivity of 0.072 $gL^{-1}h^{-1}$ (Table 2). Practically

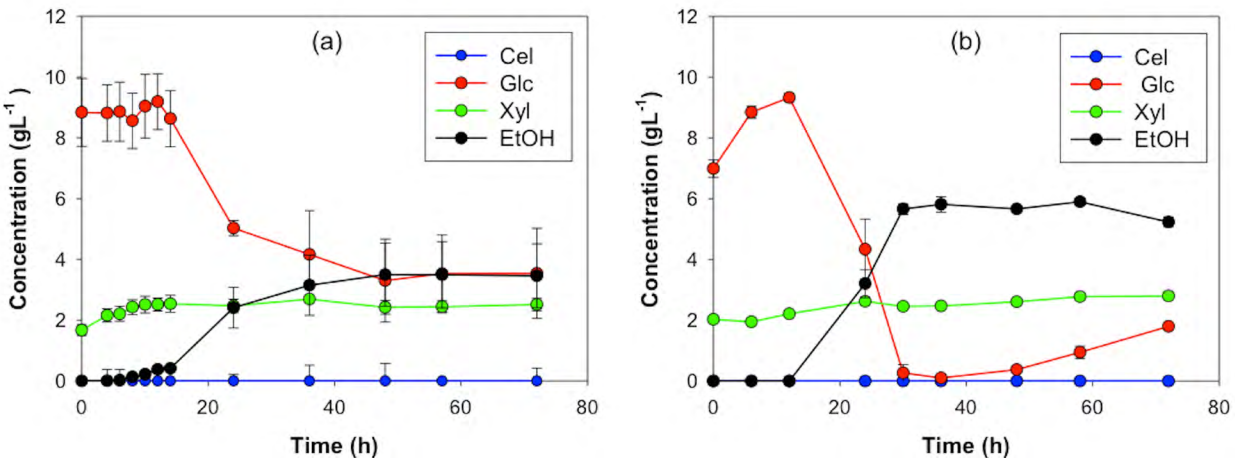

Figure 3: Time course of cellobiose (Cel), glucose (Glc), xylose (Xyl) and ethanol (EtOH) in SHF (a) and PSSF (b) of EOP-DAH, with 15% solid loading, 20 FPU and 14 CBU per g of dry biomass.

Table 2: Kinetic and stoichiometric parameters obtained in SHF and PSSF with 15% solid loading of EOP pretreated with dilute acid hydrolysis (EOP-DAH).

	SHF			PSSF		
	24h	**48h**	**72h**	**24h**	**48h**	**72h**
Fermentation yield (gg^{-1})	0.27 ± 0.04	0.38 ± 0.07	0.36 ± 0.07	0.34 ± 0.05	0.58 ± 0.01	0.46 ±0.01
Ethanol concentration (gL^{-1})	2.4 ± 0.7	3.5 ± 1.0	3.5 ± 1.1	3.2 ± 0.5	5.7 ± 0.1	5.2 ± 0.2
Productivity ($gL^{-1}h^{-1}$)	0.10 ± 0.03	0.07 ± 0.02	0.05 ± 0.01	0.13 ± 0.02	0.12 ± 0.00	0.07± 0.00

after 24 h of fermentation, the increment of ethanol production was very small; also the presence of remaining glucose (3.5 gL^{-1}) might be an indicator of the inhibitory effect of the phenols present in the fermentation media that could prevent the complete use of glucose. Ethanol concentration obtained in this work by SHF was higher than that obtained by Cuevas *et al.* (2 gL^{-1}) from olive stones pretreated by autohydrolysis with similar solids loading [15]. The fermentation yield obtained 0.38±0.07 gg^{-1} (Table 2), was also higher that the value obtained in above-mentioned work (0.23 gg^{-1}).

For PSSF process, the initial glucose concentration (7 gL^{-1}), is similar to other data reported in literature for olive pomace [13] and pulp [14] in equivalent liquid-to-solid ratio. The glucose concentration, obtained during the pre-saccharification time (15h), is very close to the concentration obtained in the SHF (8.8 gL^{-1}), indicating that all accessible cellulose was practically hydrolyzed during this time. Similarly to what was observed in SHF for *S. cerevisiae*, the *K. marxianus* strain needed some time (about 12 h) for adaptation and growth. During this time the maximum glucose concentration 9.3 gL^{-1} was reached, suggesting a higher hydrolysis of EOP-DAH in SSF mode than in SHF mode. Cellobiose (Cel) was not detected during SSF, indicating that the enzymatic reaction conditions used allowed the complete and fast conversion of the glucose dimer. An ethanol concentration of 5.7 gL^{-1} was the maximum concentration obtained after 48 h, which is higher than the value obtained in the SHF process. Also in this experiment it was noticed an accumulation of glucose after 48 h of fermentation. This result may corroborate the hypothesis that some inhibitory compound is released during the saccharification process that could making difficult to yeasts to use all available glucose present in the fermentation medium. When compared to results obtained from by-products of primary olive oil extraction, ethanol concentration was similar to the one obtained with untreated pulp (5.5 gL^{-1}) with same solids loading, that showed a fermentation yield near to 0.43 gg^{-1} against our yield that was 0.46±0.01 gg^{-1} after 72 h, and higher

than that from olive pomace pretreated by wet oxidation which had a negative impact on the fermentation process [14]. Nevertheless these results are still very low when compared with the results obtained with olive pomace pretreated by autohydrolysis [12].

4 Conclusions

DAH pre-treatment of EOP significantly removed hemicellulose. Nevertheless, the subsequent enzymatic treatments showed that the pre-treated biomass still exhibited a significant recalcitrance to cellulase action, when compared to the equivalent material pretreated by autohydrolysis. Size reduction and post-treatment of EOP-DAH with alkali solution were tested for enzymatic saccharification improvement. Alkaline post-treatment allowed a decrease in lignin content, but had little effect on enzymatic accessibility in comparison to size reduction. The PSSF process showed a relative higher fermentation yield (0.46 gg^{-1}) when compared to the SHF process, producing 5.2±0.2 gL^{-1} ethanol. Although the overall ethanol conversion efficiency from cellulose was higher for PSSF in comparison to SHF, they are rather low in both processes clearly indicating that further studies to improve EOP hydrolysis are required and thus increase ethanol production.

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Conflict of interest: Dr. Fernandes has nothing to disclose.

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