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Abstract

Olive tree pruning (OTP) represents an attractive biomass feedstock in the Mediterranean countries and worldwide. In this work, OTP has been studied as raw material for the production of advanced biofuels (i.e. bioethanol) within a biorefinery perspective. After pretreatment by water extraction and phosphoric-acid-catalyzed steam explosion, the whole pretreated slurry was completely inhibitory to the tested *Saccharomyces cerevisiae* strains. Detoxification of the liquid fraction overcame such inhibition allowing complete fermentation of both glucose and xylose by the recombinant xylose-fermenting *S. cerevisiae* F12. When reaching sugar depletion, the fermentation broth was fed with the hydrolysate resulting from enzymatic saccharification of the solid fraction at high solid loadings. This process configuration increased ethanol concentrations up to 45 g/L, reaching 80% of the theoretical conversion yields. Overall, about 180 g of ethanol per kg of extracted OTP biomass could be obtained with this process, which increases previous conversion yields by 12.5%. This strategy also enables the use of the extracted fraction for antioxidant production and offers the potential utilization of the xylose-rich fraction to obtain alternative fermentation-based bioproducts (simultaneously obtaining 125-150 g of ethanol per kg of extracted OTP biomass), thus allowing adaptation of the process to the market needs.

Keywords: Lignocellulosic biomass; olive tree pruning; biorefinery; bioethanol; *Saccharomyces cerevisiae* F12.

38 **Abbreviation list**

39 **OTP**: olive tree pruning

40 **EOTP**: extracted OTP

41 **PEOTP**: steam-exploded EOTP

42 **WIS-PEOTP**: Water Insolube Solids collected from PEOTP

43 **LF-PETOP**: Liquid Fraction collected from PEOTP

44 **DLF-PEOTP**: Detoxified LF-PEOTP

45 **SSF**: Simultaneous Saccharification and Fermentation

46 **PSSF**: Presaccharification and Simultaneous Saccharification and Fermentation

47

48 **1. Introduction**

49 The efficient conversion of biomass feedstocks is of utmost importance for the
50 development and implementation of a bio-based economy. With a global dedicated
51 surface area of 12 Mha in more than 40 countries [1], the olive sector will definitely
52 play a crucial role for the development of such bioeconomy in the Mediterranean
53 countries and worldwide. During olive cultivation, pruning of mature trees is required
54 for regeneration of the fruiting surface. This pruning process produces about 2.7-3.9 t/ha
55 of biomass residues, which are usually left on site and/or uncontrolled burnt, thus
56 causing serious environmental pollution [2]. As an attractive alternative to these
57 conventional practices, the olive tree pruning (OTP) biomass represents an excellent
58 feedstock for the production of biofuels and other value-added compounds within a
59 biorefinery context. The use of OTP biomass as a source of energy and chemicals has
60 been reviewed in detail by Ruiz et al. [3] and Negro et al. [4], listing the main
61 alternatives for biomass pretreatment and highlighting the fermentative microorganisms
62 used for the conversion of this raw material.

63 Ethanol is one of the main products to be obtained from OTP biomass due to the
64 high carbohydrate content of this material [5]. The importance of the production of
65 biofuels such as ethanol based on lignocellulosic biomasses is nowadays reinforced by
66 the objectives of recent European Energy Directive [6], which establishes a dedicated
67 target for advanced biofuels produced from a series of feedstocks among which non-
68 food lignocellulosic materials are included. Nonetheless, due to the lignocellulosic
69 nature of OTP biomass, it has a high recalcitrant structure that limits its
70 biotechnological conversion into ethanol. In order to open up the structure and easy the
71 accessibility of carbohydrates to the hydrolytic enzymes, biomass must be first
72 pretreated. Different pretreatments methods, including liquid hot water, steam
73 explosion, pretreatment with dilute acid, inorganic salts, organosolv, and extrusion have
74 been applied to OTP biomass [2, 7-10]. Among them, acid-catalyzed steam explosion is
75 probably the most commonly applied method for this feedstock. This pretreatment
76 physically breaks the fibers, solubilizes hemicelluloses, and promotes lignin
77 redistribution. The harsh conditions applied during this pretreatment process also leads
78 to biomass degradation, resulting in the formation of several by-products that inhibit the
79 subsequent saccharification and fermentation steps [11]. Another crucial step during
80 pretreatment of OTP biomass is the need of subjecting this feedstock to water extraction
81 prior to steam explosion. Extracted OTP has shown to increase the sugar recovery of
82 steam-pretreated biomass by 20% in comparison to the non-extracted material [7] and
83 offers the simultaneous revalorization of certain extracted compounds such as
84 antioxidants and manitol [5, 12].

85 Sulfuric acid has been the main acid catalyst used for steam explosion
86 pretreatment [13]. Notwithstanding, phosphoric acid has appeared as a promising
87 alternative for this pretreatment process [2]. This is mainly due to the lower

88 corrosiveness power of phosphoric acid, the presence of lower concentrations of
89 inhibitory compounds in the corresponding pretreated materials, and the potential
90 valorization of the resulting distillation streams as biofertilizers.

91 The chemical composition of OTP biomass is mainly cellulose, hemicelluloses,
92 lignin, inorganic components, and extractives [12]. Hence, enzymatic hydrolysis of
93 OTP carbohydrates mainly renders D-glucose from cellulose, and a mixture of hexoses
94 (D-glucose, D-mannose, D-galactose) and pentoses (D-xylose, L-arabinose) from
95 hemicelluloses. The utilization of all these sugars by the fermentative microorganism is
96 crucial for the economy of the biomass-to-ethanol conversion process. *Saccharomyces*
97 *cerevisiae* is the most promising candidate for lignocellulosic bioethanol production due
98 to its effective glucose fermentation, high ethanol tolerance and resistance to
99 lignocellulose-derived inhibitors. Wild type *S. cerevisiae* is however incapable of
100 fermenting xylose. Successful application of metabolic engineering has converted
101 different industrial *S. cerevisiae* strains into xylose-fermenting yeasts via the
102 introduction of the xylose reductase and xylitol dehydrogenase genes among other
103 strategies [14]. Notwithstanding, these recombinant microorganisms usually exhibit
104 difficulties for converting xylose into ethanol in glucose/xylose mixtures, especially
105 during the fermentation of highly inhibitory lignocellulosic hydrolysates [15].

106 In addition to fermenting all sugar components, working at high substrate
107 loadings is required to reach high ethanol titers, since concentrations above 40 g/L are
108 needed to make the subsequent distillation step economically viable [16]. The present
109 work targets at improving the conversion of phosphoric-acid-catalyzed steam-exploded
110 OTP biomass to maximize ethanol production from both glucose and xylose at high
111 substrate loadings. For that, different fermentation strategies were evaluated for the
112 fermentation of pretreated OTP at 15-25% (w/w) substrate loadings, using the

113 recombinant xylose-fermenting *S. cerevisiae* F12. These processes were then compared
114 in terms of final ethanol concentrations, ethanol volumetric productivities, and overall
115 process yields in order to investigate the best fermentation strategy. The results
116 presented herein will contribute for the better understanding of the crucial steps needed
117 to design an optimal biorefinery conversion process for OTP biomass, also providing
118 experimental data for future techno-economic modeling studies.

119 2. Material and Methods

120 *2.1. Raw material and pretreatment*

121 OTP was locally collected after olive harvesting in Jaén, Spain. Subsequently, OTP was
122 air dried to reach a final moisture content of about 7% and then milled with a laboratory
123 hammer mill (SM 100, Retsch, Germany) to obtain a particle size of about 4 mm.
124 Milled biomass was subjected to an aqueous extraction process at 10% (w/v) biomass
125 loading and 120 °C for 60 min [7]. Extracted material (EOTP) was then filtered and the
126 solid residue was further subjected to steam explosion in a 2-L reactor unit. Steam
127 explosion pretreatment was performed according to Negro et al. [2]. Briefly, 300 g (dry
128 basis) of EOTP (previously impregnated with 500 mL of 1% (w/w) phosphoric acid)
129 was subjected to saturated steam at 195 °C (1.4 MPa) for 10 min. After the explosion
130 (sudden depressurization), the pretreated slurry was collected in a cyclone and cooled
131 down to about 40 °C. A portion of the whole pretreated slurry (PEOTP) was stored at 4
132 °C for fermentability tests and the rest was vacuum-filtered through a Büchner funnel
133 for both solid and liquid recovery. The resulting solid (WIS-PEOTP) and liquid (LF-
134 PEOTP) fractions were then analyzed in terms of chemical composition according to
135 NREL analytical methods for biomass [17]. Hence, a small portion of the WIS-PEOTP
136 fraction was characterized in terms of glucans, hemicellulose, lignin, and inorganic
137 components, while a representative sample of the LF-PEOTP was also analyzed to

138 determine oligomeric and monomeric sugar concentrations and the biomass degradation
139 compounds (see section 2.6 for further details).

140 *2.2. Microorganisms and cultivation*

141 The recombinant xylose-fermenting *S. cerevisiae* F12 [18] was used as fermentative
142 microorganism in this study. In addition, the industrial *S. cerevisiae* Ethanol Red
143 (Lesaffre, France) was used as reference strain. Active cultures for inoculation were
144 obtained in 100-mL shake flasks containing 50 mL YPD/YPX media: yeast extract (10
145 g/L), peptone (20 g/L), and glucose/xylose (20 g/L). Cells were incubated on a rotatory
146 shaker at 35 °C and 150 rpm for 16 h. Then, cells were harvested by centrifugation at
147 10,000 *g* for 10 min and washed once with 0.9% saline solution prior to inoculation.

148 *2.3. Fermentability tests*

149 All pretreated fractions obtained after steam explosion pretreatment (slurry, WIS-
150 PEOTP, and LF-PEOTP) were subjected to fermentability tests to determine the
151 inhibitory potential of each fraction. Fermentability tests were performed in 100-mL
152 shake flasks containing 50 g of fermentation medium as follows: 15% (w/w) total solids
153 (TS) for the whole pretreated slurry, 15% and 25% TS (w/w) for WIS-PEOTP, and non-
154 diluted LF-PEOTP. *S. cerevisiae* Ethanol Red (1 g/L) was used as fermentative
155 microorganism for the fermentability tests due to its high robustness to lignocellulose-
156 derived inhibitors [19]. Prior to inoculation, assays were supplemented with the
157 following nutrients independently of the substrate used: yeast extract (2 g/L), NH_4Cl (1
158 g/L), KH_2PO_4 (1 g/L), $MgSO_4{\cdot}7H_2O$ (0.3 g/L), and the pH was adjusted to 5.5 using
159 citrate buffer 50 mM. Fermentation with 15% TS (w/w) of both slurry and WIS-PEOTP
160 were performed under a simultaneous saccharification and fermentation (SSF) strategy,
161 while 25% TS (w/w) of WIS-PEOTP was performed under presaccharification and
162 simultaneous saccharification and fermentation (PSSF) (using the same conditions

163 described in section 2.5 for *S. cerevisiae* F12). After inoculation, flasks were incubated
164 in an orbital shaker at 35 °C and 150 rpm for 120 h.

165 *2.4. Detoxification of the liquid fraction*

166 The LF-PEOTP fraction collected after steam explosion pretreatment was subjected to
167 detoxification due to its high inhibitory potential. Detoxification of LF-PEOTP was
168 performed through a glass filter holder containing 0.3 g/mL LF-PEOTP of Microionex
169 MB 200 ion exchange resin (Rohm Haas, Denmark). The pH of the resulting detoxified
170 liquid fraction (DLF-PEOTP) was then adjusted to 5.5 with 2.5 N sulfuric acid.

171 *2.5. Saccharification and fermentation processes of pretreated biomass*

172 With the aim of maximizing ethanol production, batch and fed-batch strategies were
173 studied to identify the best process configuration for converting pretreated OTP
174 biomass. This processes were performed with *S. cerevisiae* F12 (1 g/L), which is
175 capable of converting both glucose and xylose [18]. The following process strategies
176 were considered (Figure 1):

177 1) Separate batch fermentation: fermentation of DLF-PEOTP and WIS-PEOTP were
178 performed separately in 100-mL shake flasks with 50 mL of non-diluted DLF-PEOTP
179 and 25% TS (w/w) of WIS-PEOTP (both supplemented with the aforementioned
180 nutrients) at 150 rpm, 35 °C and pH 5.5 for 40 and 120 h, respectively. Fermentation of
181 DLF-PEOTP was directly inoculated with 1 g/L of *S. cerevisiae* F12. On the other hand,
182 WIS-PEOTP was subjected to PSSF. Presaccharification step was performed at 50 °C,
183 150 rpm, and pH 5 for 48 h with an enzyme loading of 15 FPU of Cellic CTec2/g of dry
184 WIS-EOTP (Cellic CTec2 enzyme preparation was provided by Novozymes, Denmark).
185 After presaccharification, the temperature was reduced to 35 °C, the pH was adjusted to
186 5.5 using 50 mM citrate buffer, and 1 g/L of *S. cerevisiae* F12 was inoculated.

187 **Note**: SSF processes of both slurry and WIS-PEOTP at 15% TS (w/w) with *S.*

188 *cerevisiae* Ethanol Red were performed by adding simultaneously hydrolytic enzymes

189 and yeast, and using the aforementioned fermentation conditions (150 rpm, 35 °C and

190 pH 5.5 for 120 h).

191 2) Fed-batch fermentation: a fed-batch fermentation strategy was also investigated with

192 the aim of improving xylose-to-ethanol conversion. In this case, DLF-PEOTP

193 (supplemented with nutrients) was first subjected to fermentation with 1 g/L of *S.*

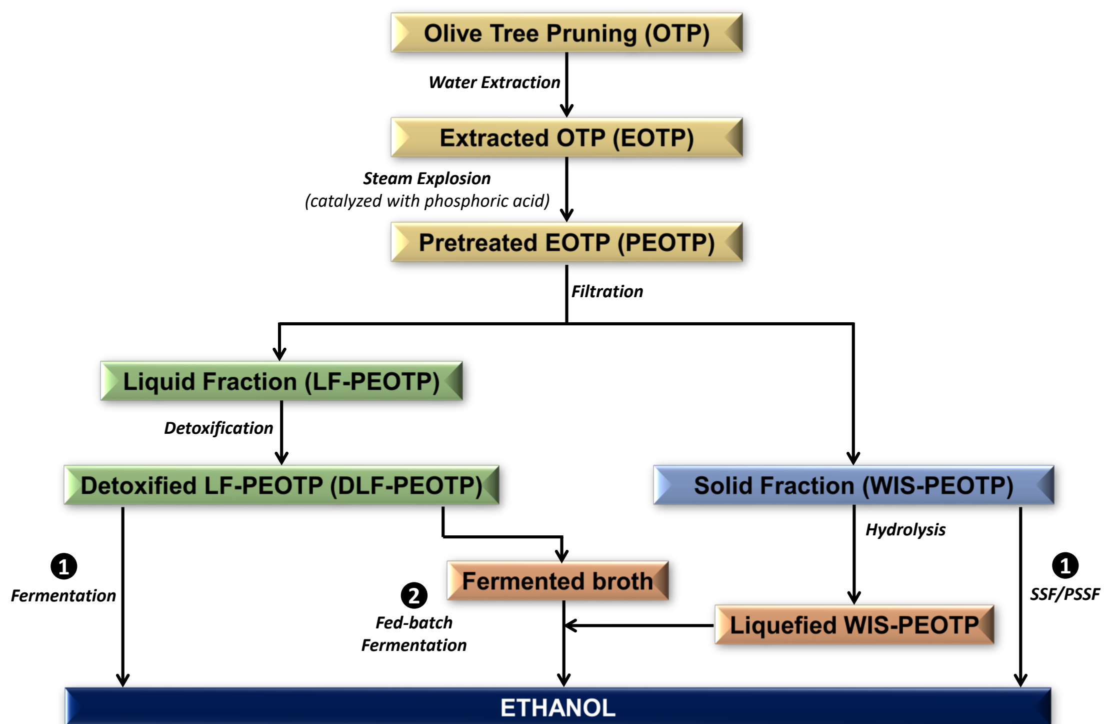
194 *cerevisiae* F12 (35 °C, pH 5.5) for xylose conversion. After 40 h of fermentation, the

195 media was fed with hydrolyzed WIS-EOTP to reach a final substrate concentration

196 equivalent to 15% TS (w/w) (prehydrolysis was performed at 25% (w/w) substrate

197 loadings with 15 FPU Cellic CTec2/g dry of WIS-EOTP at pH 5, 150 rpm, and 50 °C

198 for 48 h). The fermentation was extended for 64 h more at the same conditions.



199

200 **Figure 1.** Overall process scheme investigated in this study for OTP biomass conversion

201

202 *2.6. Analytical Methods*

203 Sugars and degradation compounds were analyzed by a Waters HPLC system (Milford,
204 MA, USA) equipped with a refractive index detector (model 2414). A Transgenomic
205 CARBOSep CHO-782 column (Omaha, NE, USA) was used for quantification of
206 glucose, xylose, galactose, arabinose, mannose, and xylitol using ultrapure water as
207 mobile phase (0.6 mL/min flow rate) and an oven temperature of 70 °C. Acetic acid,
208 formic acid, furfural, hydroxymethylfurfural (5-HMF), and phenols (vanillin and
209 syringaldehyde) were analyzed in a Hewlett-Packard 1100 HPLC system (Palo Alto,
210 CA, USA) equipped with both an Agilent 1040A Photodiode-Array detector
211 (Waldbrown, Germany) and a refractive index detector, using an ICSep ICE-COREGEL
212 87H3 column maintained at 65 °C. A mobile phase of 89% 5 mM H_2SO_4 and 11%
213 acetonitrile at flow rate of 0.7 mL/min was used for quantification of furfural, HMF and
214 phenols, while a mobile phase of 5 mM H_2SO_4 at a flow rate of 0.6 mL/ml was used for
215 quantification of aliphatic acids.

216 **3. Results and Discussion**

217 *3.1. Pretreatment of olive tree pruning biomass*

218 Within a biorefinery perspective, OTP biomass represents an important source for the
219 production of energy and fermentation-based products due to its high carbohydrate
220 content. A typical batch of OTP biomass includes about 50% thin branches, 25% wood,
221 and 25% leaves. Regarding its chemical composition, the OTP biomass used in this
222 work had 31.6 ± 1.2% glucan (22.7 ± 0.7% cellulose and 8.9 ± 0.9% starch), 18.6 ±
223 0.4% hemicelluloses (11.0 ± 0.2% xylan, 3.9 ± 0.1% arabinan, 2.6 ± 0.2% galactan, 1.1
224 ± 0.1% mannose), 23.5 ± 0.6% extractives, 18.6 ± 0.5% lignin, 4.1 ± 0.4% inorganic
225 components, and 2.1 ± 0.1% acetyl groups. After extraction and steam-explosion
226 pretreatment, this biomass composition changed according to Table 1.

227 Similar biomass compositions have been previously observed for OTP biomass
228 pretreated by combining water extraction and phosphoric-acid-catalyzed steam
229 explosion [2]. The main component in EOTP was glucan (31.3%), followed by lignin
230 (26.0%), hemicellulose (20.1%), extractives (9.0%), and inorganic components (4.8%).
231 In contrast, lignin (47.9%) was the main component in WIS-PEOTP, followed by
232 glucan (42.8%), extractives (7.9%), and hemicelluloses (1.4%). Although both EOTP
233 and WIS-PEOTP still showed some extractives in their chemical compositions, the
234 extraction step has been identified essential for the revalorization of OTP biomass in
235 future biorefineries since it improves steam-explosion efficiency [2, 7] and allows
236 revalorization of extractive components with high added-value such as antioxidants [5,
237 20]. On the other hand, hemicellulosic sugars were almost completely solubilized
238 during the pretreatment process, which is indicative of the good process performance
239 during the phosphoric-acid-catalyzed steam explosion. This solubilization of
240 hemicelluloses resulted in the accumulation of sugars (mainly glucose and xylose) and
241 certain degradation compounds (e.g. acetic acid, furfural, phenols) in the recovered LF-
242 PEOTP.

243 **Table 1.** Chemical composition of extracted olive tree pruning (EOTP) and steam-pretreated fractions
244 (WIS-PEOTP and LF-PEOTP)

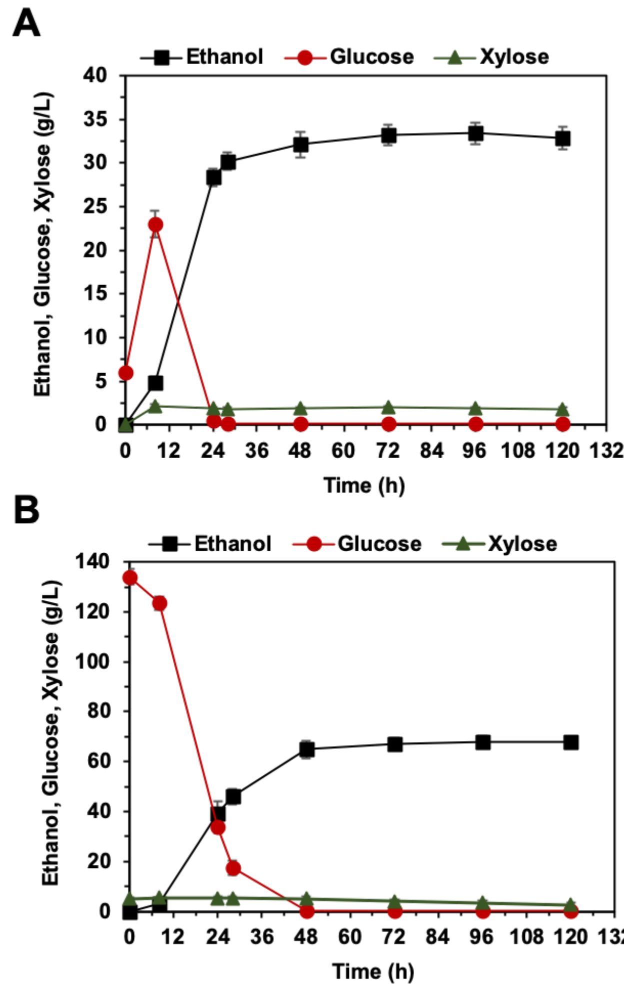
	EOTP (%)	WIS-PEOTP (%)	LF-PEOTP (g/L)			
Component			**Sugars**		**Inhibitors**	
Glucans	31.3 ± 0.8	42.8 ± 0.6	Glucose	7.5 ± 0.2	Furfural	2.3 ± 0.3
Hemicelluloses	20.1 ± 0.3	1.3 ± 0.2	Xylose	15.9 ± 0.3	5-HMF	0.5 ± 0.1
Lignin	26.0 ± 0.4	47.9 ± 0.6	Arabinose	4.2 ± 0.2	Acetic acid	3.9 ± 0.4
Inorganics	4.8 ± 0.2	n.d.	Galactose	4.1 ± 0.3	Formic acid	0.3 ± 0.6
Extractives	9.0 ± 0.8	6.9 ± 0.4	Mannose	0.9 ± 0.1	Vanillin	0.02 ± 0.01
					Syringaldehyde	0.04 ± 0.01

245 n.d., not determined; 5-HMF, 5-hydroxymethylfurfural

246 *3.2. Fermentability of pretreated olive tree pruning*

247 Steam explosion is one of the most widely applied technology for lignocellulose
248 pretreatment [13]. Nevertheless, the severe conditions required to reach high sugar

249 recoveries during the enzymatic hydrolysis of steam-exploded biomass usually results
250 in the generation of high concentrations of degradation compounds that inhibit the
251 fermentative microorganisms and limit the fermentation step. Fermentability tests of
252 steam-exploded OTP was then performed to evaluate the inhibitory potential of this
253 feedstock, using the robust industrial strain *S. cerevisiae* Ethanol Red [19]. The whole
254 PEOTP slurry (14.8-16.7% TS (w/w)) was subjected to SSF fermentation resulting in
255 complete inhibition of the fermentative microorganism (even after nutrient
256 supplementation) and no ethanol production or sugar consumption could be observed.
257 After filtration of pretreated slurry, the resulting LF-PEOTP and WIS-PEOTP
258 were also subjected to fermentation tests. As expected, the non-diluted LF-PEOTP
259 completely inhibited the fermentative microorganism since biomass degradation
260 compounds are mainly collected in this fraction. In contrast, SSF of WIS-PEOTP at
261 15% TS (w/w) substrate loadings resulted in maximum ethanol concentrations of 33.6 ±
262 1.8 g/L, and no glucose was accumulated after 120 h (Figure 2A, Table 2). Due to the
263 lower inhibitory potential of WIS-PEOTP, this fraction was also subjected to PSSF at
264 25% TS (w/w) loadings to reach higher ethanol concentrations, since ethanol titers
265 above 40 g/L are needed for an economic distillation step [16]. Under these conditions,
266 the ethanol concentration increased up to 68.8 ± 0.6 g/L (Figure 2B, Table 2).



267

268 **Figure 2.** Fermentation test of WIS-PEOTP at (**A**) 15% TS (w/w) (under SSF process configuration) and

269 (**B**) 25% TS (w/w) (under PSSF process configuration) with the robust *S. cerevisiae* Ethanol Red

270 strain.

271

272 These ethanol concentrations correspond to final ethanol yields of 0.39 ± 0.03

273 g/g and 0.42 ± 0.01 g/g, respectively, which represent about 80% of the theoretical

274 ethanol that can be obtained during these processes (estimated considering potential

275 glucose only). Furthermore, 90% of these maximum ethanol concentrations were

276 obtained within 30-48 h depending on substrate concentration.

277 It is important to highlight that overall ethanol yields remained about constant

278 after increasing substrate loadings. This result is indicative of the good pretreatment

279 performance, since the binding capacity of enzymes to cellulose usually declines after

280 increasing substrate concentration [21, 22]. For instance, Moreno et al. [15] reported a
281 reduction in ethanol yields from 0.27 g/g to 0.20 g/g after increasing the concentration
282 of steam-exploded wheat straw from 10% DM (w/v) to 20% DM (w/v). The water
283 extraction stage performed prior to steam explosion pretreatment has shown to increase
284 overall sugar recoveries up to 90% and 80% of the total glucose and xylose from the
285 raw material [7], respectively, which might be the reason for the better hydrolysability
286 of the pretreated OTP.

287 **Table 2.** Fermentation kinetics during ethanol production from different pretreated OTP fractions under
288 different process conditions and microorganisms

Microorganism	Material / Process configuration	Substrate concentration	$Ethanol_{max}$ (g/L)	$Yield_E$ (g/g)[a]	Q_E (g/L h)[b]
***S. cerevisiae* Ethanol Red**	WIS-PEOTP / Batch SSF	15% (w/w)	33.6 ± 1.8	0.39 ± 0.03$^{\phi}$	1.2 ± 0.1^{24h}
	WIS-PEOTP / Batch PSSF	25% (w/w)	68.8 ± 0.6	0.42 ± 0.01$^{\phi}$	1.6 ± 0.1^{24h}
***S. cerevisiae* F12**	DLF-PEOTP / Batch fermentation	ND	7.5 ± 0.3	0.32 ± 0.00$^{\int}$	0.2 ± 0.0^{40h}
	WIS-PEOTP / Batch PSSF	25% (w/w)	55.3 ± 0.4	0.33 ± 0.00$^{\int}$	1.0 ± 0.1^{48h}
	DLF-PEOTP + WIS-PEOTP / Fed-batch fermentation	15% (w/w)	44.9 ± 0.3	0.42 ± 0.00$^{\int}$	0.7 ± 0.0^{64h}

ND, non-diluted; WIS-PEOTP, pretreated water insoluble solid fraction; DLF-PEOTP, detoxified pretreated liquid fraction; SSF, simultaneous saccharification and fermentation; PSSF, pressaccharification and simultaneous saccharification and fermentation.
[a]Ethanol yields were determined as [Ethanol]/[sugars], considering $Ethanol_{max}$ and potential glucose (ϕ) or glucose + xylose ($\int$). Glucose from enzyme preparations is also considered
[b]Ethanol volumentric productivities were estimated at different time points (indicated in superscript) as follows: [$ethanol_t$]/tº

289

290 The use of phosphoric acid as catalyst for steam explosion pretreatment has
291 previously resulted in final yields of about 160 g of ethanol per kg of extracted OTP
292 biomass [2]. In this study, ethanol yields of 115-125 g of ethanol per kg of extracted
293 OTP were obtained independently of the substrate concentration used (Figure 3).

294 However, these processes only consider the use of glucans from the solid fraction, while

295 hemicelluloses from both solid and liquid fractions remains unused.

296 *3.3. Strategies for complete sugar fermentation of pretreated olive tree pruning*

297 The utilization of all sugar components, and in particular glucose and xylose, has been

298 considered essential for a cost-effective conversion of lignocellulosic feedstocks [23]. In

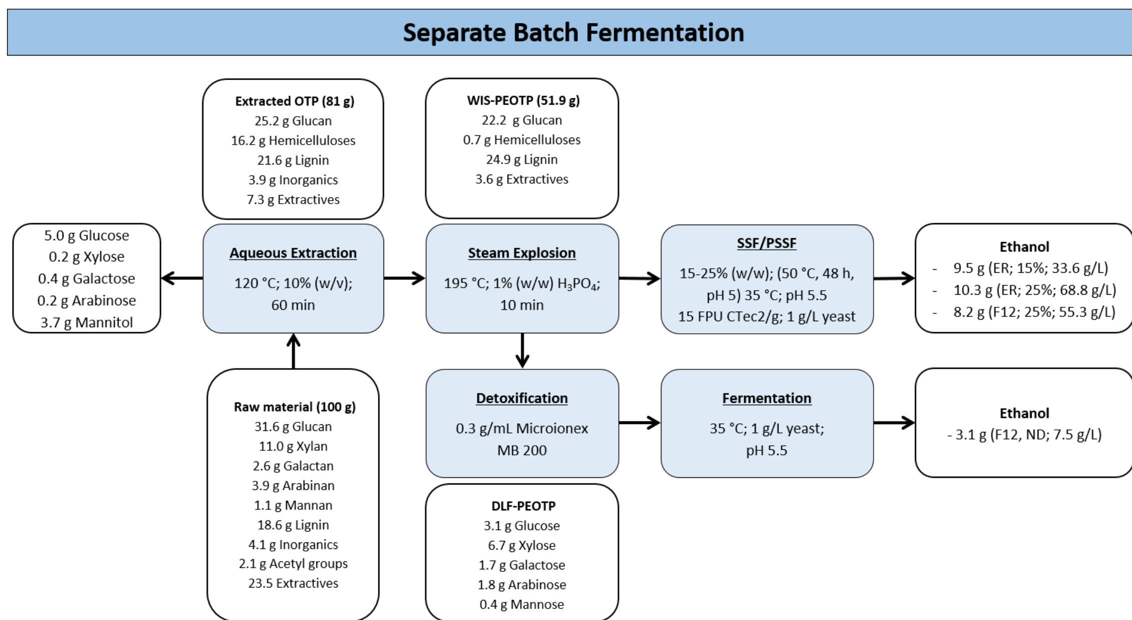
299 spite of its robustness, *S. cerevisiae* Ethanol Red is unable of fermenting xylose.

300 Therefore, the recombinant *S. cerevisiae* F12 was selected as fermentative

301 microorganism with the aim of converting both glucose and xylose into ethanol. For

302 that, both separate batch fermentation and fed-batch fermentation strategies were

303 evaluated to maximize sugar-to-ethanol conversion from pretreated OTP biomass.



304

305 **Figure 3.** Mass balance for the separate batch fermentation strategies using both *S. cerevisiae* Ethanol

306 Red (ER) and *S. cerevisiae* F12 (F12). ND: Non-diluted detoxified liquid fraction.

307

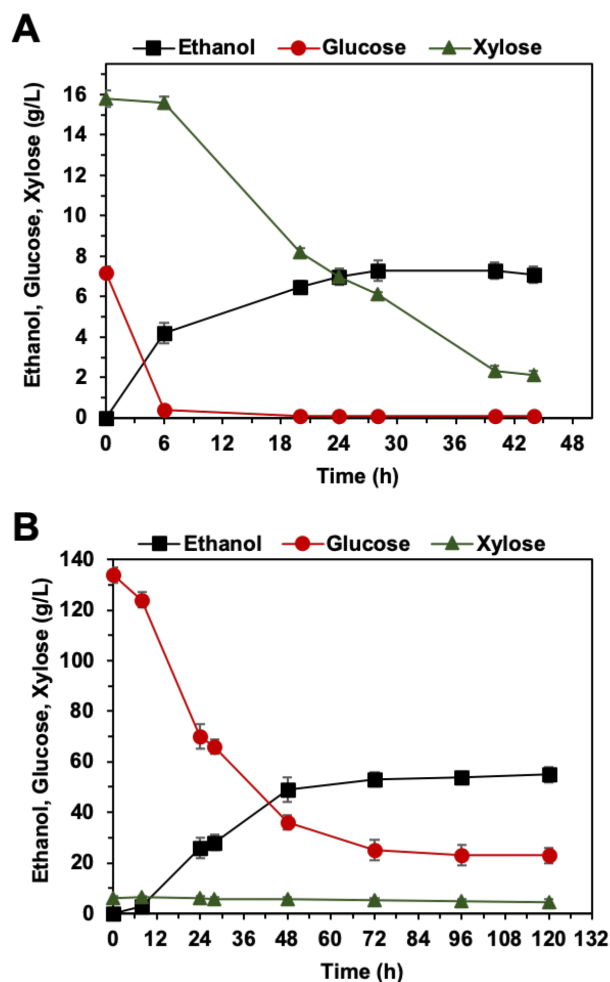
308 3.3.1. Separate batch fermentation of pretreated olive tree pruning

309 Separate batch fermentation of both liquid and solid fractions were first performed with

310 *S. cerevisiae* F12 to evaluate the fermentation performance of this yeast strain under

311 different process conditions (Figure 4, Table 2).

312



313 **Figure 4.** Batch fermentation of (**A**) DLF-PEOTP and (**B**) WIS-PEOTP with *S. cerevisiae* F12 strain.

314

315 Due to its high inhibitory potential, the LF-PEOTP was first subjected to
316 detoxification in order to trigger yeast fermentation. After detoxification, *S. cerevisiae*
317 F12 was capable of fermenting the detoxified liquid fraction (DLF-PEOTP), attaining
318 7.5 ± 0.3 g/L of ethanol. This ethanol concentration correspond to about 65% (0.32 g/g)
319 of the theoretical ethanol yield. Similar conversion yields have been obtained with this
320 yeast strain during fermentation of other lignocellulosic substrates. For instance,
321 Tomás-Pejó et al. [24] reported ethanol conversion yields of 0.27-0.31 g/g during fed-
322 batch fermentation of steam-exploded wheat straw (with a final substrate loadings of
323 11.25% w/w). It is interesting to note that the initial sugar concentration was reduced

324 by 90% after 40 h (Figure 4A), highlighting the good detoxification performance of the
325 resin used.

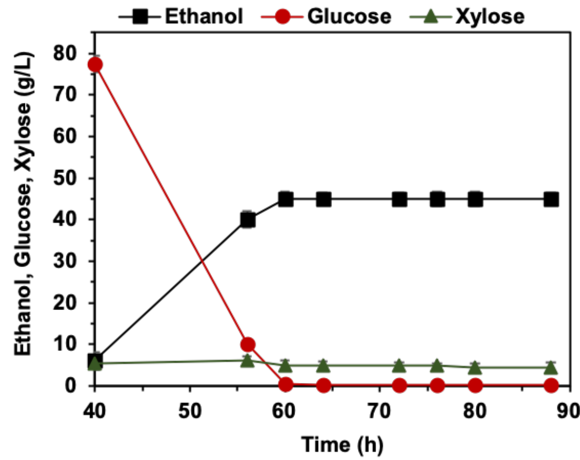
326 The detoxification capacity of this ion-exchange resin has been reported
327 previously [2, 25]. Negro et al. [2] used alkali and Microionex MB 200 for
328 detoxification of olive tree pruning prehydrolysates prior to fermentation. After
329 detoxification, these methods triggered fermentation of detoxified prehydrolysates,
330 reaching higher ethanol volumetric productivities when using the ion-exchange resin.
331 López-Linares et al. [25] also compared Microionex MB 200 with activated charcoal.
332 These authors observed higher overall inhibitor removal capacity for activated charcoal.
333 However, fermentation with *Escherichia coli* of rape straw prehydrolysates (obtained by
334 pretreatment with sulfuric acid at mild conditions) resulted in complete inhibition, even
335 after detoxification with activated charcoal. On the other hand, resin-detoxified
336 prehydrolysates showed complete sugar fermentation, even though a lag phase of 72 h
337 was observed. This result was attributed to the better phenol removal of the ion-
338 exchange resin (about 80%) in comparison to the activated charcoal (below 60%). In
339 this work, *S. cerevisiae* F12 showed no lag phase during fermentation of DLF-PEOTP
340 and a constant sugar consumption and ethanol production was observed, with ethanol
341 volumetric productivities of about 0.2 g/L h.

342 The WIS-PEOTP was also subjected to batch fermentation with *S. cerevisiae*
343 F12. This process was carried out at 25% TS (w/w) substrate concentration under PSSF
344 process configuration. As shown in Figure 4B, this pretreated fraction led to final
345 ethanol concentration of 55.3 ± 0.4 g/L, corresponding to 65% of the theoretical ethanol
346 that could be obtained (0.33 g/g) (Table 2). In comparison to *S. cerevisiae* Ethanol Red,
347 this yeast strain produced 20% lower ethanol concentrations due to incomplete sugar
348 fermentation (about 25 g/L of glucose and 5 g/L xylose still remained in the media after

349 120 h of PSSF process). Different inhibitory mechanisms involving both
350 lignocellulosic-derived compounds and the final product ethanol, combined with a
351 lower inhibitory tolerance of *S. cerevisiae* F12 towards these compounds, might be
352 responsible for the incomplete sugar fermentation. Usually, high ethanol concentrations
353 (ca. 100 g/L) are required to inhibit *S. cerevisiae* strains totally [26]. Nevertheless,
354 fermentation with *S. cerevisiae* F12 might have been terminated by the inhibitory
355 synergies between high ethanol titers and the presence of certain biomass degradation
356 compounds. When working at high substrate loadings, certain microbial inhibitors can
357 be released during saccharification of pretreated feedstocks. For instance, Alvira et al.
358 [27] reported the release of phenols, furan derivatives and weak organic acids (acetic
359 acid and formic acid) during the enzymatic hydrolysis of steam-exploded wheat straw at
360 25% TS (w/w), even after a thorough washing of the pretreated material. Similar
361 substrate loadings were investigated in this work, which might have therefore resulted
362 in the increase of inhibitory compounds during PSSF processes. Although the presence
363 of such inhibitors did not influence microbial fermentation at initial stages, the
364 synergies caused by inhibitors and the increased ethanol concentrations might have
365 exceed the stress tolerance threshold of *S. cerevisiae* F12, limiting the fermentation
366 capacity of this strain.
367 3.3.2. Fed-batch fermentation of detoxified slurry
368 Due to the lower ethanol titers obtained during fermentation of the liquid fraction,
369 alternative strategies were considered for integrating the conversion of both glucose and
370 xylose in a single process. In a first approach, DLF-PEOTP and WIS-PEOTP were
371 again combined to obtain a 'detoxified-like slurry' (15% TS (w/w) of substrate loading)
372 which was subjected to SSF fermentation with *S. cerevisiae* F12 (Supplementary Figure
373 S1). Compared to SSF fermentation at 15% TS (w/w) of WIS-PEOTP with *S. cerevisiae*

374 Ethanol Red, *S. cerevisiae* F12 increased ethanol titers to 39.4 ± 1.2 g/L with ethanol
375 volumetric productivities of 0.8 ± 0.0 g/L h (Table 2). However, limited xylose
376 conversion was observed, which reduced final ethanol yields from 0.38 to 0.34 g/g
377 (67% of the theoretical). Batch SSF/PSSF processes with glucose/xylose mixtures have
378 previously exhibited limited xylose conversion yields. Different native and recombinant
379 xylose-fermenting strains, such as *S. cerevisiae* F12 and *Candida intermedia* CBS
380 141442, have previously shown to be more prone to inhibition by biomass degradation
381 compounds during the xylose-fermenting phase [15, 23]. This effect has been attributed
382 to a drop in cell viability, which might be promoted by the stress exerted on yeast cells
383 by lignocellulose-derived inhibitors once reaching glucose depletion [15]. Although
384 most fermentative microorganisms have shown inherent oxidation and reduction
385 mechanisms for tolerating and/or converting certain degradation compounds such as
386 furan derivatives (e.g. furfural and 5-HMF), these inhibitory compounds usually act
387 synergistically and therefore represents an important limitation even at low
388 concentrations [27]. As mentioned above, certain inhibitory compounds can be released
389 during enzymatic hydrolysis of WIS-PEOTP, which combined with the non-detoxified
390 elements from DLF-PEOTP, might be inhibitory enough to hinder xylose conversion.
391 With the aim of improving xylose conversion and maximize ethanol production,
392 a fed-batch strategy was investigated to integrate the fermentation of both liquid and
393 solid fractions. This fed-batch strategy consisted on supplementing the DLF-PEOTP
394 with the hydrolyzed WIS-PEOTP after 40 h of fermentation. As can be observed in
395 Figure 5, this process configuration resulted in almost complete sugar depletion,
396 remaining only about 5 g/L of xylose at the end of the fermentation.
397 Final ethanol concentrations increased up to 44.9 ± 0.3 g/L, corresponding to an
398 overall conversion yield of 0.42 ± 0.00 g/g (ca. 80% of the theoretical) (Table 2). These

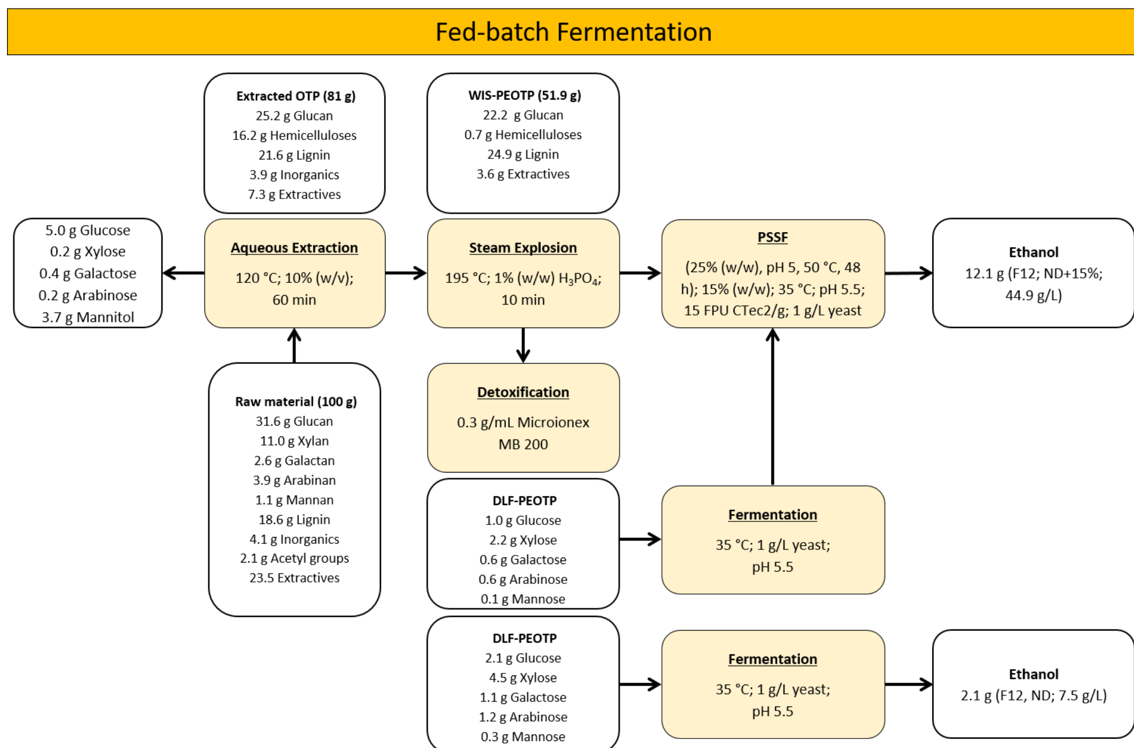
399 yields would result in the production of about 180 g of ethanol per kg of extracted OTP
400 (Figure 6), which increases previous reported yields (ca. 160 g of ethanol per kg of
401 extracted OTP) from extracted, phosphoric-acid-catalyzed OTP biomass by 12.5% [2,
402 28].



403

404 **Figure 5.** Fed-batch fermentation of detoxified pretreated OTP biomass with *S. cerevisiae* F12.

405



406

407 **Figure 6.** Mass balance for the fed-batch fermentation strategy using *S. cerevisiae* F12 (F12). ND: Non-
408 diluted detoxified liquid fraction.

It is important to mention that the fed-batch strategy also allows flexibility of an integrated OTP-based biorefinery, thus adapting the process to the market needs. In this context, in addition to use the collected water-extracted fraction as a source of antioxidants, the detoxified liquid fraction can be fermented into alternative value-added bioproducts such as xylitol [5, 20], while simultaneously producing 125-150 g of ethanol per kg of extracted OTP (by using either *S. cerevisiae* Ethanol Red or *S. cerevisiae F12* for glucose assimilation at high solid loadings) (Figure 3 and 6).

Conclusions

The fed-batch fermentation strategy presented herein allows the sequential conversion of both glucose and xylose into ethanol, demonstrating the potential of OTP biomass as an important raw material for future biorefineries. This strategy resulted in about 180 g of ethanol per g of extracted OTP, which increases previous reported yields by 12.5%. Furthermore, the present configuration offers a versatile conversion of OTP biomass to obtain multiple bioproducts, allowing flexibility of the process. This work will definitely represent an interesting base-case study for future economic assessments to determine the viability of the process at large scale.

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