1 Bioconversion of pomegranate residues into biofuels and bioactive lipids

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27 Abstract

28 Pomegranate residues (PRs) (i.e. the solid residues remaining after juice extraction),
 29 generated currently in abundance in Greece, contain a variety of carbon sources and therefore
 30 can be regarded as a potential feedstock for chemical and biotechnological processes rather
 31 than as waste materials. In the current project, the polysaccharides contained in PRs were
 32 extracted and hydrolyzed in a one-step process without the use of chemical reagents and the
 33 resulting broth was used as substrate in biotechnological applications, including ethanol and
 34 single cell oil (SCO) production. The yeasts *Meyerozyma guilliermondii*, *Scheffersomyces*
 35 *coipomoensis*, *Sugiyamaella paludigena* and especially *Saccharomyces cerevisiae*, were able
 36 to efficiently convert PR derived reducing sugars into bioethanol. Ethanol production under
 37 anaerobic conditions ranged from 3.6 to 12.5 g/L. In addition, the oleaginous yeasts
 38 *Lipomyces lipofer* and *Yarrowia lipolytica* as well as *M. guilliermondii*, *S. coipomoensis* and
 39 *S. paludigena* were tested for their ability to accumulate lipids suitable as feedstock for
 40 biodiesel production. Lipids were accumulated at concentrations up to 18% and were rich in
 41 palmitic acid (C16:0) and oleic acid (C18:1). Finally, the oleaginous fungus *Cunninghamella*
 42 *echinulata* was cultivated on PR based solid substrates for γ -linolenic acid (GLA) production.
 43 The fermented bio-products (i.e. fermented substrate plus fungal mycelia) contained up to 4.8
 44 mg GLA/g of dry weight. Phenolic removal (up to 30%) was achieved by several of the above
 45 mentioned microorganisms, including *C. echinulata*, *L. lipofer*, *M. guilliermondii*, *S.*
 46 *paludigena* and *Y. lipolytica*. We conclude that PRs can be used as a raw material for
 47 microbial growth, ethanol and SCO production, which is of economic and environmental
 48 importance.

49
 50 **Keywords:** pomegranate residues; bioethanol; *Saccharomyces cerevisiae*; modelling;
 51 microbial lipids; *Cunninghamella echinulata*

52

53 **Abbreviations:** FA, fatty acid; GLA, γ -linolenic acid; OFs, oat flakes; PRs, pomegranate
54 residues; PUFAs, polyunsaturated fatty acids; R.S., reducing sugars; SCOs, single cell oil;
55 SSF, Solid-state fermentation; TAGs, triacylglycerols; T.S., total sugars; WB, wheat bran

56 1. Introduction

57 Agriculture is a very important economic activity that boosts prosperity and contributes to
58 tackling poverty in many countries. According to The World Bank collection, agricultural
59 activity worldwide in 2017 accounted for 3.43% of the gross domestic product (GDP).
60 Specifically for Greece, in 2018 the agricultural domestic product represented 3.72%, of
61 GDP, twice as much as in other European countries, while together with processing,
62 nowadays exceeds 8% (World Bank, September 2020). However, the expansion and
63 modernization of the agricultural sector, and especially the processing of agricultural
64 products, has caused environmental concerns, due to the inevitable co-production of residues
65 and wastewaters, which are produced in huge quantities annually. Besides, water use in
66 agriculture accounts for 70% of total water use globally.

67 Two different types of agro-industrial residues exist, namely field plant residues,
68 which include remaining plant parts (e.g. leaves, straw, stalks, seeds, roots etc.), and industrial
69 residues, which include organic matter or plant parts produced by food and fruit industries
70 (e.g. molasses, peels, fruit marc and seeds and fruit pulp) (Sadh et al., 2018). Among residues,
71 lignocellulosic biomass is the most abundant residue worldwide, and although it can be
72 considered as a promising cost-effective fermentation feedstock (Diwan and Gupta, 2019;
73 Valdés et al., 2020b, 2020a), is treated as waste in many countries (Arevalo-Gallegos et al.,
74 2017). The uncontrollable disposal of agro-industrial residues, especially those produced
75 during industrial processing, to the landfills without any treatment, can cause environmental
76 pollution on the surrounded areas and harmful effects on human and animal health (Ghinea et
77 al., 2019).

78 The use of the lignocellulosic material as a substrate in biotechnological applications
79 requires additional pretreatment phases, to make it fermentable (Caporusso et al., 2021;
80 Mosier, 2005; Sarris and Papanikolaou, 2016; Valdés et al., 2020a), which increases the cost
81 of the whole process. Lignocellulosic biomass consists of cellulose, hemicellulose and lignin.
82 During the pretreatment procedure, lignin “seal” is removed and cellulose is released and
83 becomes accessible for hydrolysis (Haghighi Mood et al., 2013; Sánchez and Cardona, 2008).

Pretreatment methods are categorized in four groups, i.e. physical (i.e. grinding and milling, microwave irradiation, mechanical extrusion, freeze pretreatment), chemical (in an acid or an alkaline environment), physico-chemical (using steam explosion, hot water, wet oxidation and CO₂ explosion) and biochemical (using microorganisms and enzymes) (Haghighi Mood et al., 2013; Sarkar et al., 2012). Lately, combination of two of the above mentioned methods is proposed to increase the efficiency of the process. The first step of pretreatment involves lignin destruction, followed by hydrolysis of cellulose using acids or enzymes, resulting in the release of fermentable sugars (Sánchez and Cardona, 2008). Usually, glucose and xylose are extracted from lignocellulosic biomass. Thus, microorganisms that can utilize both C-5 and C-6 sugars are highly desired to increase the fermentation efficiency for the production of valuable metabolic products, including ethanol and lipids, from such materials (Diwan and Gupta, 2020).

The last decade the popularity of pomegranate products (mainly fresh fruit and juice) has increased tremendously thanks to their nutritional value and benefits to human health. Many reports describe the antimicrobial, antiviral, anticancer, antioxidant and antimutagenic properties of the various parts of the plant. (Akhtar et al., 2015; Bassiri-Jahromi, 2018; Hasnaoui et al., 2014; Joseph et al., 2013; Sharma et al., 2017). As a result, pomegranate cultivation has increased significantly worldwide to meet the growing demand. For instance, in Greece this crop extended up to 700-800 acres until 2007 and nowadays has reached 15.000 acres, yielding 30.000 tons of fruits per year. Consequently, the pomegranate processing industry has significantly expanded, and its negative outputs have already appeared (Kaderides et al., 2015). It has been estimated that the production of each ton of concentrated to 65 Brix pomegranate juice generates up to 5–5.5 tons of pomegranate residues (PRs) (i.e. pomegranate peels and seeds) (Hasnaoui et al., 2014). Although PRs is an excellent source of sugars, such as ribose, glucuronic acid, galacturonic acid, L-rhamnose, D-fucose, L-arabinose, D-xylose, D-mannose, D-galactose and D-glucose (Zhai et al., 2018a, 2018b) and numerous bioactive compounds (Santos et al., 2019; Sood and Gupta, 2015), in practice they are usually left unexploited and only a small amount is used as a soil conditioner

(after composting), as fuel (in the form of dry pellets), as animal feed or for the recovery of pharmaceuticals and cosmetics (Goula et al., 2017; Pathak et al., 2017; Pereira et al., 2016; Pocan et al., 2018; Siddiqui et al., 2019). On the other hand, the necessity for low-cost and environmentally friendly substrates suitable for microbial processes related to the production of high value-added products (i.e. biofuels, single cell oils, single cell protein, enzymes, pigments, organic acids, etc.), has been arisen.

Biofuels (mainly bioethanol and biodiesel) are renewable, cost-effective and environmentally friendly energy sources alternative to fossil fuels, which has attracted the interest of many countries in the context of the diminishing oil dependence and eliminating the adverse effects of its use on global warming. Therefore, the demand for biofuels is gradually increasing worldwide. Specifically, bioethanol apart from its variety of commercial applications in cosmetology and in food and pharmaceutical industry, is also widely used as an engine fuel, on its own, or blended with gasoline. However, the utilization of the so-called “1st generation” bioethanol, deriving from the fermentation of hydrolyzed corn starch or sucrose juice, runs into the “food vs fuel” dilemma (Gray et al., 2006), therefore the production of the so-called “2nd generation” bioethanol (deriving through the valorization of waste streams) is considered as a very important scientific priority worldwide (Rastogi and Shrivastava, 2017; Sarkar et al., 2012; Tian and Chen, 2016). On the other hand, biodiesel produced by a variety of renewable resources (i.e. plant or animal fats) (Banković-Ilić et al., 2014; Gutiérrez et al., 2017), is non-toxic, biodegradable, and has a favorable emission profile, but, being potentially competitive in food production, it faces similar criticism to that of the “1st generation” bioethanol. Alternatively, waste fats can be considered, with some restrictions, as a raw material for the production of biodiesel, and this option has aroused great interest in many countries. Besides, microbial oil, so-called single cell oil (SCO), produced from yeasts cultivated on agro-industrial residues, presenting a fatty acid (FA) composition similar to that of common vegetable oils, could be considered shortly as feedstock in the biodiesel manufacture (Dourou et al., 2018; Li et al., 2008; Papanikolaou and Aggelis, 2011a, 2011b; Singh and Gu, 2010; Wei et al., 2017). Contrary to yeast-derived

140 SCOs, fungi- and microalgae-derived SCOs are of pharmaceutical and dietary interest thanks
141 to the presence of polyunsaturated fatty acids (PUFAs), such as γ -linolenic acid (GLA, C18:3,
142 n-6), stearidonic (SDA, C18:4, n-3), arachidonic acid (ARA, C20:4, n- 6), eicosapentaenoic
143 acid (EPA, C20:5, n-3), docosapentaenoic acid (DPA, C22:5, n-3) and docosahexaenoic acid
144 (DHA, C22:6, n- 3) (Bellou et al., 2016; Fakas et al., 2009; Slaný et al., 2021).

145 A significant part of the production costs of both ethanol and SCO refers to the
146 substrate cost suggesting that a choice of a low-cost material as substrate, such as a carbon-
147 rich agro-industrial residue, is crucial for the process sustainability (Dourou et al., 2016;
148 Osorio-González et al., 2019; Sarris et al., 2013). Moreover, new strategies using
149 microorganisms derived after genetic engineering and/or adaptive laboratory evolution
150 approaches, used to increase the efficiency of substrate assimilation and metabolite
151 production, can contribute to the success of the process (da Silveira et al., 2020; Daskalaki et
152 al., 2019; De Melo et al., 2020; Dourou et al., 2018; Li et al., 2019).

153 The aim of the current investigation was to study the conversion of PRs into high
154 added value microbial products, i.e. ethanol and SCOs, using technologically suitable
155 microbial strains in an integrated approach, which includes an efficient and environmentally
156 friendly pretreatment followed by the production of microbial metabolic products, while
157 minimizing management issues caused by PR disposal into the environment. The yeasts
158 *Candida tropicalis*, *Lipomyces lipofer*, *Meyerozyma guilliermondii*, *Saccharomyces*
159 *cerevisiae*, *Scheffersomyces coipomoensis*, *Sugiyamaella paludigena* and *Yarrowia lipolytica*
160 were tested for their ability to grow on PR extract and produce ethanol and/or SCOs. Solid
161 state fermentations (SSFs) of the fungus *Cunninghamella echinulata* were performed using
162 PRs alone or in combination with wheat bran (WB) or oat flakes (OFs) as substrate. The final
163 fermented product was enriched with polyunsaturated FAs, thus having a potential value used
164 in animal or human nutrition. We conclude that PRs and PR extract are suitable microbial
165 substrates and can be used as raw material in various biotechnological applications.

166

167 2. Material and Methods

168 2.1 Microorganisms and culture conditions

169 The following yeast strains were used: *Saccharomyces cerevisiae* AXAZ-1, *Candida*
170 *tropicalis* NRRL Y-12968, *Lipomyces lipofer* NRRL Y-11555, *Yarrowia lipolytica* ACA DC
171 50109 (culture collection of Agricultural University of Athens, Greece) and three newly
172 isolated strains from Chilean Valdivian Forest namely *Meyerozyma guilliermondii* ACA-DC
173 5397, *Scheffersomyces coipomoensis* ACA-DC 5395, *Sugiyamaella paludigena* ACA DC
174 5396 (Valdés et al., 2020b). In addition, the fungal strain *Cunninghamella echinulata* CCF
175 2195 (culture collection of fungi, Charles University, Prague, Czech Republic) was used in
176 this research. All microbial strains were maintained at -80 °C in glycerol 30% and for short
177 term maintenance on potato dextrose agar (PDA, Himedia, Mumbai, India) slants at 4 ± 1 °C
178 and regularly sub-cultured.

179

180 2.2 PR collection, media preparation and culture conditions

181 Fresh PRs (i.e. pomegranate peel and seeds remaining after juice extraction, cultivar
182 *Wonderful*) were obtained from the Bacfresh Company located in Achaia regional unit of
183 Greece, or produced in the laboratory after juice extraction using a mechanical home juicer.
184 PR moisture was gravimetrically determined after drying at 105 °C in an oven until constant
185 weight. Polysaccharide extraction and hydrolysis were simultaneously performed from wet
186 PRs cut into pieces of 0.5×0.5 cm in water or in H₂SO₄ (Fluka, Steinheim, Germany)
187 solution used at a ratio of 40 mL of water or H₂SO₄ solution to 10 g of PRs. The treatment
188 was performed using various concentrations of H₂SO₄ (up to 0.10 M), at temperature 100 or
189 120 °C, pressure 101 or 219 kPa and time ranging from 20 to 120 min. The treated PR
190 mixture was subsequently centrifuged (Heraeus, Biofuge Stratus, Thermo Scientific,
191 Osterode, Germany) at $19,000 \times g$, 4 °C for 10 min and the supernatant, optionally diluted
192 with water, was used as culture medium. Supernatant pH was determined using an ORION
193 pH meter (Boston, USA) and adjusted before inoculation to the desirable level (ranged from
194 2.6 to 6.5 ± 0.1) by adding 4 M NaOH (Merck) or 4 M HCL (Sigma-Aldrich).

Submerged cultures under near anaerobic conditions were conducted by yeast strains in manually periodically agitated 200 mL Duran flasks (Bioengineering, Wald, Switzerland) containing 50 ± 1 mL of PR extract. The flasks were incubated in a Memmert GmbH+Co. KG, Germany incubator at temperature 28 °C. Submerged cultures under aerobic conditions were performed in 250 mL Erlenmeyer flasks containing 50 ± 1 mL of PR extract incubated in a rotary shaker (ZHICHENG ZHWY 211C, Shanghai, China) at agitation rate 180 rpm and temperature 28 °C.

After medium sterilization (at 121 °C for 20 min) the flasks were inoculated with 1 mL of a pre-culture (containing 10^8 cells), carried out in a medium containing (in g/L): yeast extract (Conda) 3; glucose (AppliChem, Darmstadt, Germany) 10; peptone (Himedia) 5; malt extract 3 (Sigma-Aldrich, Steinheim, Germany), and incubated in a rotary shaker as above.

SSFs of wet chopped peels alone or in mixture with wheat bran (WB) or oat flakes (OFs) [used in different proportions to control moisture (Klempová et al., 2020)] were performed by *C. echinulata* in high-density polyethylene (HDPE) bags (20 × 30 cm) containing 10 g of substrate at room temperature ($T = 25 \pm 2$ °C). HDPE bags ensure aseptic conditions and smooth oxygen transfer during fermentation. After sterilization (at 105 °C for 45 min) the bags were inoculated with 10^7 spores/mL obtained from a 7-day-old culture grown on polished rice (Slaný et al., 2020). After inoculation the bags were closed and the substrate was arranged on the shelves to obtain a substrate layer thickness of around 5 mm. SSFs were carried out under static conditions for up to 5 days.

215

216 2.3 Microbial growth and biomass determination

Yeast growth was estimated by enumerating the number of cells/mL using a haemocytometer cell counting chamber (Neubauer improved, Poly-Oprik, Bad Blankenburg, Germany). Dry cell mass (x, g/L) was gravimetrically determined after harvesting of yeast cells by centrifugation at $19,000 \times g$ for 15 min at 4 °C. The cells were washed twice with NaCl 0.9% (w/v) and dried at 80 °C until constant weight.

222 The fermented substrate after SSFs (around 10 g) was washed with 60 mL of distilled
223 water and filtrated through Whatman No. 1 paper. The obtained byproduct was
224 gravimetrically determined after drying at 60 °C until constant weight. The concentration of
225 reducing sugars (R.S.) was determined in the filtrate (see below) and the fungal growth was
226 indirectly estimated by calculating R.S. consumption. Besides, GLA concentration in lipids
227 (estimated as described below) of the fermented substrate was additionally used as a marker
228 to indirectly estimate fungal growth, as this FA is absent from the substrate.

229

230 2.4 Sugar and phenolics determination

231 The concentration of total sugars (T.S.) and reducing sugars (R.S.) in the liquid growth
232 medium and in the solid substrate extract was determined according to Dubois et al. (1951)
233 and DNS (Miller, 1959) methods, respectively, and expressed as glucose equivalent (in g/L).
234 Sugar concentrations were also expressed per weight of dry substrate. The total phenolic
235 compounds in the liquid growth medium and in the solid substrate extract were determined
236 according to Folin and Ciocalteu (1927). The concentration of phenolic compounds was
237 expressed as gallic acid equivalent (in mg/L or per weight of dry substrate).

238

239 2.5 Ethanol determination

240 Ethanol was determined in filtered aliquots of the culture supernatant by high performance
241 liquid chromatographer (HPLC - Ultimate 3000 Dionex, Germering, Germany) equipped with
242 a Reflective Index detector (RI-101, Shodex, Kawasaki, Kanagawa Japan) and an
243 AminexHPX-87H column (300 × 7.8 mm). H₂SO₄ (Fluka) 0.005 N was used as eluent at a
244 flow rate of 0.6 mL/min. The column temperature was 65 °C.

245

246 2.6 Lipid extraction and purification

247 Lipids from dry yeast cell mass or dry homogenized fermented substrate were extracted in
248 chloroform:methanol (Sigma-Aldrich) (2:1 v/v) according to Folch et al. (1957) method as
249 modified by Dourou et al. (2017). The extracts were filtered through Whatman No. 1 paper,

250 washed with a KCl (Sigma-Aldrich) 0.88% (w/v) solution and dried over anhydrous Na₂SO₄
 251 (Sigma-Aldrich). Finally, the solvents were evaporated under vacuum using a Rotavapor R-20
 252 device (BUCHI, Flawil, Switzerland), and the total lipids were gravimetrically determined.

253

254 2.7 Fatty acid composition of microbial lipids

255 FA moieties of total lipids were converted into their FA methyl esters (FAMES) in a two-
 256 stage reaction according to AFNOR. FAMES were analyzed in a GC apparatus (Agilent 7890
 257 A, Agilent Technologies, Shanghai, China), equipped with a flame ionization detector (FID)
 258 and an HP-88 (J&W Scientific) column (60 m × 0.32 mm). Helium was used as carrier gas at
 259 a flow rate of 1 mL/min. Injection temperature was 250 °C, the oven temperature was 200 °C
 260 and FID temperature was 280 °C. Peaks of FAMES were identified by reference to authentic
 261 standards.

262

263 2.8 Thin-layer chromatography (TLC) analysis

264 Known weight of total lipids (approximately 300 mg) were loaded in the form of narrow
 265 bands of 1 cm on Merck TLC silica gel 60 plates using a CAMAG ATS 4 apparatus and
 266 separated by a developing system for non-polar lipids using a solvent system consisting of
 267 hexane/diethyl ether/acetic acid (80:20:1, v/v/v). TLC plates were visualized using a
 268 combustion water solution containing 3.3% (v/v) sulfuric acid, 50% (v/v) methanol, 0.33%
 269 (w/v) MnCl₂·4H₂O, and dried at 130 °C for 10 min. After visualization, TLC plates were
 270 scanned by CAMAG TLC Scanner 4 at 400 nm and evaluated by winCATS software ver.
 271 1.4.8 (CAMAG) to evaluate lipidic structures such as TAGs, DAGs, FFAs, etc. in lipid
 272 extract (Gajdoš et al., 2015).

273

274 2.9 Modelling

275 The Hinshelwood model (Hinshelwood, 1947) was used to describe yeast growth and ethanol
 276 production under aerobic and anaerobic conditions. The balance equations for cell mass and
 277 ethanol production and sugar consumption are:

$$\frac{dX}{dt} = \mu_{max} \left(\frac{S}{K_{sx} + S} \right) (1 - K_{px}P) X \quad (1)$$

$$\frac{dP}{dt} = q_{max} \left(\frac{S}{K_{sp} + S} \right) (1 - K_{pp}P) X \quad (2)$$

$$\frac{dS}{dt} = -\frac{1}{Y_{xs}} \frac{dX}{dt} - \frac{1}{Y_{ps}} \frac{dP}{dt} \quad (3)$$

where X , S and P are cell mass, sugars and ethanol concentrations (g/L), respectively, t is the fermentation time (h), μ_{max} is the maximum specific growth rate (1/h), q_{max} is the maximum specific ethanol production rate (1/h), K_{sx} and K_{sp} are the saturation constants (g/L), K_{px} and K_{pp} are the ethanol inhibition constants (g/L), Y_{xs} is the yield coefficient for biomass production with respect to sugars (g biomass/g sugars) and Y_{ps} is the yield coefficient for ethanol production with respect to sugars (g ethanol/g sugars).

The fitting of the model equations on the experimental data, was performed using the numerical code Aquasim (Version 2.1d), which uses a fully implicit finite difference spatial discretization Gear scheme in conjunction with the algorithm DASSL. Aquasim uses the weighted least-squares method to estimate the optimum parameter values (Reichert, 1998).

2.10 Statistical analysis

The experimental data were treated using OriginPro 2018 Software (OriginLab Corporation, Northampton, MA, United States). One-way analysis of variance ANOVA followed by a Bonferroni post hoc test was performed to determine significant differences. The null hypothesis was rejected at a significance level of $p \leq 0.01$ or 0.05 .

3. Results and Discussion

3.1 Pomegranate juice extraction and composition of the PRs

The composition of the PRs depends not only on the cultivation conditions and the cultivar but also on the efficiency of the extraction of the juice and the extraction technology used (Catania et al., 2020; Mphahlele et al., 2016). Thus, compositional differences concerning

300 mostly initial sugar and phenolic compounds concentration were observed among samples,
301 which were used in the current investigation. The PRs resulting from the processing of the
302 fruit under laboratory conditions constitute $68.7 \pm 1.9\%$ of the total weight of the fruit, while
303 this percentage can reach up to 80% under industrial processing conditions. Consequently, the
304 yield in juice under laboratory conditions was $29.3 \pm 1.3\%$, while under industrial conditions
305 it is estimated to be less than 20%. The presence of phenolics and flavonoids in the
306 pomegranate juice is indeed suitable, due to the beneficial effects of these compounds on
307 human health. However, exhaustive fruit juicing is not generally selected by the industry to
308 avoid the exceeding enrichment of the juice in such compounds, which may affect the acidity
309 and the color of pomegranate juice and, therefore, its quality (Catania et al., 2020; Türkyılmaz
310 et al., 2013). The moisture of the solid residue produced under laboratory and industrial
311 conditions was 66% and 75%, respectively.

312

313 3.2. Pretreatment of the PRs

314 PRs were cut into small pieces to reduce their size, increase the accessible area and improve
315 the porosity. Following, PRs were treated for 20-120 min under acidic conditions with a
316 H_2SO_4 solution up to 0.10 M, at high temperature (i.e. 100 and 120 °C) and pressure (i.e. 101
317 and 213 kPa) for the simultaneous extraction and hydrolysis of the polysaccharides (Table 1).
318 The efficiency of the extraction process was evaluated by calculating the percentage of T.S.
319 extracted in the solution on the T.S. extracted under the strongest conditions (i.e. temperature
320 121 °C, pressure 219 kPa, H_2SO_4 0.10 M, for 60 min). Moreover, the efficiency of the
321 hydrolysis process in each case was evaluated by calculating the percentage of R.S. on the
322 T.S. in the extract.

323 Acid pretreatment is recommended for hardwoods and agricultural residues, while the
324 kind of acid used (e.g. HCl, H_2SO_4 , HNO_3 etc.), its concentration, temperature and reaction
325 time differ in the various protocols described in the literature (Nitsos et al., 2018; Zhang and
326 Bao, 2018). In the current investigation, we have demonstrated that the addition of a strong
327 acid to PRs is not necessary to achieve adequate extraction of polysaccharides and their

hydrolysis to R.S., probably due to the high natural acidity of PRs imparting a $\text{pH} \leq 3.5$, and to the best physicochemical properties of water, as an extractant, against sulfuric acid solutions. Specifically, the extraction efficiency achieved in water at temperature 121 °C, under 219 kPa pressure, for 60 min was no statistically different (at $p=0.01$ or 0.05) to that achieved under similar conditions in the presence of H_2SO_4 used at concentrations 0.03-0.10 M (comparisons of entries c-g, Table 1). Similarly, the utilization of H_2SO_4 did not significantly increase the hydrolysis efficiency (comparisons of entries c-g, Table 1). It is reported that the extraction efficiency of ingredients increases with fluidity and, in some cases, with solvent polarity (Xu et al., 2019). Low viscosity of the solvent is generally required to obtain high extraction yields since high viscosity hinders the mass transfer of the solvent to the target solutes. High polarity solvents are also required when polar molecules, such as polysaccharides, are targeted for extraction. Therefore, the higher polarity of water in combination with its low viscosity could explain the higher extraction yields obtained in this research in the absence of sulfuric acid. Çam and Hişil (2010) investigating polyphenols extraction from pomegranate peels, reported that water extraction was as effective as conventional methanol extraction.

The processing time is another factor that influences the extraction efficiency (Zhu and Liu, 2013). Comparing the effect of the processing time on polysaccharide extraction under the above-mentioned conditions (i.e. in water, at $T=121$ °C, $P=219$ kPa), we ascertain that the extraction yield at 20 min was higher compared to higher processing time (i.e. 30, 60, and 120 min, entries a-c and h in Table 1), although not statistically significant at $p=0.01$ and 0.05 . This apparent reduction in extraction yield is probably a result of sugar degradation. Therefore, the extraction time of 20 min was selected as a sufficient time for the extraction and hydrolysis of polysaccharides from PRs.

Trying to reduce the processing temperature more experiments were performed at 100 °C and atmospheric pressure (i.e. 101 kPa) instead of 121 °C and 219 kPa. We note that the extraction efficiency was higher and the efficiency of hydrolysis lower at 100 °C than at 121 °C (comparisons of entries h and i, Table 1). Zhu and Liu (2013) examining the effect of different temperatures (from 70 to 100 °C) on polysaccharide extraction, reported that

maximum yield was obtained at 95 - 100 °C. Finally, at T=100 °C comparing the effect of O₂ presence (entries i and j in Table 1), both T.S. and R.S. yields (in g/L) were higher (i.e. 5.3 and 4.8% respectively) when a closed system, almost in the absence of O₂ was applied.

Generally, high temperature increases solubility and diffusion of the ingredients to be extracted and decreases the viscosity of the solvent, which is intended. Though, pressure seems to have a great influence on the extraction and hydrolysis yield too. Comparing the entries h-j in Table 1, it seems that conditions reported in entry i would be appropriate. However, the high processing time is not desirable, therefore, the conditions that we conclude to use for this research are those reported in entry a: in water, for 20 min, at T=121 °C and P=219 kPa.

The selection of the pretreatment method of lignocellulosic biomass and its operating conditions are fundamental for a successful polysaccharide extraction and hydrolysis, and the subsequent fermentation. Methods of chemical pretreatment such as acid, alkaline and their combination, are the most preferred techniques in recent years, thanks to their flexibility of applications at the industrial level, and the high final yields of assimilable sugars achieved (Valdés et al., 2020a). Among them, acid pretreatment, and specifically diluted acid pretreatment, is favorable. However, regardless of the pretreatment method used, toxic compounds are generated as by-products, such as formic, acetic and levulinic acid, neutral and acidic phenolics, and other chemicals in hydrolysates, which can inhibit microbial growth (Bravo et al., 2017; Haghighi Mood et al., 2013; Janga et al., 2012; Yu et al., 2018).

376

3.3 Microbial growth on PR based media

The growth of microorganisms on PRs presents various peculiarities related to the nature of the substrate, including assimilability of the contained carbon sources, the presence of microbial inhibitors (i.e. natural such as phenolic compounds or generated during pre-treatment), and its low pH values. Therefore, the challenge is to identify microbial strains capable of growing on PRs and PR extract. In the present study, the ability of the oleaginous fungus *C. echinulata* and the yeasts *C. tropicalis*, *L. lipofer*, *M. guilliermondii*, *S. cerevisiae*,

384 *S. coipomoensis*, *S. paludigena* and *Y. lipolytica* to grow on PRs or PR extract and produce
 385 various metabolites was studied under several growth conditions (Table 2). To the best of our
 386 knowledge, the current investigation is the first report in which both pomegranate peels and
 387 seeds have been employed as fermentation medium for microbial growth, while only recently,
 388 pomegranate peels have been treated as a substrate for biodiesel production by *Bacillus*
 389 *cereus* (Kanakdande et al., 2020) and for citric acid production by the fungus *Aspergillus*
 390 *niger* (Roukas and Kotzekidou, 2020).

391 A sustainable process for microbial conversion of lignocellulosic biomass into
 392 biofuels and other products is based on the capability of the microbial strains used to
 393 assimilate/co-assimilate, the various sugars (including C-5 and C-6) released during
 394 pretreatment (Valdés et al., 2020a). All microbial strains tested in the current investigation
 395 were able to grow on PRs or PR extract showing different biochemical potential and
 396 effectiveness (Table 2). Among them, the new isolates from the Chilean Valdivian forest
 397 identified as *M. guilliermondii*, *S. coipomoensis*, and *S. paludigena* recently characterized for
 398 their ability to grow on lignocellulosic hydrolysate-model media (Valdés et al., 2020b),
 399 showed interesting ethanol production and lipid accumulation capacity, cultivated under
 400 anaerobic and aerobic conditions, respectively. The most commonly used yeast for ethanol
 401 production is *S. cerevisiae*. However this yeast is not able to metabolize pentose sugars
 402 naturally, such as xylose and arabinose, to ethanol, and therefore several strategies on
 403 engineering pentose metabolism on *S. cerevisiae* have been proposed (Fernandes and Murray,
 404 2010; Gopinarayanan and Nair, 2019; Hahn-Hägerdal et al., 2007). Interestingly, *S. cerevisiae*
 405 during submerged cultures on PR extract in this study showed remarkable growth and ethanol
 406 production ability. Contrary, growth and ethanol production by *C. tropicalis* was not
 407 satisfactory.

408 *L. lipofer* and *Y. lipolytica* were both able to accumulate lipids during submerged
 409 cultures on PRs extract. Strains belonging to the genus of *Lipomyces* have notable oleaginous
 410 capacity and are suitable for SCO production from lignocellulosic biomass and other agro-
 411 industrial wastes (Di Fidio et al., 2020; Dien et al., 2016; Dourou et al., 2016; Gong et al.,

412 2012; Liang and Jiang, 2013; Sitepu et al., 2013; Slininger et al., 2016; Vasaki et al., 2021;
 413 Zhao et al., 2008). *Y. lipolytica*, the most studied oleaginous yeast, is able to grow on a variety
 414 of agro-industrial substrates and to produce high added value metabolites (Carota et al., 2020;
 415 Chatzifragkou et al., 2011; da S. Pereira et al., 2019; Dobrowolski et al., 2016; Dourou et al.,
 416 2016; Makri et al., 2010; Patsios et al., 2020). Over the last decade, a variety of studies have
 417 focused on modifications of *Y. lipolytica* genome through genetic engineering to enable the
 418 efficient assimilation of sugars found in lignocellulosic biomass (Ledesma-Amaro and
 419 Nicaud, 2016; Niehus et al., 2018; Yook et al., 2020).

420 The oleaginous fungus *C. echinulata* was able to synthesize lipids during SSF on sole
 421 PRs or blends of PRs with WB or OFs (Table 2). WB and OFs are also produced in
 422 abundance, consisting yet again lignocellulosic residues which need management. In 2010,
 423 the National Green Tribunal established control measures to stop such biomass burning, due
 424 to air pollution, encouraging the exploitation of agricultural residues as a substrate for
 425 microbial production of high added-value products using environmentally friendly
 426 approaches. Filamentous fungi belonging to the *Cunninghamella* genus and related genera
 427 play an important role in developing sustainable biorefinery processes thanks to their ability
 428 to utilize a broad range of renewable feedstock, and waste materials (e.g. corn gluten, corn
 429 steep, orange peel and tomato waste hydrolysate) and convert them into SCOs containing
 430 significant quantities of GLA (Diwan and Gupta, 2019; Donot et al., 2014; Fakas et al., 2008;
 431 Gema et al., 2002).

432 Phenolic compounds present in agro-industrial residues are principally responsible for
 433 their phytotoxicity and microbial growth inhibition, while their breakdown is considered to be
 434 the limiting step during biotreatment (Aggelis et al., 2003; Tsioulpas et al., 2002). Moreover,
 435 phenolics can be released from lignin as by-products during the pretreatment of
 436 lignocellulosic biomass, depending on the parameters of the process, such as temperature and
 437 duration of the treatment. Phenolic compounds, when present in the growth media, penetrate
 438 biological membranes and cause loss of their integrity affecting cell growth and the whole
 439 fermentation process (Baral and Shah, 2014). Therefore, their degradation and/or removal

prior to the fermentation process is of high biotechnological interest. In this investigation, the PR extract used as a growth medium for submerged cultures of *L. lipofer* and *Y. lipolytica* was diluted in some cases with water to reduce the initial phenolic concentration and the results were compared to those obtained on undiluted PR hydrolysate (Table 2, see letters d, e, f). In addition, phenolic compounds were reduced in the case of SSF of *C. echinulata* by the incorporation of cereals. Nevertheless, remarkable phenolic removal (up to 30%) was performed in some cases by *C. echinulata*, *L. lipofer*, *M. guilliermondii*, *S. paludigena* and *Y. lipolytica*. Benzene compounds are degraded by a few stains belonging to the phylum Ascomycota (e.g. the yeasts *Debaryomyces hansenii*, *L. starkeyi*, and *S. cerevisiae*, the fungus *Geotrichum candidum*, and the yeast-like *Aureobasidium pullulans*) and the phylum Basidiomycota (*Rhodotorula*, *Trichosporon cutaneum*) (Pasha and Rao, 2009).

Low pH media are generally suitable for large scale applications since they are self-protected against bacterial contamination. Indeed, in media having exceptionally low pH, sterilization can be omitted, and the maintenance of aseptic conditions is not necessary. Thus, the fermentation cost can be significantly reduced. In the present study, the strains *M. guilliermondii*, *S. coipomoensis*, and *S. paludigena* were able to grow at pH = 2.6, *S. cerevisiae* at pH = 3.5 and 5.0, *C. tropicalis* at pH = 4.5, while *C. echinulata*, *L. lipofer* and *Y. lipolytica* were cultivated at their optimal pH value of 6.5.

3.4 Bioethanol production

Bioethanol produced using low- or negative- acquisition cost substrates, such as carbon-rich agro-industrial residues, is at the forefront of biotechnological/industrial interest for many years. Bioethanol can be used as a raw material in the food, pharmaceuticals and cosmetics industry or as biofuel. During alcoholic fermentation, the sugars in the form of hexoses or pentoses are transformed into pyruvic acid (through the metabolic path of glycolysis), which under anaerobic conditions, is converted into ethanol and CO₂. The strain AXAZ-1 of *S. cerevisiae*, as well as *M. guilliermondii*, *S. coipomoensis*, and *S. paludigena* were used in the current investigation and proved able to grow well in PR extract and to efficiently convert

sugars to ethanol cultivated under anaerobic conditions (Fig. 1 and 2). The yeast *S. cerevisiae* is widely used worldwide in winemaking, in the brewery and baking industry, as well as in many other biotechnological applications, as it possesses key features, such as quick fermentation ability, genetic stability, tolerance to low pH environments, ethanol tolerance, osmotolerance and thermotolerance (Nevoigt, 2008; Sarris et al., 2014). PR extracts derived under laboratory and industrial processing conditions were used as a fermentation medium for *S. cerevisiae*. As mentioned above, the difference between these processes is that under industrial juicing the resulting extract had a higher sugar concentration. *M. guilliermondii*, is a promising species implicated in various biotechnological applications, including the conversion of pentoses to ethanol (Martini et al., 2016; Matos et al., 2014; Yan et al., 2021). Similarly, *Scheffersomyces* and *Sugiyamaella* genera are reported as xylose-fermenting genera containing species having an ethanol production capacity (Jia et al., 2020; Lopes et al., 2018; Morais et al., 2020; Sena et al., 2017). This is the first report that describes bioethanol production from *S. coipomoensis* and *S. paludigena* strains.

Several mathematical models have been developed to describe the alcoholic fermentation process (Birol et al., 1998). Among them, the Monod (Monod, 1942) and Hinshelwood (Hinshelwood, 1947) models have been considered as suitable to describe the process under low/very low product inhibition (Birol et al., 1998; Kostov et al., 2012). In the current paper, the Monod model was initially employed to describe microbial growth, ethanol production and sugars consumption during fermentation and to calculate parameter values. However, it was observed that this model was not able to simulate the process for most of the yeast strains, probably due to the inhibitory effect of ethanol on growth and ethanol production (data not shown). Alternatively, the Hinshelwood model, which includes K_{px} and K_{pp} parameters indicating a weak inhibition on cell growth and ethanol production by ethanol (Kostov et al., 2012), was employed to simulate the process. Fig. 1 and 2 show experimental data and model fitting, while the estimated values of the kinetic parameters along with the correlation coefficient (R^2) are shown in Table 3.

495 The μ_{max} values ranged between 0.0038 and 0.0079 1/h, except for the value
 496 obtained from the yeast *S. coipomoensis* in anaerobic conditions, which was 0.00024 1/h. The
 497 corresponding values in the literature using the Hinshelwood model range from 0.231 to
 498 0.289 1/h by using free or immobilized cells of yeast *S. cerevisiae* growing on glucose
 499 (Kostov et al., 2012), sweet sorghum stalk juice (Jin et al., 2012) and corn stover (Tian and
 500 Chen, 2016). The low μ_{max} values in the present study may be due to the cell growth
 501 inhibition by ethanol and/or inhibitory compounds generated during PRs pretreatment. It has
 502 been reported that the presence in the fermentation medium of phenolics at 2.0 g/L negatively
 503 affects both cell viability of *S. cerevisiae* and ethanol production (Liu et al., 2016). In the
 504 present study, the initial concentration of phenolic compounds was about 2.0 g/L and
 505 remained almost constant during fermentation of *S. cerevisiae*. Y_{ps} calculated under anaerobic
 506 conditions ranged from 0.389 (for *M. guilliermondii*) to 0.364 (for *S. paludigena*), while the
 507 experimental values are close to those predicted by the model (Table 3). For Y_{sx} , the values
 508 predicted by the model were in all cases higher compared to the experimental values for all
 509 yeast strains. These discrepancies may be due to the presence of growth inhibitors on PR
 510 extract that are not included in the Hinshelwood model. The estimated values of K_{pp} , K_{px} ,
 511 K_{sx} and K_{sp} are in the range of values reported in the literature (Birol et al., 1998; Jin et al.,
 512 2012; Kostov et al., 2012; Tian and Chen, 2016).

513 In all experiments, the mathematical model adequately predicts the biomass and
 514 ethanol production as well as sugar consumption, which was confirmed by the high R^2 values
 515 in most of the cases. The kinetic parameters values vary among strains indicating that each of
 516 them exhibits a different growth behavior on PR extract under aerobic and anaerobic
 517 conditions (Table 3). Most yeast strains produced ethanol ranged between 3.6 and 12.5 g/L
 518 showing high productivities evidenced by the high values of the parameter q_{max} (ranged
 519 between 0.592 and 0.841 g/g·h). Exceptionally, *S. coipomoensis* cultivated under aerobic
 520 conditions did not produce significant ethanol quantities (up to 0.186 g/L at the end of
 521 fermentation), thus low q_{max} value was observed. Kostov et al. (2012) found that q_{max} ,

calculated using the Hinshelwood model fitted on the experimental data, of free cells of *S. cerevisiae* was 0.452 g/g h when cultivated on 110 g/L of glucose, while this value increased above 1 g/g h using immobilized cells of *S. cerevisiae*.

Worthy of mentioning is that all fermentations were conducted under low pH values. Specifically, *S. cerevisiae* was cultivated at pH=3.5 (natural pH of PR extract) and pH=5.0 (after adjustment) on PR extract derived under laboratory processing conditions (Table 2), and ethanol and cell mass production was similar. Moreover, the pH during fermentation of *M. guilliermondii*, *S. paludigena* and *S. coipomoensis* was 2.6. The above-mentioned strains, having inherent tolerance of low pH, are suitable for industrial ethanol production.

Ethanol production by *S. cerevisiae* is reported using as substrate a variety of agro-industrial residues, such as enriched pasteurized grape musts, blends of molasses and olive mill wastewaters, olive mill wastewater enriched with glucose, sugarcane bagasse, dried fruits etc., reporting yields up to 0.5 g/g (Behera et al., 2011; Sarris et al., 2014, 2013, 2009; Singh et al., 2013). Other yeasts such as *C. shehatei*, *S. stipitis* and *Pachysolen tannophilus* have been reported as ethanol producers from xylose or cellobiose. Given the fact that *S. cerevisiae* cannot utilize xylose as part of its natural metabolism, the concurrent use of more than one microorganism, for instance, *S. stipitis* and *S. cerevisiae*, has gained popularity in the context of lignocellulose valorization (De Bari et al., 2013; Karagoz et al., 2019; Ntaikou et al., 2018; Santosh et al., 2017).

3.5 Single cell oil production

When undiluted PR extract was used as a culture medium for *L. lipofer*, lipid accumulation (L/x%, w/w) was 7.0%, probably restricted by the presence of phenolics or other inhibitors, while when the extract was diluted by 50%, L/x increased to 13.5% (Table 2). Dien et al. (2016) reported that the same strain of *L. lipofer* cultivated in sugar-rich media (i.e. 100 g/L) accumulated 61.6% lipids in the dry cell mass cultivated on glucose, 54.8% on xylose and 56.9% on arabinose. Therefore the low lipid accumulation reported in the current paper should be attributed, in addition to the presence of phenolics, to the low sugar content in the

550 growth medium. On the other hand, yeast growth seems to be unaffected by the concentration
551 of phenolics, as biomass production was proportional to the initial sugar concentration. The
552 biotechnological potential of *M. guilliermondii*, *S. coipomoensis*, and *S. paludigena* to convert
553 lignocellulosic sugars to oily biomass was first reported by Valdés et al. (2020b). In the
554 present study, the above-mentioned strains, cultivated in undiluted PR extract presented an
555 oleaginous capacity depending on the growth conditions (Table 2). Specifically, *M.*
556 *guilliermondii* was able to produce 2.1 g/L dry cell mass and accumulate 18.1% lipids, while
557 increased lipid accumulation was achieved by *S. paludigena* cultivated under aerobic
558 conditions compared to anaerobic conditions (i.e. 15.4 and 9.0% respectively). Low lipid
559 accumulation was achieved by *S. coipomoensis* under both aerobic and anaerobic conditions
560 (i.e. $L/x\%$ = 8.3 and 4.2%), although this yeast was able to accumulate almost 18% of lipids
561 cultivated on glucose and xylose, and 24% on mannose (Valdés et al., 2020b). Although *Y.*
562 *lipolytica* can grow on a variety of substrates and accumulate lipid in high percentages
563 (Dourou et al., 2018; Papanikolaou and Aggelis, 2011a, 2011b, 2002), during submerged
564 cultures on undiluted PR extract, $X_{\max}$ did not exceed 1.7 g/L containing only 7.0% of lipids.
565 However, when PR extract diluted by 50% lipid accumulation increased to 11.0% in the dry
566 cell mass.

567 Oleic acid (C18:1) was the major FA in all lipids produced by the yeasts, followed by
568 palmitic acid (C16:0) in the lipids of *M. guilliermondii*, *S. coipomoensis*, and *S. paludigena*
569 (Table 4). In the lipids produced by *Y. lipolytica*, C16:0, palmitoleic (C16:1), and linoleic acid
570 (C18:2) participated in non-negligible percentages. Stearic acid (C18:0) was detected in
571 limited amounts in all lipids. According to these data, the oil produced seems to be suitable as
572 raw material in the biodiesel industry. *Y. lipolytica* presented a similar FA profile to that
573 previously reported when cultivated on olive mill wastewater or glycerol (Dourou et al., 2016;
574 Makri et al., 2010; Papanikolaou and Aggelis, 2002). Contrary to the present study, Valdés et
575 al. (2020b) reported that lipids of *M. guilliermondii* and *S. coipomensis* were rich in C16:1,
576 and lipids of *S. paludigena* were rich in C18:2, suggesting that the nature of the carbon source
577 affects the FA profile of these strains.

578 SSF, a fermentation technique suitable for low moisture solid substrates, is frequently
579 used as an effective technology, alternative to submerged fermentation, for the valorization of
580 various agro-industrial by-products (Čertík et al., 2012). PUFA-rich fermented substrates
581 derived from SSFs, after the necessary processing, can be utilized in human diet or as a feed
582 supplement (Čertík et al., 2013; Economou et al., 2010; Gema et al., 2002; Slaný et al., 2021).

583 Though the presence of R.S. in PRs was high (up to 160 mg/g of substrate, Fig. 3A),
584 *C. echinulata* presented a low ability to grow on PRs used as sole substrate in SSF. This is
585 probably the result of the presence of phenolic compounds in high amounts (i.e. up to 14.0
586 mg/g substrate, Fig. 3B) or other inhibitors, the high substrate humidity (Fig. 3C) and/or the
587 loss of nutrients during sterilization process. To reduce moisture to adequate levels, blends of
588 PRs with two cereal substrates, WB and OFs, were used. These cereals, treated also as wastes,
589 are a good source of carbon and other nutrients, supporting fungal proliferation (Čertík et al.,
590 2013; Slaný et al., 2020). Actually, their incorporation in high percentage, positively affected
591 fungal growth as R.S. consumption (Fig. 3A) and % substrate utilization (Fig. 3C) confirm.
592 Specifically, when cereals were present at 70 to 90%, % substrate utilization was satisfactory.
593 On the contrary, when PRs were present at a level higher than 30%, R.S. consumption and %
594 substrate utilization were not satisfactory. During mixed cultures, R.S. originated from PRs (0
595 day in Fig. 3A) were partly utilized by the fungus. Moreover, the addition of WB and OFs
596 resulted in a reduction of water content (Fig. 3C), as well as, of phenolic compounds (up to
597 3.5 mg/g substrate, Fig. 3B). In all cases, the percentage of substrate utilization and the level
598 of GLA in intracellular lipid gradually increased and maximized on the 5th day (Fig. 3C and
599 Fig. 4). *C. echinulata* cultivated on blends of 90% WB and 10% PRs, produced the highest
600 GLA quantities (i.e. 4.8 mg GLA/g fermented substrate) corresponding to 13.6% GLA in total
601 lipids, while increasing PRs incorporation resulted in decreased GLA production. The
602 addition of 80% OFs resulted in the higher GLA production among OFs-blends (i.e. 3.1 mg
603 GLA/g of fermented substrate representing 4.3% in total lipids). As in the case of WB, when
604 PRs used in a higher percentage (up to 50%) *C. echinulata* formed a low amount of GLA (i.e.
605 0.5-0.8 mg/g). When PRs were incorporated in low percentages GLA produced was higher

606 than that reported for *C. echinulata* ATHUM 4411 cultivated on orange peel, where GLA
607 production was 1.2-1.5 mg/g fermented substrate (Gema et al., 2002).

608 The FA composition of lipids extracted from the fermented substrate was similar
609 regardless of the starting substrate (PR or blends of PRs with WB or OFs) (Tables 5 and 6).
610 C18:2 was the dominant FA and its concentration reduced with time. The percentage of C16:0
611 in total lipids also decreased with time. On the contrary, the percentage of C18:1 increased
612 with time, mostly in WB-blends, suggesting biosynthesis of this FA by *C. echinulata* and/or
613 selective uptake of other FAs resulting in change of lipid profile. As expected GLA was not
614 detected in the initial substrate and therefore was synthesized by the fungus during substrate
615 assimilation. Some quantities of C18:2 were probably converted to GLA. In all cases, the
616 final fermented substrate containing C18:2, GLA and C18:3n-3, even in small quantities, is of
617 high nutritional value for animal or human consumption. The necessity for cereal-based
618 products enriched with essential FAs and other compounds (such as pigments) has been
619 reported (Čertík et al., 2013; Certik and Adamechova, 2009) and the use of oleaginous fungi
620 during SSF seems like a promising approach.

621 TLC analysis of total lipids by *C. echinulata* is depicted in Fig. 5. The lipids of the
622 fermented PRs contained various species of TAGs, while the absence of 1,3-DAG and
623 ergosterol is noticed. Lipids from SSF performed on WB and OF alone contained ergosterol
624 in significant percentages and TAGs were present in greater diversity. As the incorporation of
625 PRs increased in the substrate, specific species of TAGs (i.e. TAG1) also increased, while on
626 the contrary, the ergosterol content decreased. There are several distinct patterns of sterols
627 among fungi, depending on the phylum and/or environmental conditions (Volkman, 2003).
628 The presence of ergosterol in fungal or yeast lipids is essential for their response to stress by
629 inhibitors and therefore to their growth (Dupont et al., 2012). Phytosterol content was similar
630 in lipids produced during SSF on individual substrates (approximately 11%, w/w) and
631 decreased in blends of PRs with cereals, with exception of the substrate in which OFs
632 participated at a percentage of 80%, in which phytosterol remained in high levels. Moreover,
633 the incorporation of WB resulted in a higher amount of free FAs on the final product, while

the percentage of polar lipids slightly increased in the case of blends of PRs with cereals (ranging from 6.2 to 11.5%). During SSF of WB by *Umbelopsis isabellina* the percentage of free FAs and sterols increased (Slaný et al., 2021).

4. Conclusions

Agro-industrial residues, such as PRs, are suitable low-cost substrates for microbial fermentation processes leading to a “green” and cost-effective production of value-added products. These residues are largely available and abundant in nutrients. Researchers are focused on pretreatment methods of lignocellulosic biomass, while they have to overcome a variety of peculiarities to use such materials. In this investigation, efficient extraction and hydrolysis of the polysaccharides found in PRs were conducted in a one-step process and the resulted broth was successfully utilized by several yeast strains, able to grow into such extreme conditions, for either ethanol or SCO production. Moreover, PRs combined with cereals, were effectively operated by an oleaginous fungus for the production of a final fermented substrate enriched with PUFAs. We conclude that new perspectives are emerging for the use of PRs as a raw material to produce valuable microbial metabolites, using environmentally friendly approaches.

Declaration of interests

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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Table 1: Polysaccharide extraction and hydrolysis from PRs under various experimental conditions of H₂SO₄ concentration, processing time and temperature. The asterisk (*) indicates one set experiments performed using an open vessel assuring the presence of O₂. Data are presented as mean values of three replications. Letters refer to comparisons at vertical reading only; different letters indicate that differences of means are statistically significant at $p \leq 0.05$. Data were treated by one-way ANOVA test followed by Bonferroni post hoc test.

Set	Time (min)	Temperature (°C)	H ₂ SO ₄ (M)	Total Sugars (g/L)	Reducing Sugars (g/L)	Extraction (%)	Hydrolysis (%)
a	20	121	0.00	34.7 ^a ± 2.3	28.3 ^a ± 0.9	100.6	81.6
b	30	121	0.00	30.3 ^a ± 1.6	29.0 ^a ± 1.6	86.6	95.7
c	60	121	0.00	32.5 ^a ± 2.6	29.8 ^a ± 2.6	94.2	91.7
d	60	121	0.03	32.5 ^a ± 2.2	31.6 ^a ± 3.0	94.2	97.2
e	60	121	0.05	33.5 ^a ± 2.7	32.5 ^b ± 3.4	97.1	97.0
f	60	121	0.07	30.4 ^a ± 2.0	28.5 ^a ± 3.3	88.1	93.8
g	60	121	0.10	34.5 ^a ± 1.9	28.6 ^a ± 3.1	100.0	82.9
h	120	121	0.00	30.9 ^a ± 0.8	31.1 ^a ± 0.3	89.6	100.6
i	120	100	0.00	31.7 ^a ± 0.2	26.1 ^a ± 0.6	91.9	82.3
j*	120	100	0.00	30.1 ^a ± 0.5	24.9 ^b ± 0.2	87.2	82.7

Table 2: Cell mass, ethanol production, lipid accumulation and phenolic removal by different microorganisms cultivated on PR extract or solid residue under different culture conditions. Abbreviations: R.S., reducing sugars; nd, not determined; ND, not detected

Microbial strain	Culture conditions			Initial R.S. concentration (g/L or mg/g by-product)	X _{max} (g/L)	Ethanol _{max} (g/L)	L/X _{max} %	Initial phenolic concentration (g/L or mg/g by-product)	Phenolic removal (% w/w)
	Fermentation	Growth	pH						
<i>C. echinulata</i>	Solid state	Aerobic	6.5	163.7 ^a	nd	-	11.7	14.0	16.8
			6.5	22.3 ^b	nd	-	4.8	0.8	44.3
			6.5	7.5 ^c	nd	-	7.1	2.5	59.9
<i>C. tropicalis</i>	Submerged	Aerobic	4.5	37.0 ^{d, #}	nd	4.9	-	nd	nd
		Anaerobic	4.5	32.1 ^{d, *}	nd	6.8	-	nd	nd
<i>L. lipofer</i>	Aerobic	Aerobic	6.5	22.5 ^{d, #}	2.1	-	7.0	1.6	29.8
			6.5	15.3 ^{e, #}	1.6	-	3.9	0.9	18.3
			6.5	9.6 ^{f, #}	1.0	-	13.5	0.5	36.5
<i>M. guilliermondii</i>	Aerobic	Aerobic	2.6	18.0 ^{d, #}	2.1	3.2	18.1	2.3	48.8
			2.6	19.3 ^{d, #}	1.7	5.8	4.7	2.3	22.3
<i>S. cerevisiae</i>	Anaerobic	Anaerobic	3.5	30.8 ^{d, #}	2.7	7.4	-	2.6	ND
			3.5	37.4 ^{d, *}	2.8	12.5	-	nd	nd
			3.5	32.3 ^{d, *}	2.7	9.3	-	nd	nd
			5.0	33.3 ^{d, #}	2.8	9.7	-	2.5	ND
<i>S. coipomoensis</i>	Aerobic	Aerobic	2.6	20.8 ^{d, #}	0.7	0.2	8.3	2.0	22.7
			2.6	21.5 ^{d, #}	1.0	3.6	4.2	2.0	30.6
<i>S. paludigena</i>	Aerobic	Aerobic	2.6	18.0 ^{d, #}	2.1	1.6	15.4	2.3	61.0
			2.6	19.3 ^{d, #}	1.5	5.3	9.0	2.3	22.1
<i>Y. lipolytica</i>	Aerobic	Aerobic	6.5	21.8 ^{d, #}	1.7	-	7.0	2.6	45.0
			6.5	7.6 ^{f, #}	0.9	-	11.0	1.3	64.5

^aFermentation on 100% PRs; ^bFermentation on blend 10% PRs and 90% WB; ^cFermentation on blend 20% PRs and 80% OFs; ^d100% PR extract; ^e80% PR extract and 20% water; ^f50% PR extract and 50% water; [#]laboratory processing conditions; ^{*} industrial processing conditions

678 **Table 3:** Kinetic parameter values of yeast strains growing under aerobic and anaerobic conditions in PRs extract. The asterisk (*) indicates the experimental
679 values.

Yeast strain	Culture condition	Parameter									R ²	
		K _{pp} (g/L)	K _{px} (g/L)	K _{sp} (g/L)	K _{sx} (g/L)	q _{max} (1/h)	μ _{max} (1/h)	Y _{ps} (g/g)	Y _{sx} (g/g)			
<i>M. guilliermondii</i>	Aerobic	0.762	0.586	1.478	3.097	0.841	0.0074	0.297	0.240*	0.199	0.149*	0.679
	Anaerobic	0.196	0.209	10.601	1.864	0.728	0.0050	0.389	0.333*	0.242	0.099*	0.815
<i>S. cerevisiae</i>	Anaerobic	0.109	0.095	0.128	2.122	0.592	0.0038	0.373	0.321*	0.357	0.080*	0.844
<i>S. coipomoensis</i>	Aerobic	10.055	9.397	13.750	5.003	0.101	0.0079	0.150	0.022*	0.096	0.072*	0.579
	Anaerobic	0.480	0.437	1.961	0.039	0.706	0.00024	0.369	0.369*	0.197	0.102*	0.654
<i>S. paludigena</i>	Aerobic	0.741	0.413	0.165	1.847	0.734	0.0042	0.304	0.120*	0.208	0.152*	0.636
	Anaerobic	0.242	0.249	14.290	4.641	0.736	0.0055	0.364	0.364*	0.277	0.104*	0.641

680

Table 4: Fatty acid composition (% w/w) of total lipids produced by oleaginous yeasts cultivated on PRs. Data represent the mean of two determinations. Abbreviations: ND, not detected.

Yeast strain	Fatty acid composition (% w/w)					
	C16:0	C16:1	C18:0	C18:1 n-9	C18:2 n-6	Others
<i>M. guilliermondii</i>	35.1 ± 0.1	ND	ND	61.9 ± 0.9	ND	3.0 ± 0.2
<i>S. coipomoensis</i>	35.5 ± 0.3	ND	ND	62.5 ± 1.0	ND	2.0 ± 0.9
<i>S. paludigena</i>	26.0 ± 0.2	11.6 ± 0.3	3.9 ± 0.0	37.5 ± 0.5	4.5 ± 0.0	16.7 ± 1.1
<i>Y. lipolytica</i>	12.7 ± 0.1	14.3 ± 0.2	4.5 ± 0.1	43.2 ± 0.3	19.6 ± 0.2	1.7 ± 0.5

Table 5: Fatty acid composition (%) of total lipids of *Cunninghamella echinulata* during solid-state fermentation on PRs and WB used in different ratios. Data represent the mean of two determinations. Abbreviations: ND, not detected

Ratio of PRs:WB (%)		Day of SSF	Fatty acid composition (%)							
PRs	WB		C16:0	C16:1	C18:0	C18:1 n-9	C18:2 n-6	C18:3 n-6	C18:3 n-3	Others
0	100	0	19.6 ± 0.2	ND	3.7 ± 0.3	24.5 ± 0.9	48.6 ± 1.3	ND	3.3 ± 0.2	0.3 ± 0.1
		5	14.6 ± 0.1	0.6 ± 0.0	2.9 ± 0.1	26.4 ± 1.1	39.8 ± 1.1	14.4 ± 0.4	1.1 ± 0.0	0.2 ± 0.0
		10	20.1 ± 0.7	0.6 ± 0.0	1.3 ± 0.0	25.2 ± 1.2	48.6 ± 0.6	ND	3.6 ± 0.1	0.3 ± 0.1
10	90	0	19.9 ± 0.5	0.2 ± 0.0	2.2 ± 0.0	26.0 ± 0.9	48.5 ± 1.2	ND	3.0 ± 0.2	0.2 ± 0.0
		3	16.6 ± 0.4	0.5 ± 0.0	5.5 ± 0.4	30.9 ± 0.2	37.2 ± 1.0	7.3 ± 0.1	1.6 ± 0.0	0.4 ± 0.1
		5	16.5 ± 0.5	0.8 ± 0.1	5.9 ± 0.6	33.9 ± 0.7	33.3 ± 0.4	8.1 ± 0.2	1.1 ± 0.1	0.4 ± 0.1
20	80	0	19.9 ± 1.0	1.0 ± 0.3	2.5 ± 0.1	25.3 ± 0.3	47.6 ± 0.9	ND	3.5 ± 0.3	0.2 ± 0.0
		3	18.0 ± 0.2	0.4 ± 0.0	4.3 ± 0.1	26.5 ± 0.5	44.2 ± 0.7	3.6 ± 0.0	2.7 ± 0.2	0.3 ± 0.0
		5	17.6 ± 0.1	1.0 ± 0.0	5.9 ± 0.3	34.5 ± 1.1	32.2 ± 0.7	6.8 ± 0.5	1.6 ± 0.1	0.4 ± 0.1
30	70	0	21.2 ± 1.1	0.5 ± 0.1	3.0 ± 0.2	23.8 ± 0.6	48.2 ± 0.2	ND	3.1 ± 0.2	0.2 ± 0.0
		3	20.2 ± 0.9	0.3 ± 0.0	2.4 ± 0.2	25.1 ± 0.5	47.4 ± 1.4	1.3 ± 0.0	3.0 ± 0.9	0.3 ± 0.0
		5	17.3 ± 0.3	0.4 ± 0.0	4.3 ± 0.7	30.3 ± 1.0	40.3 ± 1.1	4.6 ± 0.2	2.5 ± 0.7	0.3 ± 0.1
40	60	0	19.3 ± 0.4	0.3 ± 0.1	2.1 ± 0.1	23.6 ± 0.2	52.6 ± 0.9	ND	0.2 ± 0.0	1.9 ± 0.3
		3	16.2 ± 0.1	0.2 ± 0.0	3.2 ± 0.0	23.3 ± 0.5	51.7 ± 0.6	5.0 ± 0.1	0.2 ± 0.0	0.2 ± 0.0
		5	17.9 ± 0.3	0.8 ± 0.2	3.4 ± 0.2	24.6 ± 0.4	46.1 ± 1.0	2.2 ± 0.1	4.1 ± 0.2	0.9 ± 0.2
50	50	0	19.3 ± 0.4	0.3 ± 0.1	2.1 ± 0.1	23.6 ± 0.2	52.6 ± 0.9	ND	0.2 ± 0.0	1.9 ± 0.3
		3	16.2 ± 0.1	0.2 ± 0.0	3.2 ± 0.0	23.3 ± 0.5	51.7 ± 0.6	5.0 ± 0.1	0.2 ± 0.0	0.2 ± 0.0
		5	17.9 ± 0.3	0.8 ± 0.2	3.4 ± 0.2	24.6 ± 0.4	46.1 ± 1.0	2.2 ± 0.1	4.1 ± 0.2	0.9 ± 0.2

688

Table 6: Fatty acid composition (%) of total lipids of *Cunninghamella echinulata* during solid-state fermentation on PRs and OFs used in different ratios. Data represent the mean of two determinations.

Ratio of PRs:OFs (%)		Day of SSF	Fatty acid composition (%)							
PRs	OFs		C16:0	C16:1	C18:0	C18:1 n-9	C18:2 n-6	C18:3 n-6	C18:3 n-3	Others
0	100	1	15.7	0.9	1.3	38.9	41.3	ND	1.5	0.4
			± 1.1	± 0.1	± 0.2	± 1.1	± 0.2		± 0.1	± 0.1
		5	14.5	1.4	2.5	35.4	34.3	9.5	2.1	0.3
10	90	0	16.9	0.3	1.5	37.7	41.5	ND	1.9	0.2
			± 0.9	± 0.0	± 0.1	± 0.7	± 1.0		± 0.2	± 0.0
		3	16.5	0.6	2.6	38.2	37.4	2.9	1.6	0.2
20	80	0	16.1	0.7	1.3	37.8	41.8	ND	2.1	0.2
			± 0.3	± 0.0	± 0.0	± 1.1	± 0.3		± 0.2	± 0.1
		3	15.5	0.5	2.3	37.0	38.6	3.8	2.0	0.3
30	70	0	16.6	0.7	4.0	40.4	32.8	4.3	0.9	0.3
			± 0.5	± 0.2	± 0.6	± 0.5	± 0.5	± 0.1	± 0.0	± 0.1
		3	17.2	0.6	1.9	38.1	39.6	ND	2.4	0.2
40	60	0	16.8	0.2	2.3	37.3	39.7	1.6	1.8	0.3
			± 0.9	± 0.0	± 0.3	± 0.6	± 0.2	± 0.0	± 0.1	± 0.1
		3	16.4	0.4	2.1	38.2	40.0	1.1	1.6	0.2
50	50	0	16.9	0.8	2.4	36.5	41.2	ND	2.1	0.1
			± 0.9	± 0.2	± 0.1	± 0.5	± 0.8		± 0.3	± 0.0
		3	17.2	0.7	3.0	39.7	35.7	1.9	1.5	0.3
60	40	0	17.8	0.2	1.6	37.4	40.4	0.6	1.7	0.3
			± 0.4	± 0.0	± 0.5	± 0.8	± 0.2	± 0.0	± 0.2	± 0.1
		3	19.7	0.5	5.0	37.7	34.4	ND	1.9	0.8
70	30	0	16.9	0.5	3.3	35.4	39.3	2.2	2.2	0.2
			± 0.6	± 0.1	± 0.4	± 0.3	± 0.1	± 0.2	± 0.1	± 0.0
		3	19.0	0.6	3.8	36.9	38.1	1.4	0.1	0.1
80	20	0	19.7	0.5	5.0	37.7	34.4	ND	1.9	0.8
			± 1.1	± 0.1	± 0.9	± 0.2	± 0.4		± 0.2	± 0.2
		3	16.9	0.5	3.3	35.4	39.3	2.2	2.2	0.2
90	10	0	16.9	0.3	1.5	37.7	41.5	ND	1.9	0.2
			± 0.9	± 0.0	± 0.1	± 0.7	± 1.0		± 0.2	± 0.0
		3	16.5	0.6	2.6	38.2	37.4	2.9	1.6	0.2
100	0	0	16.1	0.7	1.3	37.8	41.8	ND	2.1	0.2
			± 0.3	± 0.0	± 0.0	± 1.1	± 0.3		± 0.2	± 0.1
		3	15.5	0.5	2.3	37.0	38.6	3.8	2.0	0.3

692

693 **Figure captions**

694 **Fig. 1:** Kinetics of growth, ethanol production and substrate consumption of *Saccharomyces*
695 *cerevisiae* under anaerobic conditions, cultivated at pH=3.5 and laboratory processing
696 conditions (i), at pH=3.5 and industrial processing conditions (ii), (iii) and at pH=5.0 and
697 laboratory processing conditions (iv). Points and lines represent experimental data and model
698 prediction, respectively.

699
700 **Fig. 2:** Kinetics of growth, ethanol production and substrate consumption of *Meyerozyma*
701 *guilliermondii* (A), *Scheffersomyces coipomoensis* (B), and *Sugiyamaella paludigena* (C)
702 cultivated under anaerobic conditions at pH=2.6 and laboratory processing conditions. Points
703 and lines represent experimental data and model prediction, respectively.

704
705 **Fig. 3:** Content of reducing (mg/g by-product) and total sugars (g/g by-product) (A), phenolic
706 compounds (mg/g by-product) (B), and substrate humidity and utilization (%) (C) after solid-
707 state fermentation using *Cunninghamella echinulata* on sole PRs (i), different amounts of PRs
708 and WB (ii), and different amounts of PRs and OFs (iii).

709
710 **Fig. 4:** Content of total lipids (%), GLA on total lipids (%), yield of GLA (mg/g by-product)
711 after solid-state fermentation using *Cunninghamella echinulata* on sole PRs (i), different
712 amounts of PRs and WB (ii), and different amounts of PRs and OFs (iii).

713
714 **Fig. 5:** TLC analysis of non-polar lipids of *Cunninghamella echinulata*.

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