Bioconversion of pomegranate residues into biofuels and bioactive lipids

Marianna Dourou[a], Christina N. Economou[b], Lida Aggeli[a,b], Miroslav Janák[a,#], Gabriela Valdés[a,&], Nefeli Elezi[a], Dimitrios Kakavas[a], Theodore Papageorgiou[a], Dimitrios V. Vayenas[b], Milan Certik[c], George Aggelis[a,*]

[a]Division of Genetics, Cell and Developmental Biology, Department of Biology, University of Patras 26504, Patras, Greece

[b]Department of Chemical Engineering, University of Patras 26504, Patras, Greece

[c]Institute of Biotechnology, Faculty of Chemical and Food Technology, Slovak University of Technology, Bratislava 81237, Slovak Republic

[*]Corresponding author: Email address: George.Aggelis@upatras.gr; Tel (+30) 2610969232

[#]Current address: Institute of Biotechnology, Faculty of Chemical and Food Technology, Slovak University of Technology, Bratislava 81237, Slovak Republic

[&]Current address: Centro Regional de Investigación e Innovación para la Sostenibilidad de la Agricultura y los Territorios Rurales (CERES), Pontificia Universidad Católica de Valparaíso, Valparaíso 2340000, Chile.

Author contributions: M.D.: Methodology; Validation; Formal analysis; Writing - Original Draft; Visualization; C.N.E.: Validation; Formal analysis; Writing - Original Draft; L.A.: Methodology; Formal analysis; Investigation; M.J.: Methodology; Investigation; G.V.: Investigation; N.E.: Investigation; D.K.: Investigation; Th.P.: Investigation; D.V.V.: Supervision; M.C.: Supervision; G.A.: Conceptualization; Writing - Review & Editing; Supervision

Abstract

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

Keywords: pomegranate residues; bioethanol; *Saccharomyces cerevisiae*; modelling; microbial lipids; *Cunnighamella echinulata*

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

1. Introduction

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

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

The use of the lignocellulosic material as a substrate in biotechnological applications requires additional pretreatment phases, to make it fermentable (Caporusso et al., 2021; Mosier, 2005; Sarris and Papanikolaou, 2016; Valdés et al., 2020a), which increases the cost of the whole process. Lignocellulosic biomass consists of cellulose, hemicellulose and lignin. During the pretreatment procedure, lignin “seal” is removed and cellulose is released and 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 CO_2 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

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

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

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

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 H_2SO_4 (Fluka, Steinheim, Germany)
187 solution used at a ratio of 40 mL of water or H_2SO_4 solution to 10 g of PRs. The treatment
188 was performed using various concentrations of H_2SO_4 (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 × 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 ± 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.

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 × 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.

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

2.4 Sugar and phenolics determination

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

2.5 Ethanol determination

Ethanol was determined in filtered aliquots of the culture supernatant by high performance liquid chromatographer (HPLC - Ultimate 3000 Dionex, Germering, Germany) equipped with a Reflective Index detector (RI-101, Shodex, Kawasaki, Kanagawa Japan) and an AminexHPX-87H column (300 × 7.8 mm). H_2SO_4 (Fluka) 0.005 N was used as eluent at a flow rate of 0.6 mL/min. The column temperature was 65 °C.

2.6 Lipid extraction and purification

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

washed with a KCl (Sigma-Aldrich) 0.88% (w/v) solution and dried over anhydrous Na_2SO_4 (Sigma-Aldrich). Finally, the solvents were evaporated under vacuum using a Rotavapor R-20 device (BUCHI, Flawil, Switzerland), and the total lipids were gravimetrically determined.

2.7 Fatty acid composition of microbial lipids

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

2.8 Thin-layer chromatography (TLC) analysis

Known weight of total lipids (approximately 300 mg) were loaded in the form of narrow bands of 1 cm on Merck TLC silica gel 60 plates using a CAMAG ATS 4 apparatus and separated by a developing system for non-polar lipids using a solvent system consisting of hexane/diethyl ether/acetic acid (80:20:1, v/v/v). TLC plates were visualized using a combustion water solution containing 3.3% (v/v) sulfuric acid, 50% (v/v) methanol, 0.33% (w/v) $MnCl_2.4H_2O$, and dried at 130 °C for 10 min. After visualization, TLC plates were scanned by CAMAG TLC Scanner 4 at 400 nm and evaluated by winCATS software ver. 1.4.8 (CAMAG) to evaluate lipidic structures such as TAGs, DAGs, FFAs, etc. in lipid extract (Gajdoš et al., 2015).

2.9 Modelling

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

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

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

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

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

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

3.2. Pretreatment of the PRs

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

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

328 hydrolysis to R.S., probably due to the high natural acidity of PRs imparting a pH ≤3.5, and to
329 the best physicochemical properties of water, as an extractant, against sulfuric acid solutions.
330 Specifically, the extraction efficiency achieved in water at temperature 121 °C, under 219 kPa
331 pressure, for 60 min was no statistically different (at p=0.01 or 0.05) to that achieved under
332 similar conditions in the presence of H_2SO_4 used at concentrations 0.03-0.10 M (comparisons
333 of entries c-g, Table 1). Similarly, the utilization of H_2SO_4 did not significantly increase the
334 hydrolysis efficiency (comparisons of entries c-g, Table 1). It is reported that the extraction
335 efficiency of ingredients increases with fluidity and, in some cases, with solvent polarity (Xu
336 et al., 2019). Low viscosity of the solvent is generally required to obtain high extraction
337 yields since high viscosity hinders the mass transfer of the solvent to the target solutes. High
338 polarity solvents are also required when polar molecules, such as polysaccharides, are
339 targeted for extraction. Therefore, the higher polarity of water in combination with its low
340 viscosity could explain the higher extraction yields obtained in this research in the absence of
341 sulfuric acid. Çam and Hişil (2010) investigating polyphenols extraction from pomegranate
342 peels, reported that water extraction was as effective as conventional methanol extraction.
343 The processing time is another factor that influences the extraction efficiency (Zhu
344 and Liu, 2013). Comparing the effect of the processing time on polysaccharide extraction
345 under the above-mentioned conditions (i.e. in water, at T=121 °C, P=219 kPa), we ascertain
346 that the extraction yield at 20 min was higher compared to higher processing time (i.e. 30, 60,
347 and 120 min, entries a-c and h in Table 1), although not statistically significant at p=0.01 and
348 0.05. This apparent reduction in extraction yield is probably a result of sugar degradation.
349 Therefore, the extraction time of 20 min was selected as a sufficient time for the extraction
350 and hydrolysis of polysaccharides from PRs.
351 Trying to reduce the processing temperature more experiments were performed at 100
352 °C and atmospheric pressure (i.e. 101 kPa) instead of 121 °C and 219 kPa. We note that the
353 extraction efficiency was higher and the efficiency of hydrolysis lower at 100 °C than at 121
354 °C (comparisons of entries h and i, Table 1). Zhu and Liu (2013) examining the effect of
355 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 O_2 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 O_2 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).

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. coipomensis*, 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 CO_2. 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

468 sugars to ethanol cultivated under anaerobic conditions (Fig. 1 and 2). The yeast *S. cerevisiae*
469 is widely used worldwide in winemaking, in the brewery and baking industry, as well as in
470 many other biotechnological applications, as it possesses key features, such us quick
471 fermentation ability, genetic stability, tolerance to low pH environments, ethanol tolerance,
472 osmotolerance and thermotolerance (Nevoigt, 2008; Sarris et al., 2014). PR extracts derived
473 under laboratory and industrial processing conditions were used as a fermentation medium for
474 *S. cerevisiae*. As mentioned above, the difference between these processes is that under
475 industrial juicing the resulting extract had a higher sugar concentration. *M. guilliermondii*, is a
476 promising species implicated in various biotechnological applications, including the
477 conversion of pentoses to ethanol (Martini et al., 2016; Matos et al., 2014; Yan et al., 2021).
478 Similarly, *Scheffersomyces* and *Sugiyamaella* genera are reported as xylose-fermenting
479 genera containing species having an ethanol production capacity (Jia et al., 2020; Lopes et al.,
480 2018; Morais et al., 2020; Sena et al., 2017). This is the first report that describes bioethanol
481 production from *S. coipomoensis* and *S. paludigena* strains.

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

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

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

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

Oleic acid (C18:1) was the major FA in all lipids produced by the yeasts, followed by palmitic acid (C16:0) in the lipids of *M. guilliermondii*, *S. coipomoensis*, and *S. paludigena* (Table 4). In the lipids produced by *Y. lipolytica*, C16:0, palmitoleic (C16:1), and linoleic acid (C18:2) participated in non-negligible percentages. Stearic acid (C18:0) was detected in limited amounts in all lipids. According to these data, the oil produced seems to be suitable as raw material in the biodiesel industry. *Y. lipolytica* presented a similar FA profile to that previously reported when cultivated on olive mill wastewater or glycerol (Dourou et al., 2016; Makri et al., 2010; Papanikolaou and Aggelis, 2002). Contrary to the present study, Valdés et al. (2020b) reported that lipids of *M. guilliermondii* and *S. coipomensis* were rich in C16:1, and lipids of *S. paludigena* were rich in C18:2, suggesting that the nature of the carbon source 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 5^{th} 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

than that reported for *C. echinulata* ATHUM 4411 cultivated on orange peel, where GLA production was1.2-1.5 mg/g fermented substrate (Gema et al., 2002).

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

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

Funding

This work was financial supported by a) the project "INVALOR: Research Infrastructure for Waste Valorization and Sustainable Management" (MIS 5002495), which is implemented under the Action "Reinforcement of the Research and Innovation Infrastructure," funded by the Operational Program "Competitiveness, Entrepreneurship and Innovation" (NSRF 2014–2020) and co-financed by Greece and the European Union (European Regional Development

662 Fund), b) the project “Optimization of pomegranate tree cultivation in Achaia and
663 development of innovative products”, funded by the Regional Operational Program of
664 Western Greece 2014-2020 and The Bacfresh Company and c) the grant VEGA 1/0323/19
665 from Ministry of Education, Science, Research and Sports, Slovak Republic.

Table 1: Polysaccharide extraction and hydrolysis from PRs under various experimental conditions of H_2SO_4 concentration, processing time and temperature. The asterisk (*) indicates one set experiments performed using an open vessel assuring the presence of O_2. 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_2S0_4 (M)	Total Sugars (g/L)	Reducing Sugars (g/L)	Extraction (%)	Hydrolysis (%)
a	20	121	0.00	$34.7^{a} \pm 2.3$	$28.3^{a} \pm 0.9$	100.6	81.6
b	30	121	0.00	$30.3^{a} \pm 1.6$	$29.0^{a} \pm 1.6$	86.6	95.7
c	60	121	0.00	$32.5^{a} \pm 2.6$	$29.8^{a} \pm 2.6$	94.2	91.7
d	60	121	0.03	$32.5^{a} \pm 2.2$	$31.6^{a} \pm 3.0$	94.2	97.2
e	60	121	0.05	$33.5^{a} \pm 2.7$	$32.5^{b} \pm 3.4$	97.1	97.0
f	60	121	0.07	$30.4^{a} \pm 2.0$	$28.5^{a} \pm 3.3$	88.1	93.8
g	60	121	0.10	$34.5^{a} \pm 1.9$	$28.6^{a} \pm 3.1$	100.0	82.9
h	120	121	0.00	$30.9^{a} \pm 0.8$	$31.1^{a} \pm 0.3$	89.6	100.6
i	120	100	0.00	$31.7^{a} \pm 0.2$	$26.1^{a} \pm 0.6$	91.9	82.3
j*	120	100	0.00	$30.1^{a} \pm 0.5$	$24.9^{b} \pm 0.2$	87.2	82.7

674 **Table 2**: Cell mass, ethanol production, lipid accumulation and phenolic removal by different microorganisms cultivated on PR extract or solid residue under

675 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						
C. echinulata	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
C. tropicalis	Submerged	Aerobic	4.5	37.0[d, #]	nd	4.9	-	nd	nd
		Anaerobic	4.5	32.1[d, *]	nd	6.8	-	nd	nd
L. lipofer		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
M. guilliermondii		Aerobic	2.6	18.0[d, #]	2.1	3.2	18.1	2.3	48.8
		Anaerobic	2.6	19.3[d, #]	1.7	5.8	4.7	2.3	22.3
S. cerevisiae		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
S. coipomoensis		Aerobic	2.6	20.8[d, #]	0.7	0.2	8.3	2.0	22.7
		Anaerobic	2.6	21.5[d, #]	1.0	3.6	4.2	2.0	30.6
S. paludigena		Aerobic	2.6	18.0[d, #]	2.1	1.6	15.4	2.3	61.0
		Anaerobic	2.6	19.3[d, #]	1.5	5.3	9.0	2.3	22.1
Y. lipolytica		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

676 [a]Fermentation on 100% PRs; [b]Fermentation on blend 10% PRs and 90% WB; [c]Fermentation on blend 20% PRs and 80% OFs; [d]100% PR extract; [e]80% PR

677 extract and 20% water; [f]50% 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)		
M. guilliermondii	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
S. cerevisiae	Anaerobic	0.109	0.095	0.128	2.122	0.592	0.0038	0.373	0.321*	0.357	0.080*	0.844
S. coipomoensis	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
S. paludigena	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

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

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

684

685 **Table 5:** Fatty acid composition (%) of total lipids of *Cunninghamella echinulata* during
686 solid-state fermentation on PRs and WB used in different ratios. Data represent the mean of
687 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	90	0	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
		3	15.7 ± 0.3	0.9 ± 0.1	4.4 ± 0.5	29.7 ± 0.7	40.5 ± 0.7	5.0 ± 0.1	3.3 ± 0.2	0.5 ± 0.2
		5	13.9 ± 0.1	0.8 ± 0.2	3.5 ± 0.2	32.1 ± 1.0	34.1 ± 0.3	13.6 ± 0.2	1.7 ± 0.0	0.3 ± 0.0
20	80	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
30	70	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
40	60	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
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

689 **Table 6:** Fatty acid composition (%) of total lipids of *Cunninghamella echinulata* during
690 solid-state fermentation on PRs and OFs used in different ratios. Data represent the mean of
691 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 ± 1.1	0.9 ± 0.1	1.3 ± 0.2	38.9 ± 1.1	41.3 ± 0.2	ND	1.5 ± 0.1	0.4 ± 0.1
		5	14.5 ± 1.2	1.4 ± 0.2	2.5 ± 0.2	35.4 ± 1.3	34.3 ± 0.4	9.5 ± 0.5	2.1 ± 0.5	0.3 ± 0.1
10	90	0	16.9 ± 0.9	0.3 ± 0.0	1.5 ± 0.1	37.7 ± 0.7	41.5 ± 1.0	ND	1.9 ± 0.2	0.2 ± 0.0
		3	16.5 ± 1.0	0.6 ± 0.1	2.6 ± 0.3	38.2 ± 0.6	37.4 ± 0.9	2.9 ± 0.1	1.6 ± 0.1	0.2 ± 0.0
		5	14.8 ± 0.5	1.2 ± 0.1	4.4 ± 0.5	29.4 ± 0.3	39.9 ± 0.4	5.8 ± 0.3	4.3 ± 0.6	0.2 ± 0.0
20	80	0	16.1 ± 0.3	0.7 ± 0.0	1.3 ± 0.0	37.8 ± 1.1	41.8 ± 0.3	ND	2.1 ± 0.2	0.2 ± 0.1
		3	15.5 ± 0.8	0.5 ± 0.0	2.3 ± 0.2	37.0 ± 1.2	38.6 ± 0.8	3.8 ± 0.2	2.0 ± 0.3	0.3 ± 0.0
		5	16.6 ± 0.5	0.7 ± 0.2	4.0 ± 0.6	40.4 ± 0.5	32.8 ± 0.5	4.3 ± 0.1	0.9 ± 0.0	0.3 ± 0.1
30	70	0	17.2 ± 0.2	0.6 ± 0.1	1.9 ± 0.4	38.1 ± 0.2	39.6 ± 0.6	ND	2.4 ± 0.2	0.2 ± 0.0
		3	16.8 ± 0.9	0.2 ± 0.0	2.3 ± 0.3	37.3 ± 0.6	39.7 ± 0.2	1.6 ± 0.0	1.8 ± 0.1	0.3 ± 0.1
		5	16.4 ± 1.1	0.4 ± 0.0	2.1 ± 0.1	38.2 ± 0.7	40.0 ± 1.0	1.1 ± 0.1	1.6 ± 0.1	0.2 ± 0.0
40	60	0	16.9 ± 0.9	0.8 ± 0.2	2.4 ± 0.1	36.5 ± 0.5	41.2 ± 0.8	ND	2.1 ± 0.3	0.1 ± 0.0
		3	17.2 ± 0.5	0.7 ± 0.0	3.0 ± 0.6	39.7 ± 1.1	35.7 ± 0.3	1.9 ± 0.1	1.5 ± 0.3	0.3 ± 0.0
		5	17.8 ± 0.4	0.2 ± 0.0	1.6 ± 0.5	37.4 ± 0.8	40.4 ± 0.2	0.6 ± 0.0	1.7 ± 0.2	0.3 ± 0.1
50	50	0	19.7 ± 1.1	0.5 ± 0.1	5.0 ± 0.9	37.7 ± 0.2	34.4 ± 0.4	ND	1.9 ± 0.2	0.8 ± 0.2
		3	16.9 ± 0.6	0.5 ± 0.1	3.3 ± 0.4	35.4 ± 0.3	39.3 ± 0.1	2.2 ± 0.2	2.2 ± 0.1	0.2 ± 0.0
		5	19.0 ± 0.8	0.6 ± 0.2	3.8 ± 0.2	36.9 ± 0.4	38.1 ± 0.1	1.4 ± 0.1	0.1 ± 0.0	0.1 ± 0.0

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*.

715 **References**

716 https://www.worldbank.org/en/topic/agriculture/overview

717 Aggelis, G., Iconomou, D., Christou, M., Bokas, D., Kotzailias, S., Christou, G., Tsagou, V.,
718 Papanikolaou, S., 2003. Phenolic removal in a model olive oil mill wastewater using
719 *Pleurotus ostreatus* in bioreactor cultures and biological evaluation of the process.
720 Water Res. 37, 3897–3904. https://doi.org/10.1016/S0043-1354(03)00313-0

721 Akhtar, S., Ismail, T., Fraternale, D., Sestili, P., 2015. Pomegranate peel and peel extracts:
722 Chemistry and food features. Food Chem. 174, 417-425.
723 https://doi.org/10.1016/j.foodchem.2014.11.035

724 Arevalo-Gallegos, A., Ahmad, Z., Asgher, M., Parra-Saldivar, R., Iqbal, H.M.N., 2017.
725 Lignocellulose: A sustainable material to produce value-added products with a zero
726 waste approach—A review. Int. J. Biol. Macromol. 99, 308–318.
727 https://doi.org/10.1016/j.ijbiomac.2017.02.097

728 Banković-Ilić, I.B., Stojković, I.J., Stamenković, O.S., Veljkovic, V.B., Hung, Y.T., 2014.
729 Waste animal fats as feedstocks for biodiesel production. Renew. Sustain. Energy Rev.
730 32, 238-254. https://doi.org/10.1016/j.rser.2014.01.038

731 Baral, N.R., Shah, A., 2014. Microbial inhibitors: formation and effects on acetone-butanol-
732 ethanol fermentation of lignocellulosic biomass. Appl. Microbiol. Biotechnol. 98, 9151–
733 9172. https://doi.org/10.1007/s00253-014-6106-8

734 Bassiri-Jahromi, S., 2018. *Punica granaturn* (Pomegranate) activity in health promotion and
735 cancer prevention. Oncol. Rev. https://doi.org/10.4081/oncol.2018.345

736 Behera, S., Mohanty, R.C., Ray, R.C., 2011. Ethanol production from mahula (*Madhuca*
737 *latifolia L.*) flowers with immobilized cells of *Saccharomyces cerevisiae* in *Luffa*
738 *cylindrica L.* sponge discs. Appl. Energy. 88, 212-215.
739 https://doi.org/10.1016/j.apenergy.2010.07.035

740 Bellou, S., Triantaphyllidou, I.E., Aggeli, D., Elazzazy, A.M., Baeshen, M.N., Aggelis, G.,
741 2016. Microbial oils as food additives: Recent approaches for improving microbial oil
742 production and its polyunsaturated fatty acid content. Curr. Opin. Biotechnol. 37, 24–35.

https://doi.org/10.1016/j.copbio.2015.09.005

Birol, G., Doruker, P., Kirdar, B., Önsan, Z.I., Ülgen, K., 1998. Mathematical description of ethanol fermentation by immobilised *Saccharomyces cerevisiae*. Process Biochem. 33, 763-771. https://doi.org/10.1016/S0032-9592(98)00047-8

Bravo, C., Garcés, D., Faba, L., Sastre, H., Ordóñez, S., 2017. Selective arabinose extraction from *Pinus* sp. sawdust by two-step soft acid hydrolysis. Ind. Crops Prod. 104, 229-236. https://doi.org/10.1016/j.indcrop.2017.04.027

Çam, M., Hişil, Y., 2010. Pressurised water extraction of polyphenols from pomegranate peels. Food Chem. 123, 878–885. https://doi.org/10.1016/j.foodchem.2010.05.011

Caporusso, A., De Bari, I., Valerio, V., Albergo, R., Liuzzi, F., 2021. Conversion of cardoon crop residues into single cell oils by *Lipomyces tetrasporus* and *Cutaneotrichosporon curvatus*: process optimizations to overcome the microbial inhibition of lignocellulosic hydrolysates. Ind. Crops Prod. 159, 113030. https://doi.org/10.1016/j.indcrop.2020.113030

Carota, E., Petruccioli, M., D'Annibale, A., Crognale, S., 2020. Mixed glycerol and orange peel-based substrate for fed-batch microbial biodiesel production. Heliyon 6, e04801. https://doi.org/10.1016/j.heliyon.2020.e04801

Catania, P., Comparetti, A., De Pasquale, C., Morello, G., Vallone, M., 2020. Effects of the extraction technology on pomegranate juice quality. Agronomy 10, 1483. https://doi.org/10.3390/agronomy10101483

Certik, M., Adamechova, Z., 2009. Cereal-based bioproducts containing polyunsaturated fatty acids. Lipid Technol. 21, 250-253. https://doi.org/10.1002/lite.200900058

Čertík, M., Adamechová, Z., Guothová, L., 2013. Simultaneous enrichment of cereals with polyunsaturated fatty acids and pigments by fungal solid state fermentations. J. Biotechnol. 168, 130–134. https://doi.org/10.1016/j.jbiotec.2013.03.016

Čertík, M., Adamechová, Z., Laoteng, K., 2012. Microbial production of γ-linolenic acid: Submerged versus solid-state fermentations. Food Sci. Biotechnol. 21, 921–926. https://doi.org/10.1007/s10068-012-0121-2

771 Chatzifragkou, A., Makri, A., Belka, A., Bellou, S., Mavrou, M., Mastoridou, M., Mystrioti,
772 P., Onjaro, G., Aggelis, G., Papanikolaou, S., 2011. Biotechnological conversions of
773 biodiesel derived waste glycerol by yeast and fungal species. Energy 36, 1097–1108.
774 https://doi.org/10.1016/j.energy.2010.11.040
775 da S. Pereira, A., Fontes-Sant'Ana, G.C., Amaral, P.F.F., 2019. Mango agro-industrial wastes
776 for lipase production from *Yarrowia lipolytica* and the potential of the fermented solid
777 as a biocatalyst. Food Bioprod. Process. 115, 68–77.
778 https://doi.org/10.1016/j.fbp.2019.02.002
779 da Silveira, F.A., de Oliveira Soares, D.L., Bang, K.W., Balbino, T.R., de Moura Ferreira,
780 M.A., Diniz, R.H.S., de Lima, L.A., Brandão, M.M., Villas-Bôas, S.G., da Silveira,
781 W.B., 2020. Assessment of ethanol tolerance of *Kluyveromyces marxianus* CCT 7735
782 selected by adaptive laboratory evolution. Appl. Microbiol. Biotechnol. 104, 7483–
783 7494. https://doi.org/10.1007/s00253-020-10768-9
784 Daskalaki, A., Perdikouli, N., Aggeli, D., Aggelis, G., 2019. Laboratory evolution strategies
785 for improving lipid accumulation in *Yarrowia lipolytica*. Appl. Microbiol. Biotechnol.
786 103, 8585–8596. https://doi.org/10.1007/s00253-019-10088-7
787 De Bari, I., De Canio, P., Cuna, D., Liuzzi, F., Capece, A., Romano, P., 2013. Bioethanol
788 production from mixed sugars by *Scheffersomyces stipitis* free and immobilized cells,
789 and co-cultures with *Saccharomyces cerevisiae*. N. Biotechnol. 30, 591-597.
790 https://doi.org/10.1016/j.nbt.2013.02.003
791 De Melo, A.H.F., Lopes, A.M.M., Dezotti, N., Santos, I.L., Teixeira, G.S., Goldbeck, R.,
792 2020. Evolutionary engineering of two robust Brazilian industrial yeast strains for
793 thermotolerance and second-generation biofuels. Ind. Biotechnol. 16, 91-98.
794 https://doi.org/10.1089/ind.2019.0031
795 Di Fidio, N., Dragoni, F., Antonetti, C., De Bari, I., Raspolli Galletti, A.M., Ragaglini, G.,
796 2020. From paper mill waste to single cell oil: Enzymatic hydrolysis to sugars and their
797 fermentation into microbial oil by the yeast *Lipomyces starkeyi*. Bioresour. Technol.
798 315, 123790. https://doi.org/10.1016/j.biortech.2020.123790

799 Dien, B.S., Slininger, P.J., Kurtzman, C.P., Moser, B.R., O'Bryan, P.J., 2016. Identification
800 of superior lipid producing *Lipomyces* and *Myxozyma* yeasts. AIMS Environ. Sci. 3, 1–
801 20. https://doi.org/10.3934/environsci.2016.1.1
802 Diwan, B., Gupta, P., 2020. Conversion of rice straw to caprylic acid-rich microbial oils by
803 oleaginous yeast isolates. Biomass Convers. Biorefinery.
804 https://doi.org/10.1007/s13399-020-01039-8
805 Diwan, B., Gupta, P., 2019. Lignocellulosic biomass to fungal oils: A radical bioconversion
806 toward establishing a prospective resource, in: Yadav, A., Singh, S., Mishra, S., Gupta,
807 A. (Eds.), Recent advancement in white biotechnology through fungi. Springer, pp.
808 407–440. https://doi.org/10.1007/978-3-030-14846-1_14
809 Dobrowolski, A., Mituła, P., Rymowicz, W., Mirończuk, A.M., 2016. Efficient conversion of
810 crude glycerol from various industrial wastes into single cell oil by yeast *Yarrowia*
811 *lipolytica*. Bioresour. Technol. 207, 237-243.
812 https://doi.org/10.1016/j.biortech.2016.02.039
813 Donot, F., Fontana, A., Baccou, J.C., Strub, C., Schorr-Galindo, S., 2014. Single cell oils
814 (SCOs) from oleaginous yeasts and moulds: Production and genetics. Biomass and
815 Bioenergy 68, 135–150. https://doi.org/10.1016/j.biombioe.2014.06.016
816 Dourou, M., Aggeli, D., Papanikolaou, S., Aggelis, G., 2018. Critical steps in carbon
817 metabolism affecting lipid accumulation and their regulation in oleaginous
818 microorganisms. Appl. Microbiol. Biotechnol. 102, 2509–2523.
819 https://doi.org/10.1007/s00253-018-8813-z
820 Dourou, M., Kancelista, A., Juszczyk, P., Sarris, D., Bellou, S., Triantaphyllidou, I.E.,
821 Rywinska, A., Papanikolaou, S., Aggelis, G., 2016. Bioconversion of olive mill
822 wastewater into high-added value products. J. Clean. Prod. 139, 957–969.
823 https://doi.org/10.1016/j.jclepro.2016.08.133
824 Dourou, M., Mizerakis, P., Papanikolaou, S., Aggelis, G., 2017. Storage lipid and
825 polysaccharide metabolism in *Yarrowia lipolytica* and *Umbelopsis isabellina*. Appl.
826 Microbiol. Biotechnol. 101, 7213–7226. https://doi.org/10.1007/s00253-017-8455-6

827 Dubois, M., Gilles, K., Hamilton, J.K., Rebers, P.A., Smith, F., 1951. A colorimetric method
828 for the determination of sugars. Nature 168, 167. https://doi.org/10.1038/168167a0
829 Dupont, S., Lemetais, G., Ferreira, T., Cayot, P., Gervais, P., Beney, L., 2012. Ergosterol
830 biosynthesis: A fungal pathway for life on land? Evolution. 66, 2961-2968.
831 https://doi.org/10.1111/j.1558-5646.2012.01667.x
832 Economou, C.N., Makri, A., Aggelis, G., Pavlou, S., Vayenas, D. V., 2010. Semi-solid state
833 fermentation of sweet sorghum for the biotechnological production of single cell oil.
834 Bioresour. Technol. 101, 1385–1388. https://doi.org/10.1016/j.biortech.2009.09.028
835 Fakas, S., Čertik, M., Papanikolaou, S., Aggelis, G., Komaitis, M., Galiotou-Panayotou, M.,
836 2008. γ-Linolenic acid production by *Cunninghamella echinulata* growing on complex
837 organic nitrogen sources. Bioresour. Technol. 99, 5986–5990.
838 https://doi.org/10.1016/j.biortech.2007.10.016
839 Fakas, S., Makri, A., Mavromati, M., Tselepi, M., Aggelis, G., 2009. Fatty acid composition
840 in lipid fractions lengthwise the mycelium of *Mortierella isabellina* and lipid production
841 by solid state fermentation. Bioresour. Technol. 100, 6118–6120.
842 https://doi.org/10.1016/j.biortech.2009.06.015
843 Fernandes, S., Murray, P.G., 2010. Metabolic engineering for improved microbial pentose
844 fermentation. Bioeng. Bugs 1, 424–428. https://doi.org/10.4161/bbug.1.6.12724
845 Folch, J., Lees, M., Stanley, G.H.S., 1957. A simple method for the isolation and purification
846 of total lipides from animal tissues. J. Biol. Chem. 226, 497–509.
847 Folin, O., Ciocalteau, V., 1927. Tyrosine and tryptophane in proteins. J. Biol. Chem. 73, 627–
848 648.
849 Gajdoš, P., Nicaud, J.M., Rossignol, T., Čertík, M., 2015. Single cell oil production on
850 molasses by *Yarrowia lipolytica* strains overexpressing *DGA2* in multicopy. Appl.
851 Microbiol. Biotechnol. 99, 8065-8074. https://doi.org/10.1007/s00253-015-6733-8.
852 Gema, H., Kavadia, A., Dimou, D., Tsagou, V., Komaitis, M., Aggelis, G., 2002. Production
853 of γ-linolenic acid by *Cunninghamella echinulata* cultivated on glucose and orange peel.
854 Appl. Microbiol. Biotechnol. 58, 303–307. https://doi.org/10.1007/s00253-001-0910-7

855 Ghinea, C., Apostol, L.C., Prisacaru, A.E., Leahu, A., 2019. Development of a model for food
856 waste composting. Environ. Sci. Pollut. Res. 26, 4056–4069.
857 https://doi.org/10.1007/s11356-018-3939-1
858 Gong, Z., Wang, Q., Shen, H., Hu, C., Jin, G., Zhao, Z.K., 2012. Co-fermentation of
859 cellobiose and xylose by *Lipomyces starkeyi* for lipid production. Bioresour. Technol.
860 117, 20–24. https://doi.org/10.1016/j.biortech.2012.04.063
861 Gopinarayanan, V.E., Nair, N.U., 2019. Pentose metabolism in *Saccharomyces cerevisiae*:
862 The need to engineer global regulatory systems. Biotechnol. J. 14, 1800364.
863 https://doi.org/10.1002/biot.201800364
864 Goula, A.M., Ververi, M., Adamopoulou, A., Kaderides, K., 2017. Green ultrasound-assisted
865 extraction of carotenoids from pomegranate wastes using vegetable oils. Ultrason.
866 Sonochem. 34, 821-830. https://doi.org/10.1016/j.ultsonch.2016.07.022
867 Gray, K.A., Zhao, L., Emptage, M., 2006. Bioethanol. Curr. Opin. Chem. Biol. 10, 141–146.
868 https://doi.org/10.1016/j.cbpa.2006.02.035
869 Gutiérrez, C.D.B., Serna, D.L.R., Alzate, C.A.C., 2017. A comprehensive review on the
870 implementation of the biorefinery concept in biodiesel production plants. Biofuel Res. J.
871 4, 691-703. https://doi.org/10.18331/BRJ2017.4.3.6
872 Haghighi Mood, S., Hossein Golfeshan, A., Tabatabaei, M., Salehi Jouzani, G., Najafi, G.H.,
873 Gholami, M., Ardjmand, M., 2013. Lignocellulosic biomass to bioethanol, a
874 comprehensive review with a focus on pretreatment. Renew. Sustain. Energy Rev. 27,
875 77–93. https://doi.org/10.1016/j.rser.2013.06.033
876 Hahn-Hägerdal, B., Karhumaa, K., Fonseca, C., Spencer-Martins, I., Gorwa-Grauslund, M.F.,
877 2007. Towards industrial pentose-fermenting yeast strains. Appl. Microbiol. Biotechnol.
878 74, 937–953. https://doi.org/10.1007/s00253-006-0827-2
879 Hasnaoui, N., Wathelet, B., Jiménez-Araujo, A., 2014. Valorization of pomegranate peel from
880 12 cultivars: Dietary fibre composition, antioxidant capacity and functional properties.
881 Food Chem. 160, 196–203. https://doi.org/10.1016/j.foodchem.2014.03.089
882 Hinshelwood, C.N., 1947. The chemical kinetics of the bacterial cell. J. Am. Chem. Soc.

883 https://doi.org/10.1021/ja01201a035

884 Janga, K.K., Hägg, M.B., Moe, S.T., 2012. Influence of acid concentration, temperature, and
885 time on decrystallization in two-stage concentrated sulfuric acid hydrolysis of pinewood
886 and aspenwood: A statistical approach. BioResources. 7, 391-411.
887 http://ncsu.edu/bioresources ISSN: 1930-2126

888 Jia, R., Lv, S., Chai, C., Hui, F., 2020. Three new *Scheffersomyces* species associated with
889 insects and rotting wood in China. MycoKeys 71, 87–99.
890 https://doi.org/10.3897/mycokeys.71.56168

891 Jin, H., Liu, R., He, Y., 2012. Kinetics of batch fermentations for ethanol production with
892 immobilized *Saccharomyces cerevisiae* growing on sweet sorghum stalk juice. Procedia
893 Environ. Sci. 12, 137-145. https://doi.org/10.1016/j.proenv.2012.01.258

894 Joseph, M.M., Aravind, S.R., George, S.K., Varghese, S., Sreelekha, T.T., 2013. A
895 galactomannan polysaccharide from *Punica granatum* imparts in vitro and in vivo
896 anticancer activity. Carbohydr. Polym. 98, 1466-1475.
897 https://doi.org/10.1016/j.carbpol.2013.07.023

898 Kaderides, K., Goula, A.M., Adamopoulos, K.G., 2015. A process for turning pomegranate
899 peels into a valuable food ingredient using ultrasound-assisted extraction and
900 encapsulation. Innov. Food Sci. Emerg. Technol. 31, 204–215.
901 https://doi.org/10.1016/j.ifset.2015.08.006

902 Kanakdande, A.P., Khobragade, C.N., Mane, R.S., 2020. Utilization of pomegranate waste-
903 peel as a novel substrate for biodiesel production by: *Bacillus cereus* (MF908505).
904 Sustain. Energy Fuels 4, 1199–1207. https://doi.org/10.1039/c9se00584f

905 Karagoz, P., Bill, R.M., Ozkan, M., 2019. Lignocellulosic ethanol production: Evaluation of
906 new approaches, cell immobilization and reactor configurations. Renew. Energy. 143,
907 741-752. https://doi.org/10.1016/j.renene.2019.05.045

908 Klempová, T., Slaný, O., Šišmiš, M., Marcinčák, S., Čertík, M., 2020. Dual production of
909 polyunsaturated fatty acids and beta-carotene with *Mucor wosnessenskii* by the process
910 of solid-state fermentation using agro-industrial waste. J. Biotechnol. 311, 1–11.

911 https://doi.org/10.1016/j.jbiotec.2020.02.006

912 Kostov, G., Popova, S., Gochev, V., Koprinkova-Hristova, P., Angelov, M., Georgieva, A.,

913 2012. Modeling of batch alcohol fermentation with free and immobilized yeasts

914 *Saccharomyces cerevisiae* 46 EVD. Biotechnol. Biotechnol. Equip. 26, 3021-3030.

915 https://doi.org/10.5504/bbeq.2012.0025

916 Ledesma-Amaro, R., Nicaud, J.-M., 2016. Metabolic engineering for expanding the substrate

917 range of *Yarrowia lipolytica*. Trends Biotechnol. 34, 798–809.

918 https://doi.org/10.1016/j.tibtech.2016.04.010

919 Li, Q., Du, W., Liu, D., 2008. Perspectives of microbial oils for biodiesel production. Appl.

920 Microbiol. Biotechnol. 80, 749–756. https://doi.org/10.1007/s00253-008-1625-9

921 Li, W.C., Zhu, J.Q., Zhao, X., Qin, L., Xu, T., Zhou, X., Li, X., Li, B.Z., Yuan, Y.J., 2019.

922 Improving co-fermentation of glucose and xylose by adaptive evolution of engineering

923 xylose-fermenting *Saccharomyces cerevisiae* and different fermentation strategies.

924 Renew. Energy. 139, 1176-1183. https://doi.org/10.1016/j.renene.2019.03.028

925 Liang, M.H., Jiang, J.G., 2013. Advancing oleaginous microorganisms to produce lipid via

926 metabolic engineering technology. Prog. Lipid Res. 52, 395–408.

927 https://doi.org/10.1016/j.plipres.2013.05.002

928 Liu, X., Xu, W., Mao, L., Zhang, C., Yan, P., Xu, Z., Zhang, Z.C., 2016. Lignocellulosic

929 ethanol production by starch-base industrial yeast under PEG detoxification. Sci. Rep. 6,

930 20361. https://doi.org/10.1038/srep20361

931 Lopes, M.R., Batista, T.M., Franco, G.R., Ribeiro, L.R., Santos, A.R.O., Furtado, C., Moreira,

932 R.G., Goes-Neto, A., Vital, M.J.S., Rosa, L.H., Lachance, M.-A., Rosa, C.A., 2018.

933 *Scheffersomyces stambukii* f.a., sp. nov., a d-xylose-fermenting species isolated from

934 rotting wood. Int. J. Syst. Evol. Microbiol. 68, 2306–2312.

935 https://doi.org/10.1099/ijsem.0.002834

936 Makri, A., Fakas, S., Aggelis, G., 2010. Metabolic activities of biotechnological interest in

937 *Yarrowia lipolytica* grown on glycerol in repeated batch cultures. Bioresour. Technol.

938 101, 2351–2358. https://doi.org/10.1016/j.biortech.2009.11.024

939 Martini, C., Tauk-Tornisielo, S.M., Codato, C.B., Bastos, R.G., Ceccato-Antonini, S.R., 2016.
940 A strain of *Meyerozyma guilliermondii* isolated from sugarcane juice is able to grow and
941 ferment pentoses in synthetic and bagasse hydrolysate media. World J. Microbiol.
942 Biotechnol. 32, 80. https://doi.org/10.1007/s11274-016-2036-1
943 Matos, I.T.S.R., Cassa-Barbosa, L.A., Galvão, R. de S.M., Nunes-Silva, C.G., Filho, S.A.,
944 2014. Isolation, taxonomic identification and investigation of the biotechnological
945 potential of wild-type *Meyerozyma guilliermondii* associated with Amazonian termites
946 able to ferment D-xylose. Biosci. J. 30, 260-266.
947 Miller, G.L., 1959. Use of dinitrosalicylic acid reagent for determination of reducing sugar.
948 Anal. Chem. 31, 426–428. https://doi.org/10.1021/ac60147a030
949 Monod, J., 1942. Recherches sur la croissance des cultures bacteriennes. Ann. Inst. Pasteur.
950 Morais, C.G., Sena, L.M.F., Lopes, M.R., Santos, A.R.O., Barros, K.O., Alves, C.R.,
951 Uetanabaro, A.P.T., Lachance, M.A., Rosa, C.A., 2020. Production of ethanol and
952 xylanolytic enzymes by yeasts inhabiting rotting wood isolated in sugarcane bagasse
953 hydrolysate. Fungal Biol. 124, 639–647. https://doi.org/10.1016/j.funbio.2020.03.005
954 Mosier, N., 2005. Features of promising technologies for pretreatment of lignocellulosic
955 biomass. Bioresour. Technol. 96, 673–686.
956 https://doi.org/10.1016/j.biortech.2004.06.025
957 Mphahlele, R.R., Fawole, O.A., Mokwena, L.M., Opara, U.L., 2016. Effect of extraction
958 method on chemical, volatile composition and antioxidant properties of pomegranate
959 juice. South African J. Bot. 103, 135–144. https://doi.org/10.1016/j.sajb.2015.09.015
960 Nevoigt, E., 2008. Progress in metabolic engineering of *Saccharomyces cerevisiae*.
961 Microbiol. Mol. Biol. Rev. 72, 379–412. https://doi.org/10.1128/MMBR.00025-07
962 Niehus, X., Crutz-Le Coq, A.-M., Sandoval, G., Nicaud, J.-M., Ledesma-Amaro, R., 2018.
963 Engineering *Yarrowia lipolytica* to enhance lipid production from lignocellulosic
964 materials. Biotechnol. Biofuels 11, 11. https://doi.org/10.1186/s13068-018-1010-6
965 Nitsos, C., Matsakas, L., Triantafyllidis, K., Rova, U., Christakopoulos, P., 2018.
966 Investigation of different pretreatment methods of Mediterranean-type ecosystem

967 agricultural residues: characterisation of pretreatment products, high-solids enzymatic
968 hydrolysis and bioethanol production. Biofuels 9, 545–558.
969 https://doi.org/10.1080/17597269.2017.1378988
970 Ntaikou, I., Menis, N., Alexandropoulou, M., Antonopoulou, G., Lyberatos, G., 2018.
971 Valorization of kitchen biowaste for ethanol production via simultaneous
972 saccharification and fermentation using co-cultures of the yeasts *Saccharomyces*
973 *cerevisiae* and *Pichia stipitis*. Bioresour. Technol. 263, 75-83.
974 https://doi.org/10.1016/j.biortech.2018.04.109
975 Osorio-González, C.S., Hegde, K., Brar, S.K., Kermanshahipour, A., Avalos-Ramírez, A.,
976 2019. Challenges in lipid production from lignocellulosic biomass using
977 *Rhodosporidium* sp.; A look at the role of lignocellulosic inhibitors. Biofuels, Bioprod.
978 Biorefining. 13, 740-759. https://doi.org/10.1002/bbb.1954
979 Papanikolaou, S., Aggelis, G., 2011a. Lipids of oleaginous yeasts. Part I: Biochemistry of
980 single cell oil production. Eur. J. Lipid Sci. Technol. 113, 1031–1051.
981 https://doi.org/10.1002/ejlt.201100014
982 Papanikolaou, S., Aggelis, G., 2011b. Lipids of oleaginous yeasts. Part II: Technology and
983 potential applications. Eur. J. Lipid Sci. Technol. 113, 1052–1073.
984 https://doi.org/10.1002/ejlt.201100015
985 Papanikolaou, S., Aggelis, G., 2002. Lipid production by *Yarrowia lipolytica* growing on
986 industrial glycerol in a single-stage continuous culture. Bioresour. Technol. 82, 43–49.
987 https://doi.org/10.1016/S0960-8524(01)00149-3
988 Pasha, C., Rao, L.V., 2009. Yeast biotechnology: Diversity and applications. Springer
989 Netherlands, Dordrecht. pp. 551-588. https://doi.org/10.1007/978-1-4020-8292-4
990 Pathak, P.D., Mandavgane, S.A., Kulkarni, B.D., 2017. Valorization of pomegranate peels: A
991 biorefinery approach. Waste and biomass valorization. 8, 1127-1137.
992 https://doi.org/10.1007/s12649-016-9668-0
993 Patsios, S.I., Dedousi, A., Sossidou, E.N., Zdragas, A., 2020. Sustainable animal feed protein
994 through the cultivation of *Yarrowia lipolytica* on agro-industrial wastes and by-products.

995 Sustainability 12, 1398. https://doi.org/10.3390/su12041398
996 Pereira, P.H.F., Oliveira, T.Í.S., Rosa, M.F., Cavalcante, F.L., Moates, G.K., Wellner, N.,
997 Waldron, K.W., Azeredo, H.M.C., 2016. Pectin extraction from pomegranate peels with
998 citric acid. Int. J. Biol. Macromol. 88, 373-379.
999 https://doi.org/10.1016/j.ijbiomac.2016.03.074
1000 Pocan, P., Bahcegul, E., Oztop, M.H., Hamamci, H., 2018. Enzymatic hydrolysis of fruit
1001 peels and other lignocellulosic biomass as a source of sugar. Waste and Biomass
1002 Valorization. 9, 929-937. https://doi.org/10.1007/s12649-017-9875-3
1003 Rastogi, M., Shrivastava, S., 2017. Recent advances in second generation bioethanol
1004 production: An insight to pretreatment, saccharification and fermentation processes.
1005 Renew. Sustain. Energy Rev. 80, 330–340. https://doi.org/10.1016/j.rser.2017.05.225
1006 Reichert, P., 1998. AQUASIM 2.0 - Computer Program for the identification and simulation
1007 of aquatic systems - User Manual, Water Science and Technology.
1008 Roukas, T., Kotzekidou, P., 2020. Pomegranate peel waste: a new substrate for citric acid
1009 production by *Aspergillus niger* in solid-state fermentation under non-aseptic conditions.
1010 Environ. Sci. Pollut. Res. 27, 13105–13113. https://doi.org/10.1007/s11356-020-07928-
1011 9
1012 Sadh, P.K., Duhan, S., Duhan, J.S., 2018. Agro-industrial wastes and their utilization using
1013 solid state fermentation: a review. Bioresour. Bioprocess. 5, 1.
1014 https://doi.org/10.1186/s40643-017-0187-z
1015 Sánchez, Ó.J., Cardona, C.A., 2008. Trends in biotechnological production of fuel ethanol
1016 from different feedstocks. Bioresour. Technol. 99, 5270–5295.
1017 https://doi.org/10.1016/j.biortech.2007.11.013
1018 Santos, M.P., Souza, M.C., Sumere, B.R., da Silva, L.C., Cunha, D.T., Bezerra, R.M.N.,
1019 Rostagno, M.A., 2019. Extraction of bioactive compounds from pomegranate peel
1020 (*Punica granatum L.*) with pressurized liquids assisted by ultrasound combined with an
1021 expansion gas. Ultrason. Sonochem. 54, 11–17.
1022 https://doi.org/10.1016/j.ultsonch.2019.02.021

1023 Santosh, I., Ashtavinayak, P., Amol, D., Sanjay, P., 2017. Enhanced bioethanol production
1024 from different sugarcane bagasse cultivars using co-culture of *Saccharomyces cerevisiae*
1025 and *Scheffersomyces* (*Pichia*) *stipitis*. J. Environ. Chem. Eng. 5, 2861-2868.
1026 https://doi.org/10.1016/j.jece.2017.05.045
1027 Sarkar, N., Ghosh, S.K., Bannerjee, S., Aikat, K., 2012. Bioethanol production from
1028 agricultural wastes: An overview. Renew. Energy 37, 19–27.
1029 https://doi.org/10.1016/j.renene.2011.06.045
1030 Sarris, D., Giannakis, M., Philippoussis, A., Komaitis, M., Koutinas, A.A., Papanikolaou, S.,
1031 2013. Conversions of olive mill wastewater-based media by *Saccharomyces cerevisiae*
1032 through sterile and non-sterile bioprocesses. J. Chem. Technol. Biotechnol. 88, 958–
1033 969. https://doi.org/10.1002/jctb.3931
1034 Sarris, D., Kotseridis, Y., Linga, M., Galiotou-Panayotou, M., Papanikolaou, S., 2009.
1035 Enhanced ethanol production, volatile compound biosynthesis and fungicide removal
1036 during growth of a newly isolated *Saccharomyces cerevisiae* strain on enriched
1037 pasteurized grape musts. Eng. Life Sci. 9, 29–37.
1038 https://doi.org/10.1002/elsc.200800059
1039 Sarris, D., Matsakas, L., Aggelis, G., Koutinas, A.A., Papanikolaou, S., 2014. Aerated vs non-
1040 aerated conversions of molasses and olive mill wastewaters blends into bioethanol by
1041 *Saccharomyces cerevisiae* under non-aseptic conditions. Ind. Crops Prod. 56, 83–93.
1042 https://doi.org/10.1016/j.indcrop.2014.02.040
1043 Sarris, D., Papanikolaou, S., 2016. Biotechnological production of ethanol: Biochemistry,
1044 processes and technologies. Eng. Life Sci. 16, 307–329.
1045 https://doi.org/10.1002/elsc.201400199
1046 Sena, L.M.F., Morais, C.G., Lopes, M.R., Santos, R.O., Uetanabaro, A.P.T., Morais, P.B.,
1047 Vital, M.J.S., de Morais, M.A., Lachance, M.A., Rosa, C.A., 2017. d-Xylose
1048 fermentation, xylitol production and xylanase activities by seven new species of
1049 *Sugiyamaella*. Antonie van Leeuwenhoek, Int. J. Gen. Mol. Microbiol. 110, 53–67.
1050 https://doi.org/10.1007/s10482-016-0775-5

1051 Sharma, P., McClees, S.F., Afaq, F., 2017. Pomegranate for prevention and treatment of
1052 cancer: An update. Molecules. 22, 177. https://doi.org/10.3390/molecules22010177
1053 Siddiqui, M.T.H., Nizamuddin, S., Mubarak, N.M., Shirin, K., Aijaz, M., Hussain, M.,
1054 Baloch, H.A., 2019. Characterization and process optimization of biochar produced
1055 using novel biomass, waste pomegranate peel: A response surface methodology
1056 approach. Waste and Biomass Valorization 10, 521–532.
1057 https://doi.org/10.1007/s12649-017-0091-y
1058 Singh, A., Sharma, P., Saran, A.K., Singh, N., Bishnoi, N.R., 2013. Comparative study on
1059 ethanol production from pretreated sugarcane bagasse using immobilized
1060 *Saccharomyces cerevisiae* on various matrices. Renew. Energy. 50, 488-493.
1061 https://doi.org/10.1016/j.renene.2012.07.003
1062 Singh, J., Gu, S., 2010. Commercialization potential of microalgae for biofuels production.
1063 Renew. Sustain. Energy Rev. 14, 2596–2610. https://doi.org/10.1016/j.rser.2010.06.014
1064 Sitepu, I.R., Sestric, R., Ignatia, L., Levin, D., German, J.B., Gillies, L.A., Almada, L.A.G.,
1065 Boundy-Mills, K.L., 2013. Manipulation of culture conditions alters lipid content and
1066 fatty acid profiles of a wide variety of known and new oleaginous yeast species.
1067 Bioresour. Technol. 144, 360–369. https://doi.org/10.1016/j.biortech.2013.06.047
1068 Slaný, O., Klempová, T., Marcinčák, S., Čertík, M., 2020. Production of high-value
1069 bioproducts enriched with γ-linolenic acid and β-carotene by filamentous fungi
1070 *Umbelopsis isabellina* using solid-state fermentations. Ann. Microbiol. 70, 17.
1071 https://doi.org/10.1186/s13213-020-01559-8
1072 Slaný, O., Klempová, T., Shapaval, V., Zimmermann, B., Kohler, A., Čertík, M., 2021.
1073 Animal fat as a substrate for production of n-6 fatty acids by fungal solid-state
1074 fermentation. Microorganisms 9, 170. https://doi.org/10.3390/microorganisms9010170
1075 Slininger, P.J., Dien, B.S., Kurtzman, C.P., Moser, B.R., Bakota, E.L., Thompson, S.R.,
1076 O'Bryan, P.J., Cotta, M.A., Balan, V., Jin, M., Sousa, L. da C., Dale, B.E., 2016.
1077 Comparative lipid production by oleaginous yeasts in hydrolyzates of lignocellulosic
1078 biomass and process strategy for high titers. Biotechnol. Bioeng. 113, 1676-1690.

https://doi.org/10.1002/bit.25928

Sood, A., Gupta, M., 2015. Extraction process optimization for bioactive compounds in pomegranate peel. Food Biosci. 12, 100–106. https://doi.org/10.1016/j.fbio.2015.09.004

Tian, S.-Q., Chen, Z.-C., 2016. Dynamic analysis of bioethanol production from corn stover and immobilized yeast. BioResources 11. https://doi.org/10.15376/biores.11.3.6040-6049

Tsioulpas, A., Dimou, D., Iconomou, D., Aggelis, G., 2002. Phenolic removal in olive oil mill wastewater by strains of *Pleurotus* spp. in respect to their phenol oxidase (laccase) activity. Bioresour. Technol. 84, 251–257. https://doi.org/10.1016/S0960-8524(02)00043-3

Türkyılmaz, M., Tağı, Ş., Dereli, U., Özkan, M., 2013. Effects of various pressing programs and yields on the antioxidant activity, antimicrobial activity, phenolic content and colour of pomegranate juices. Food Chem. 138, 1810–1818. https://doi.org/10.1016/j.foodchem.2012.11.100

Valdés, G., Mendonça, R.T., Aggelis, G., 2020a. Lignocellulosic biomass as a substrate for oleaginous microorganisms: A review. Appl. Sci. 10, 7698. https://doi.org/10.3390/app10217698

Valdés, G., Mendonça, R.T., Parra, C., Aggelis, G., 2020b. Patterns of lignocellulosic sugar assimilation and lipid production by newly isolated yeast strains from Chilean Valdivian Forest. Appl. Biochem. Biotechnol. 192, 1124–1146. https://doi.org/10.1007/s12010-020-03398-4

Vasaki, M., Karri, R.R., Ravindran, G., Paramasivan, B., 2021. Predictive capability evaluation and optimization of sustainable biodiesel production from oleaginous biomass grown on pulp and paper industrial wastewater. Renew. Energy 168, 204–215. https://doi.org/10.1016/j.renene.2020.12.038

Volkman, J.K., 2003. Sterols in microorganisms. Appl. Microbiol. Biotechnol. 60, 495–506. https://doi.org/10.1007/s00253-002-1172-8

Wei, Y., Siewers, V., Nielsen, J., 2017. Cocoa butter-like lipid production ability of non-

oleaginous and oleaginous yeasts under nitrogen-limited culture conditions. Appl. Microbiol. Biotechnol. 101, 3577–3585. https://doi.org/10.1007/s00253-017-8126-7

Xu, M., Ran, L., Chen, N., Fan, X., Ren, D., Yi, L., 2019. Polarity-dependent extraction of flavonoids from citrus peel waste using a tailor-made deep eutectic solvent. Food Chem. 297, 124970. https://doi.org/10.1016/j.foodchem.2019.124970

Yan, W., Gao, H., Qian, X., Jiang, Y., Zhou, J., Dong, W., Xin, F., Zhang, W., Jiang, M., 2021. Biotechnological applications of the non-conventional yeast *Meyerozyma guilliermondii*. Biotechnol. Adv. 46, 107674. https://doi.org/10.1016/j.biotechadv.2020.107674

Yook, S. Do, Kim, J., Gong, G., Ko, J.K., Um, Y., Han, S.O., Lee, S., 2020. High□yield lipid production from lignocellulosic biomass using engineered xylose□utilizing *Yarrowia lipolytica*. GCB Bioenergy 12, 670–679. https://doi.org/10.1111/gcbb.12699

Yu, Z., Du, Y., Shang, X., Zheng, Y., Zhou, J., 2018. Enhancing fermentable sugar yield from cassava residue using a two-step dilute ultra-low acid pretreatment process. Ind. Crops Prod. 124, 555-562. https://doi.org/10.1016/j.indcrop.2018.08.029

Zhai, X., Zhu, C., Li, Y., Zhang, Y., Duan, Z., Yang, X., 2018a. Optimization for pectinase-assisted extraction of polysaccharides from pomegranate peel with chemical composition and antioxidant activity. Int. J. Biol. Macromol. 109, 244–253. https://doi.org/10.1016/j.ijbiomac.2017.12.064

Zhai, X., Zhu, C., Zhang, Y., Sun, J., Alim, A., Yang, X., 2018b. Chemical characteristics, antioxidant capacities and hepatoprotection of polysaccharides from pomegranate peel. Carbohydr. Polym. 202, 461–469. https://doi.org/10.1016/j.carbpol.2018.09.013

Zhang, J., Bao, J., 2018. Lignocellulose pretreatment using acid as catalyst, in: Handbook of biorefinery research and technology. https://doi.org/10.1007/978-94-007-6724-9_3-1

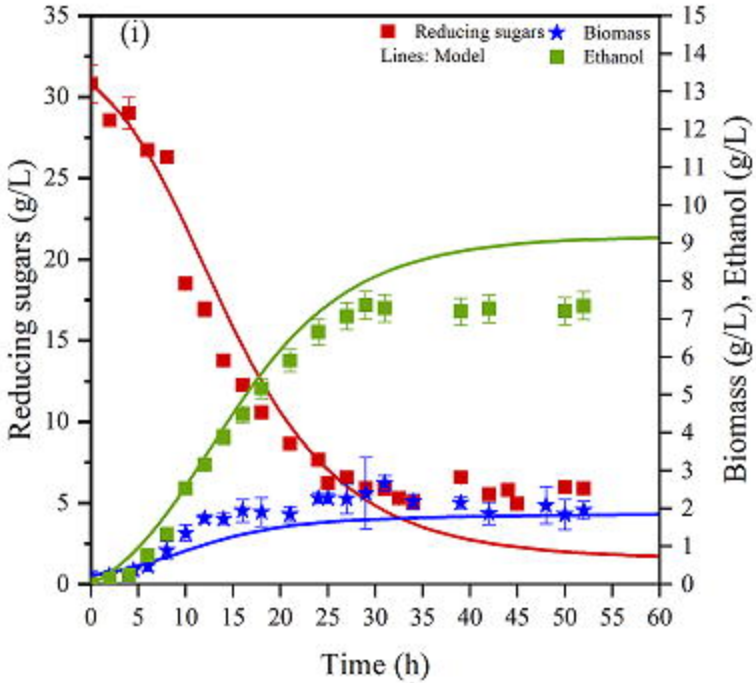
Zhao, X., Kong, X., Hua, Y., Feng, B., Zhao, Z., 2008. Medium optimization for lipid production through co-fermentation of glucose and xylose by the oleaginous yeast *Lipomyces starkeyi*. Eur. J. Lipid Sci. Technol. 110, 405-412. https://doi.org/10.1002/ejlt.200700224

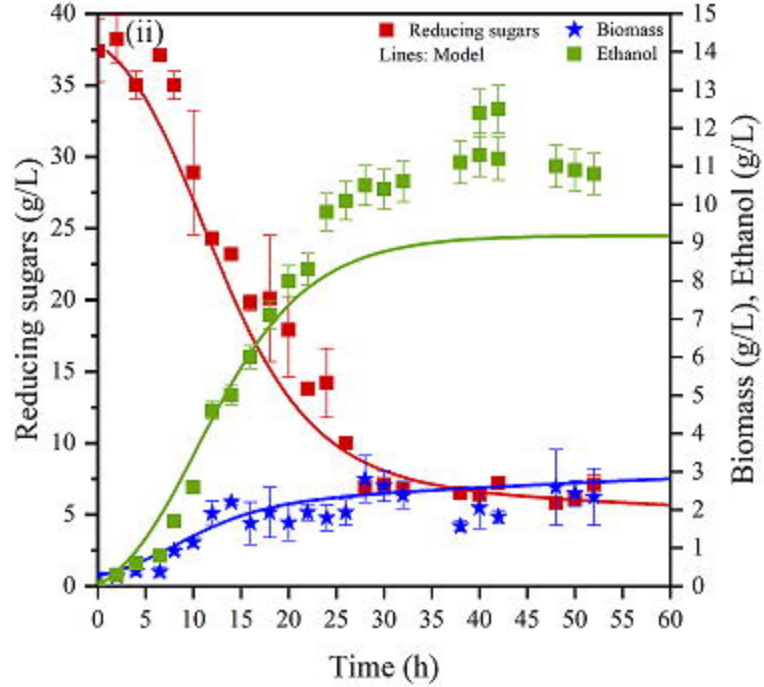
1135 Zhu, C., Liu, X., 2013. Optimization of extraction process of crude polysaccharides from

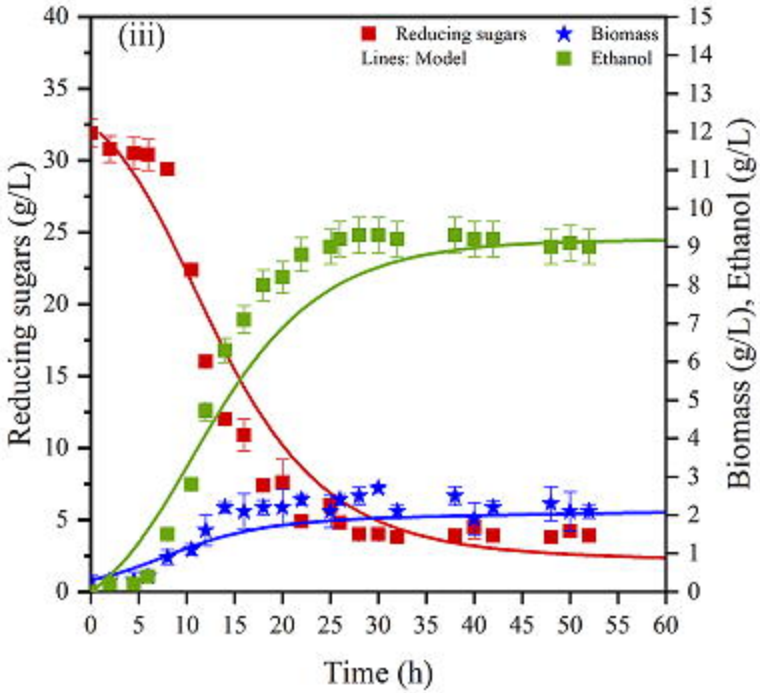
1136 Pomegranate peel by response surface methodology. Carbohydr. Polym. 92, 1197–1202.

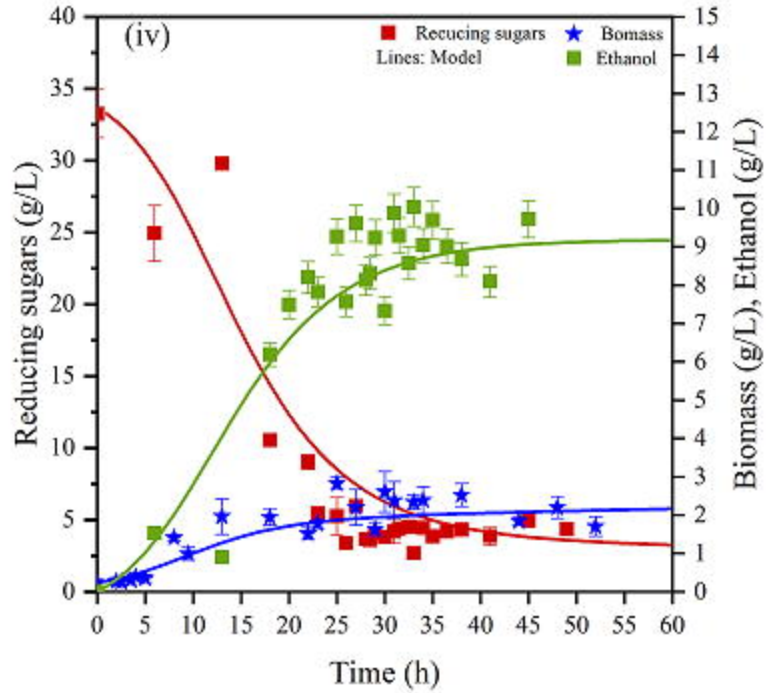
1137 https://doi.org/10.1016/j.carbpol.2012.10.073

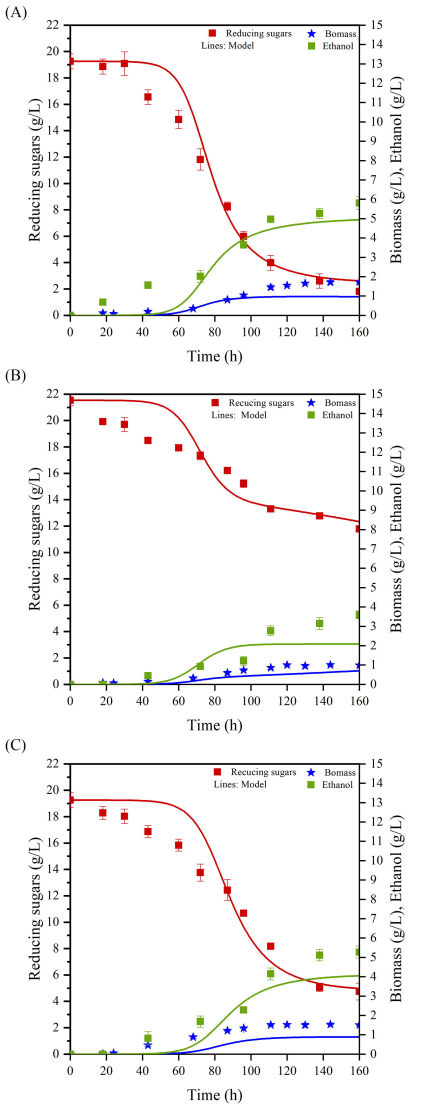
1138


(i)
Reducing sugars
Biomass
Lines: Model
Ethanol
Reducing sugars (g/L)
Biomass (g/L), Ethanol (g/L)
Time (h)

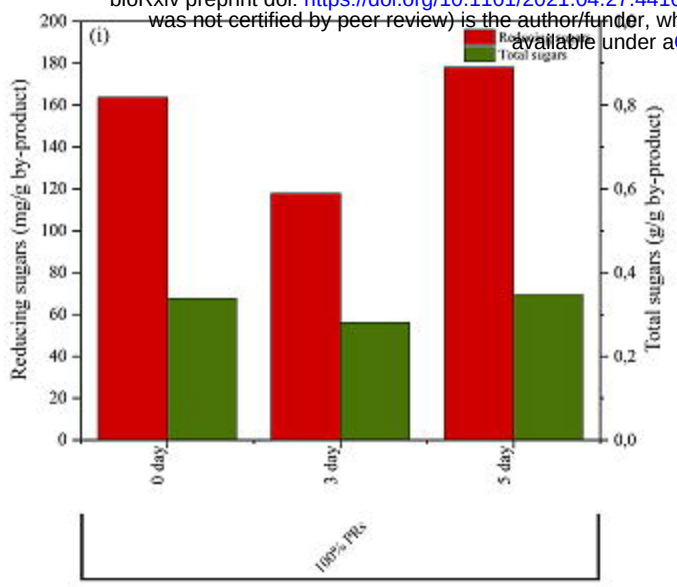

(ii)
Reducing sugars
Biomass
Lines: Model
Ethanol
Reducing sugars (g/L)
Biomass (g/L), Ethanol (g/L)
Time (h)

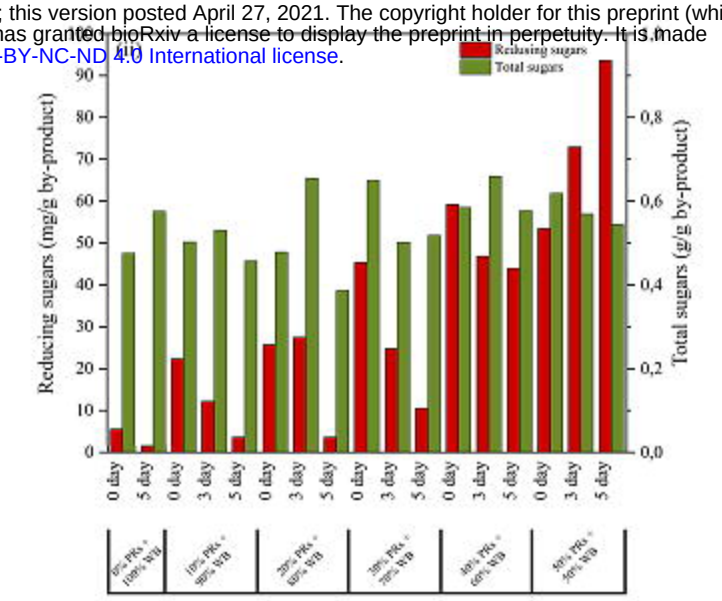

(iii)
Reducing sugars
Biomass
Lines: Model
Ethanol
Reducing sugars (g/L)
Biomass (g/L), Ethanol (g/L)
Time (h)

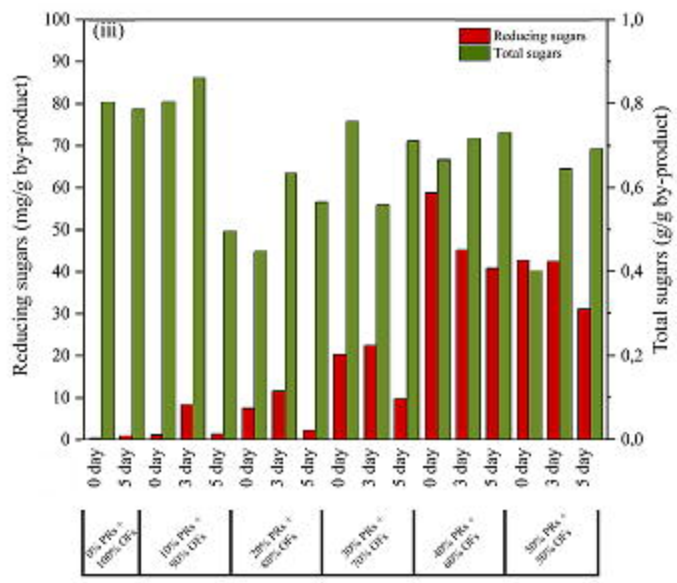

(iv)
Recucing sugars
Bomass
Lines: Model
Ethanol
Reducing sugars (g/L)
Biomass (g/L), Ethanol (g/L)
Time (h)


(A)
Reducing sugars
Bomass
Lines: Model
Ethanol
Reducing sugars (g/L)
Biomass (g/L), Ethanol (g/L)
Time (h)
(B)
Recucing sugars
Bomass
Lines: Model
Ethanol
Reducing sugars (g/L)
Biomass (g/L), Ethanol (g/L)
Time (h)
(C)
Recucing sugars
Bomass
Lines: Model
Ethanol
Reducing sugars (g/L)
Biomass (g/L), Ethanol (g/L)
Time (h)

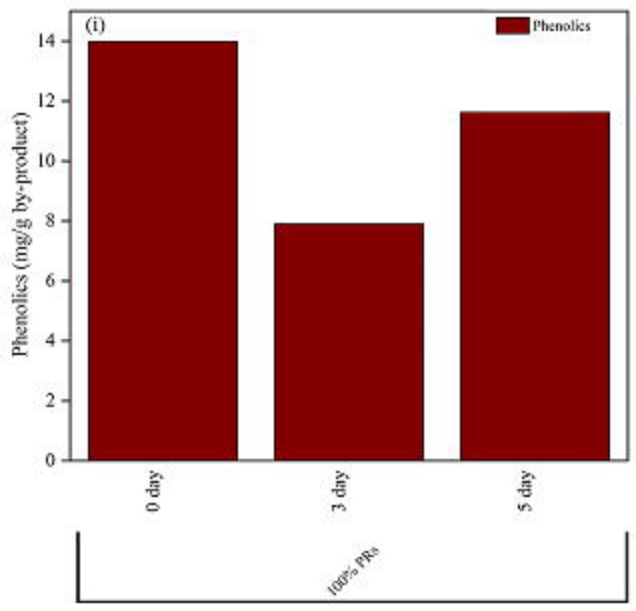
(A)

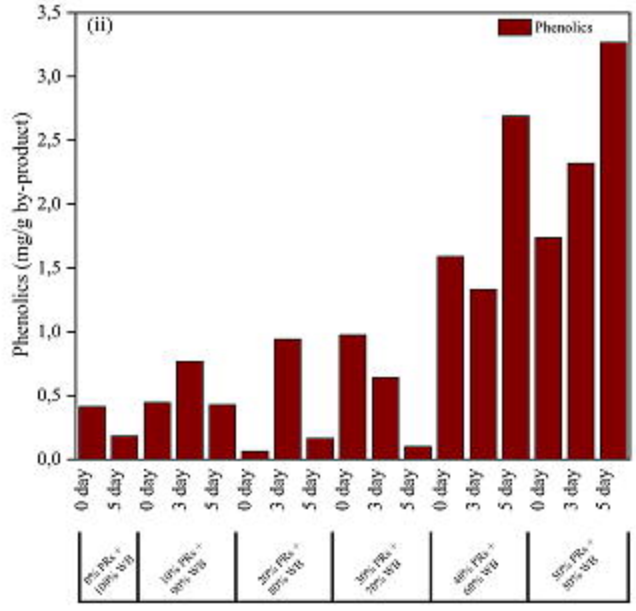


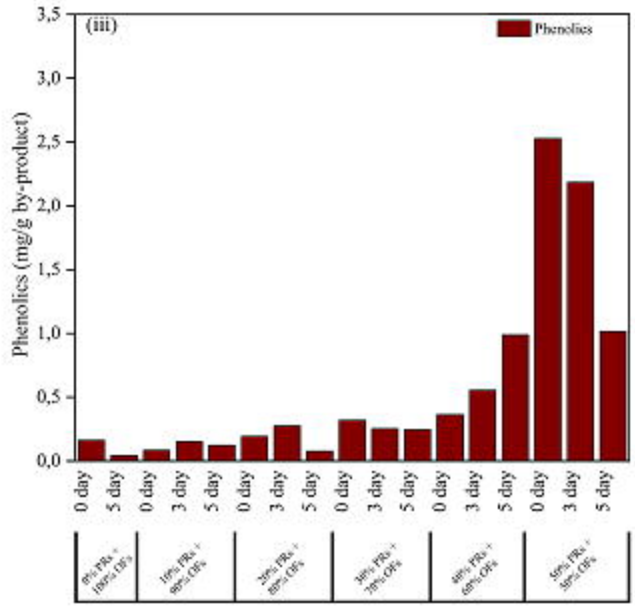




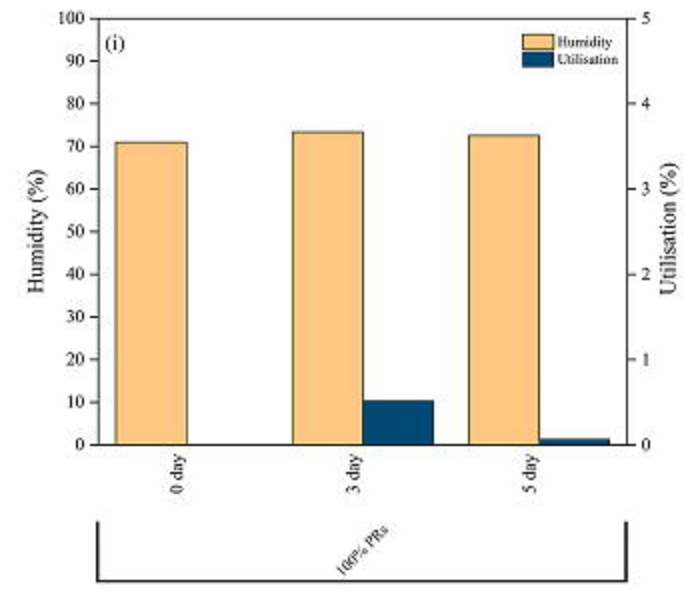
(B)

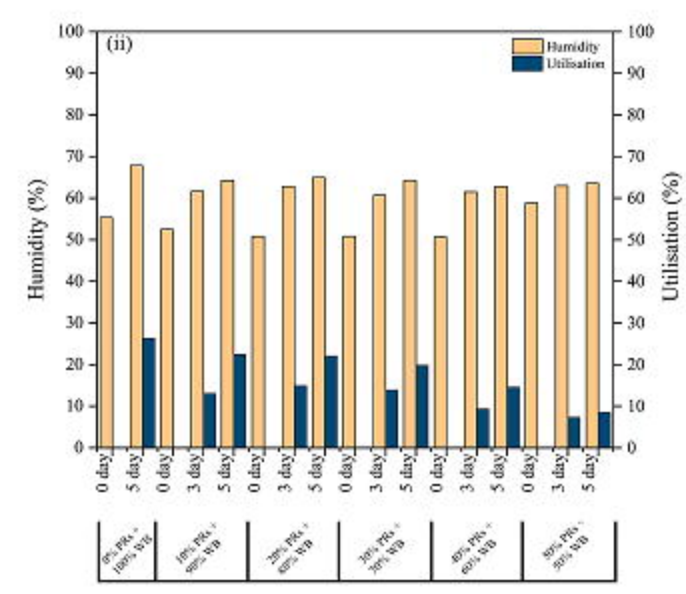


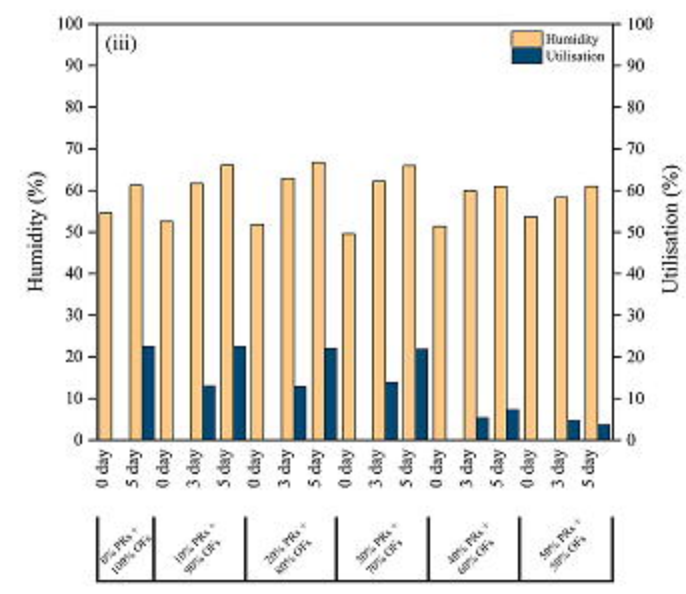


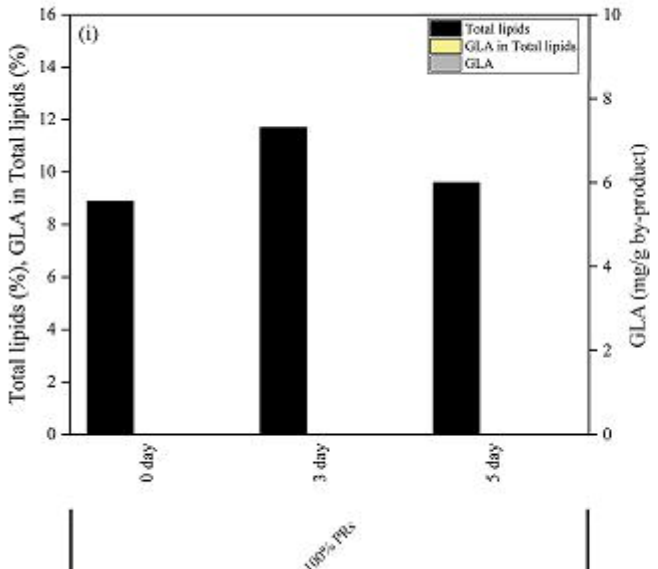


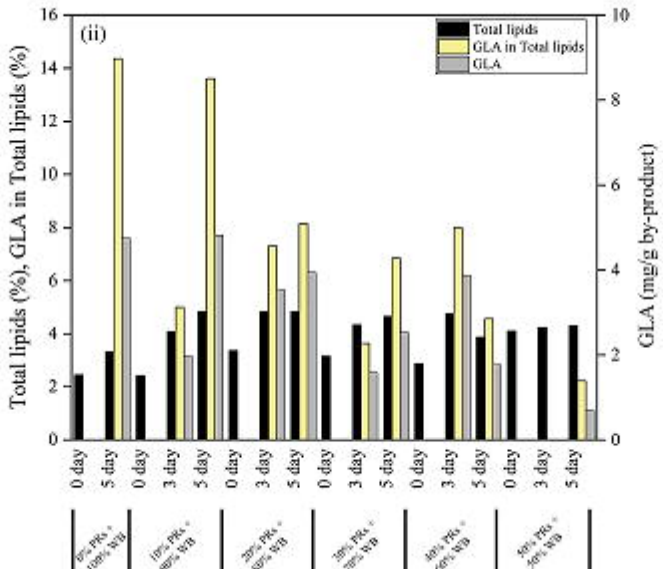
(C)

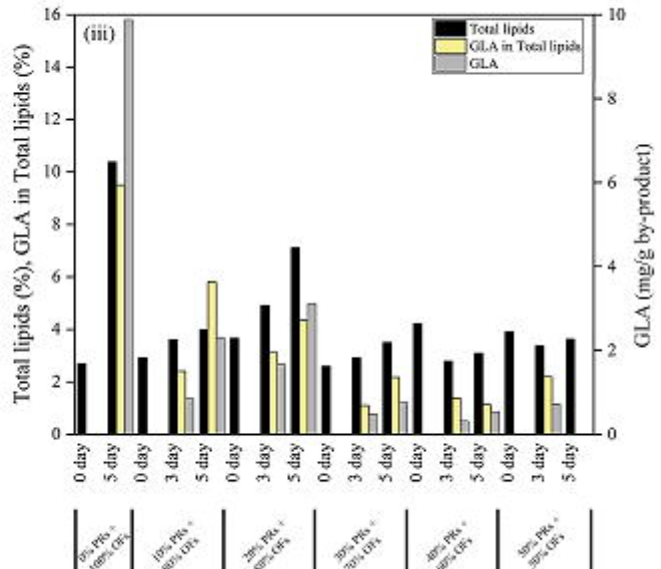







(i)
Total lipids
GLA in Total lipids
GLA
Total lipids (%), GLA in Total lipids (%)
GLA (mg/g by-product)
0 day
3 day
5 day
100% PRs


(ii)
Total lipids
GLA in Total lipids
GLA
Total lipids (%), GLA in Total lipids (%)
GLA (mg/g by-product)
0 day
5 day
3 day
5 day
0% PRs + 100% WB
10% PRs + 90% WB
20% PRs + 80% WB
30% PRs + 70% WB
40% PRs + 60% WB
50% PRs + 50% WB


(iii)
Total lipids
GLA in Total lipids
GLA
Total lipids (%), GLA in Total lipids (%)
GLA (mg/g by-product)
0 day
5 day
3 day
5 day
0% PRs + 100% CFs
10% PRs + 90% CFs
20% PRs + 80% CFs
30% PRs + 70% CFs
40% PRs + 60% CFs
50% PRs + 50% CFs