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Is pyrolysis bio-oil prone to microbial conversion into added-value products?

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1 **Is pyrolysis bio-oil prone to microbial conversion into added-value products?**

2

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Abstract

In view of the potential application of pyrolysis-based biotechnologies, it is crucial to look for novel microorganisms able to convert pyrolysis-derived products, in particular bio-oil water-soluble constituent, into valuable compounds. For the first time, this paper proposed a survey on a collection of bacterial, yeast, and fungal strains with well-known industrial properties as well as new bacterial isolates in order to select microbes able to both tolerate bio-oil inhibitors and convert bio-oil into valuable products. This survey found that bio-oil aqueous phase (BOAP) obtained from intermediate pyrolysis could be metabolized as it is by fungal strains whereas several dilutions are needed to do not hamper cell viability of many tested yeast and bacterial isolates.

To process BOAP into valuable products, the yeast *Saccharomyces cerevisiae* L13, selected as the most industrially relevant tested strain, was adopted to convert bio-oil aqueous fraction hydrolysate into ethanol without any detoxification step. The fermenting performances were much greater than those of the benchmark yeast strain and *S. cerevisiae* L13 proved to be a strong candidate for bioethanol production from BOAP hydrolysates.

This study demonstrated that the search for microorganisms is a promising approach to the future development of pyrolysis oil-based biorefinery platforms.

Keywords: microbial valorization; pyrolysis; wood; bioethanol production; hybrid thermochemical-biological treatment; industrial yeast;

55 **1. Introduction**

56 Residual lignocellulose represents a relevant problem and an inviting opportunity at the same time.
57 The problem lies on the need to safely dispose of huge amounts of organic wastes, while the
58 opportunity is the possible extraction of fermentable sugars to be used for a number of different
59 applications, such as the production of starch- and lignocellulose-based bioethanol [Cripwell et al.,
60 2020; Favaro et al., 2019a; Hamelinck et al., 2005; Karagoz et al., 2019; Salehi Jouzani and
61 Taherzadeh, 2015] and other bio-products [Alibardi et al., 2017; de Paula et al., 2019; Favaro et al.,
62 2019b; Kucharska et al., 2018; Lopes et al., 2018; Schirru et al., 2014; Shah et al., 2016; Sindhu et
63 al., 2016a; Taherzadeh-Ghahfarokhi et al., 2019]. However, a series of challenges are still
64 hampering the development of the process to commercial scale. Indeed, in order to release cellulose
65 and hemicellulose from the lignocellulosic material, several costly pre-treatments of the biomass are
66 required [Sindhu et al., 2016b], followed by chemical or enzymatic hydrolysis to convert the
67 polymers into simple sugars. Moreover, enzymatic saccharification occurs at a slow rate, and,
68 during the most common pre-treatments, inhibitory compounds, such as furans, weak acids, and
69 phenolics, are often produced. These inhibitors slow down or even prevent microbial fermentation,
70 thus limiting the feasibility of the process [Larsson et al., 2000; Favaro et al., 2013a].

71 Pyrolysis could represent an unconventional way to release sugars from lignocellulosic material,
72 making them available for microbial fermentation purposes. This is an anaerobic process, carried
73 out at high temperatures, transforming the biomass into char, gas, and bio-oil [Bridgwater et al.,
74 2002; Lü et al., 2018]. Most of the biomass energy concentrates into bio-oil that, for this reason, is
75 considered as a second-generation biofuel, suitable for combustion and used to produce electricity
76 and heat in small-medium plants [Jacobson et al, 2013]. Bio-oils fraction obtained after pyrolysis
77 contains a wide range of water-soluble organic molecules such as sugars, organic acids, alcohols,
78 aldehydes, ketones, and phenolic components [Piskorz et al., 1989, Cordella et al., 2012] and could
79 be alternatively exploited as a carbon source for microorganisms in fermentative processes to obtain
80 biomass or high-value products [Arnold et al., 2019a; Islam et al., 2015; Jarboe et al, 2011; Torri et
81 al., 2020].

82 Unfortunately, microbial valorization of bio-oil is an arduous challenge for both the chemical nature
83 of the sugars obtained after the catalytic processes and the presence of inhibitors of microbial
84 growth such as furans, phenolic compounds, and ketones [Chi et al., 2013; Davis et al., 2019;
85 Jarboe et al., 2011; Prosen et al., 1993]. Thus, for the microbial utilization of molecules derived
86 from pyrolysis, the strains should not only be able to degrade them, but also tolerate the inhibitory
87 substances that are present in bio- oils.

88 Since very little information is available on the utilization and degradation of the pyrolysis oil by
89 microorganisms [Yang et al., 2011; Islam et al., 2015; Arnold et al., 2019b; Arnold et al., 2019c], in
90 the present study, a survey on microbial strain collection and new isolates has been carried out in
91 order to select microbes able both to tolerate the concentration of inhibitors and to use the pyrolysis
92 derived sugars potentially available in the bio-oil aqueous phase (BOAP) obtained from
93 intermediate pyrolysis. The possible production of added-value products by such microbes would
94 be a further important trait to be selected.

95

96 **2. Materials and methods**

97 *2.1 Microbial strains*

98 Two hundred and three bacterial, and fungal strains, previously isolated and/or characterized for
99 their promising industrial phenotypes (wine, H₂, bioethanol, biopolymers, bacteriocins, enzymes),
100 were used in this study (Table 1 and 2). Bacterial, yeast, and fungal isolates were grown on NA
101 (Nutrient Agar, Oxoid), PDA (Potato Dextrose Agar, Oxoid) and YPD (Yeast Peptone Dextrose,
102 Sigma), respectively.

103

104 *2.2 Biomass feedstock and pyrolysis*

105 Bio-oil was obtained by intermediate pyrolysis of fir wood pellet. Pyrolysis equipment consisted of
106 an auger pyrolyzer with 1-10 kg/h capacity, as described elsewhere [Torri et al., 2020]. Briefly, it
107 consists of a pyrolysis reactor with an external diameter of 114 mm, 6 mm thickness, and a length
108 of 1350 mm. The central part of the system was equipped with a single 65 mm screw and 4 electric
109 jackets (total power 4 kW) that maintained the external temperature of the heated zone measured at
110 the top of the pyrolysis chamber at the set value of 400 °C for a length equal to 600 mm. By
111 considering that the electric jackets heated up from the bottom, this corresponded to a maximum
112 measured temperature of about 550 °C at the bottom of the reactor, with an average residence time
113 equal to 30 min. For safety reasons, a flow of N₂ at 0.1 L/min was provided nearby the airlock shaft
114 coupling. The reactor was coaxially attached to a U-tube heat exchanger (stainless steel, AISI 304)
115 with a bio-oil collection tank in the bottom part, and biomass/biochar flowed by means of two
116 opposite radial openings for entrance of biomass from airlock feed, and biochar discharge opposed
117 to shaft coupling.

Phenotype Genus/Species	Tolerant strains (n.) at different dilution levels (v/v)								References
	tested strains (n.)	Undiluted	1:2.5	1:5	1:10	1:30	1:50		
BACTERIA									
PHAs producers									
<i>Acidovorax temperans</i> PE1	1	-	-	-	-	1	1	1	Povolo et al., 2012
<i>Acinetobacter</i> sp. BT1	1	-	-	-	-	-	1	1	Povolo et al., 2012
<i>Cupravidus necator</i> DSM 545	1	-	-	-	-	-	-	-	Gamero et al., 2018
<i>Delftia acidovorans</i> DSM 39	1	-	-	-	-	-	-	-	Romanelli et al., 2014
<i>Hydrogenophaga pseudoflava</i> DSM 1034	1	-	-	-	-	-	1	1	Povolo et al., 2013
<i>Pseudomonas hydrogenovora</i> DSM 1749	1	-	-	-	-	1	1	1	Samori et al., 2014
<i>Pseudomonas oleovorans</i> DSM 1045	1	-	-	-	-	1	1	1	Favaro et al., 2019c
	7	-	-	-	-	3	5	5	
Bacteriocins producers									
<i>Enterococcus faecium</i>	6	-	-	-	-	1	5	5	Todorov et al., 2011; Favaro et al., 2014a
H2-producers									
<i>Bacillus</i> sp.	30	-	-	-	-	-	-	22	Alibardi et al., 2012
<i>Bacillus badius</i>	20	-	-	-	-	-	-	10	Shah et al., 2016
<i>Bacillus berjingsensis</i>	6	-	-	-	-	-	-	-	
<i>Bacillus farraginis</i>	8	-	-	-	-	-	-	-	
<i>Bacillus flexus</i>	1	-	-	-	-	-	-	-	
<i>Bacillus licheniformis</i>	3	-	-	-	-	-	1	1	
<i>Bacillus megaterium</i>	3	-	-	-	-	-	-	-	
<i>Bacillus subtilis</i>	3	-	-	-	-	-	3	3	
<i>Bacillus tequilensis</i>	4	-	-	-	-	-	-	-	
<i>Brevibacillus</i> sp.	3	-	-	-	-	-	-	2	
<i>Brevibacillus agri</i>	3	-	-	-	-	-	-	-	
<i>Brevibacillus brevis</i>	2	-	-	-	-	-	1	1	
<i>Brevibacillus parabrevis</i>	1	-	-	-	-	-	-	-	
<i>Enterobacter</i> sp.	3	-	-	-	-	-	-	1	
<i>Enterobacter cloacae</i>	1	-	-	-	-	-	-	-	
<i>Lysinibacillus</i> sp.	16	-	-	-	-	-	-	5	
<i>Paenibacillus</i> sp.	6	-	-	-	-	-	-	2	
<i>Paenibacillus cookii</i>	3	-	-	-	-	-	-	1	
<i>Sporosarcina</i> sp.	4	-	-	-	-	-	-	1	
<i>Staphylococcus saprophyticus</i>	1	-	-	-	-	-	-	1	
	121	-	-	-	-	-	-	50	
total	134	-	-	-	-	4	60	60	
% tolerant strains						3	45	45	

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Table 1. Bacterial strains with promising industrial phenotypes screened for their ability to grow in the presence of different BOAP dilutions. The number of tolerant strains not showing growth inhibition haloes at the tested dilution is indicated. “-“ means “no growth”.

Phenotype Genus/Species	Tolerant strains (n.) at different dilution levels (v/v)							References
	tested strains (n.)	Undiluted	1:2.5	1:5	1:10	1:30	1:50	
YEAST								
Wine producers								
<i>Candida glabrata</i>	12	-	-	4	7	12	12	DAFNAE
<i>Candida zemplinina</i>	10	-	-	1	8	9	10	DAFNAE
<i>Issatchenkia orientalis</i>	12	-	-	-	6	11	12	DAFNAE
<i>S. cerevisiae</i>	4	-	-	-	2	4	4	DAFNAE
<i>Saccharomyces ludwigii</i> DSM 70551	1	-	-	-	-	1	1	DSMZ
	39	-	-	5	23	37	39	
Bioethanol producers								
<i>S. cerevisiae</i>	22	-	-	1	6	22	22	Favaro et al., 2013a, 2014b
<i>S. cerevisiae</i> DSM 70449	1	-	-	-	1	1	1	DSMZ
<i>S. cerevisiae</i> Ethanol Red™	1	-	-	-	-	1	1	Fermentis
	24	-	-	1	7	24	24	
FUNGI								
Enzymes producers								
<i>Armillaria</i> sp.	1	-	-	-	-	-	-	TESAF
<i>Biscognauxia mediterranea</i>	1	-	-	1	1	1	1	TESAF
<i>Ganoderma appianatum</i>	1	1	1	1	1	1	1	TESAF
<i>Lepiota procera</i>	1	-	-	-	-	1	1	TESAF
<i>Pleurotus ostreatus</i>	1	1	1	1	1	1	1	TESAF
<i>Schizophium comune</i>	1	-	-	1	1	1	1	TESAF
<i>Trametes versicolor</i>	1	1	1	1	1	1	1	TESAF
	7	3	3	5	5	6	6	
total	70	3	3	11	35	67	69	
% tolerant strains		4	4	16	50	96	98	

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Table 2. Fungal strains with promising industrial phenotypes screened for their ability to grow in the presence of different BOAP dilutions. The number of tolerant strains not showing growth inhibition haloes at the tested dilution is indicated. “-“ means “no growth”.

DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen); DAFNAE (Department of Agronomy Food Natural resources Animals and Environment) and TESAF (Department of Land, Environment, Agriculture and Forestry) are Departments of Padova University

129 Bio-oil, consisting of a biphasic liquid, was collected from the heat exchanger, and, after
130 fractionation, was found to be composed by 85% aqueous phase (BOAP) and 15% of the heavy
131 water-insoluble phase (WIP), mainly formed by pyrolytic lignin. Chemical characterization of
132 pyrolysis oil (bio-oil) and BOAP is described below in the 2.6 section.

133

134 2.3 *Antimicrobial activity of BOAP*

135 The effect of BOAP on the growth of microbial strains was studied using the agar well diffusion
136 method. Samples (100 μ L) of calibrated suspensions ($OD_{600} = 0.8$, corresponding to an average
137 concentration of 10^6 cells /mL) of bacterial and yeast cells, grown 24 h at 30°C on agar plates, were
138 used to spread plates containing 20 mL of the appropriate media described below and purified agar
139 (Sigma, Italy). In the case of fungal isolates, a sample of 72 h old fungal colony has been
140 transferred onto the centre of PDA plates.

141 Five holes (diameter of 4 mm) were then made in the agar using a sterile glass pipette. To each
142 hole, samples 20 μ L of BOAP or its specific dilutions obtained with sterile distilled water (1 to 2.5,
143 5, 10, 30 and 50, v/v) were introduced using a sterile micropipette. pH values were adjusted to 5.0
144 using KOH 5M. Sterile distilled water was used as the negative control. Petri dishes were incubated
145 for 48 h at 30°C, in the case of yeast/bacterial strains. Fungal strains were incubated for 120 h at
146 25°C.

147 After incubation, zones of inhibition were measured and recorded. The experiments were
148 conducted in triplicates.

149

150 2.4 *Isolation and genetic identification of microbial strains able to use BOAP as a carbon source*

151 Urban compost from domestic organic waste was used as biodiversity source to look for
152 microbial strains able to use BOAP as carbon source. Samples of compost (1 g) have been inserted
153 into 500 mL Erlenmeyer flasks containing 150 mL of Enrichment Medium (EM, yeast extract 2
154 g/L, 0.05 M phosphate buffer pH 7) and 5 mL of BOAP and incubated under shaking (150 rpm) for
155 20 days at 30°C. Then, 10 mL samples from each flask have been used to i) inoculate fresh 150 mL
156 EM with 5 mL of BOAP, for a second incubation period at 30°C of 20 days, ii) perform microbial
157 isolation procedure as follows. Ten mL of EM were dispersed in 100 mL of sterile physiological
158 water (0.85% NaCl), plated, after appropriate decimal dilutions, on NA, PDA and BHI (Brain Heart
159 Infusion, Oxoid) plates and incubated at 30 °C for 72 h.

160 After the second incubation period, aliquots of 10 mL from each flask have been used to i)
161 inoculate fresh 150 mL EM containing 5 mL of BOAP, for a third incubation at 30°C of 20 days, ii)

162 perform microbial isolation procedure as described above. At the end of the third incubation,
163 microbial isolation method was also carried out.

164 After isolation, microbial colonies were purified by growing on the respective solid medium at
165 30 °C for 72 h. Isolates were maintained at -80 °C in the respective medium containing 20% (v/v)
166 glycerol.

167 Newly isolated bacterial strains were genetically identified by 16S rDNA sequencing as
168 previously described [Rahman et al., 2014]. In short, genomic DNA was extracted as follows: a
169 small colony of each strain, grown for 24 h on NA plates, was picked up with a sterile toothpick and
170 dissolved in 50 µL of lysis solution (0.05 M NaOH, 0.25% sodium dodecyl sulfate). The suspension
171 was heated at 94°C (15 min) and then centrifuged (10,000 g, 15 min).

172 Prokaryotic small rDNA subunits were amplified using bacterial universal primers 1389r and 63F
173 as previously described [Hongoh et al., 2003]. Amplification products were visualized by agarose
174 gel electrophoresis and then subjected to sequencing.

175 QIAquick PCR Purification kit (Quiagen) was used for PCR product purification which was then
176 resuspended in 30 µL deionised water. The dideoxy chain termination method was subsequently
177 used for DNA sequencing by an ABI Prism 3100 DNA Analyzer, using an ABI Prism Big Dye
178 Terminator Cycle Sequencing Ready Reaction kit (PE Biosystems) according to the manufacturer.

179 Comparisons of sequences with those included in GenBank were performed with the BLASTN
180 interface (<http://www.ncbi.nlm.nih.gov/BLAST/>) in order to obtain the closest neighbours. A
181 minimum sequence similarity level of 98% was considered for taxonomic attribution.

182

183 2.5 *Small-scale fermentation studies*

184 To determine if BOAP could be effectively converted into ethanol, fermentation tests were
185 performed using *S. cerevisiae* L13, selected as one of the most tolerant strains. *S. cerevisiae*
186 DSM70449, used in many papers for the ethanol production from different lignocellulosic
187 substrates [Almeida et al., 2007, Favaro et al., 2013b, Liu et al., 2004], was also included as
188 benchmark yeast.

189 Before entering fermentation experiments, BOAP has been pre-treated with H₃PO₄ (0.3% w/v in
190 water) to yield glucose from levoglucosan and oligosaccharides. Hydrolysis was performed as
191 follows: the BOAP was 1:5 diluted with 0.3% w/w H₃PO₄, then placed in a closed pyrex vessel at
192 95°C. Levoglucosan hydrolysis to glucose was monitored over time by silylation and GC-MS of
193 aliquots of hydrolysate (neutralized with CaCO₃ and dried). The reaction was then stopped, the
194 solution neutralized with ammonia and vacuum filtrated onto a Buckner filter.

195 Fermentation performances were assessed in Synthetic Complete (SC) medium (Difco™)
196 supplemented with a dilution 1:5 (v/v) of hydrolysed BOAP. Since hydrolysis of BOAP involved
197 1:5 dilution, this corresponds to final 1:25 dilution of BOAP. In view of reducing chemical inputs
198 and costs, fermentations were also performed without SC medium supplementation. pH was
199 adjusted to 5.0 with NaOH 5 M. Reference fermentations using SC with an equivalent amount of
200 glucose (16.5 g/L) were also included.

201 Pre-cultures of yeast strains grown to early stationary phase in SC broth containing 20 g/L
202 glucose were used as inoculum. Cells were collected by centrifugation for 5 min at 4000 g, washed
203 twice in sterile distilled water, and used to inoculate 50 mL medium to an initial OD₆₀₀ of 0.3 in
204 triplicate experiments using 55 mL glass serum bottles. The small-scale fermentations were carried
205 out under oxygen-limited conditions. The bottles were sealed with rubber stoppers, incubated at
206 30°C and mixed on a magnetic stirrer (300 rpm). Syringe needles pierced through the bottle stopper
207 served for sampling purposes and carbon dioxide removal. Samples obtained before and during
208 fermentation were analyzed for glucose, ethanol, and glycerol content using HPLC.

209

210 *2.6 Analytical methods, calculations, and statistical analysis*

211 Bio-oils were characterized using previously published procedures [Busetto et al., 2011;
212 Cordella et al., 2012]. Briefly, the water content of the pyrolysis oil was determined through Karl-
213 Fischer titration. Volatile organic compounds (e.g. methanol, ethanol, acetic acid) were evaluated
214 by solid-phase micro-extraction (Supelco SPME with PDMS coating 75 µm) and GC-MS analysis.
215 Active aldehydes (acetaldehyde, hydroxyacetaldehyde, methylglyoxal) were determined by GC-
216 MS after derivatization into the corresponding dimethyl acetals by catalytic methanolysis.

217 For anhydrosugars determination, BOAP was dried, an aliquot was silylated with BSTF+TMCS,
218 and analyzed with GC-MS for determination of small polar compounds and anhydrosugars. For the
219 determination of oligo and polysaccharides, another aliquot of BOAP (100 mg) was dried and
220 subjected to methanolysis with 3.5 mL anhydrous methanol over Amberlyst® (0.5 g) at 64 °C for
221 24 h. Then, the solution was evaporated under nitrogen at room temperature, and the residue
222 subjected to the same derivatization procedure described above, thus obtaining the methyl-O-
223 glycosides derived from hydrolysis of levoglucosan and polysaccharides. The amount of
224 oligosaccharides was then determined by subtracting the content of anhydrosugars from the total
225 value of methyl-O-glycosides derived from hydrolysis.

226 From the small scale fermentations, sugars, glycerol and ethanol were detected in samples,
227 filtered through 0.22-µm, and diluted prior to HPLC analysis as previously described [Favaro et al.,
228 2017]. In short, liquid chromatography analysis was accomplished using a Shimadzu Nexera HPLC

229 system, with a RID-10A refractive index detector (Shimadzu, Kyoto, Japan) and a Phenomenex
230 Rezex ROA-Organic Acid H⁺ (8%) column (300mm×7.8mm). The column temperature was set at
231 65 °C and the flow rate was 0.6 mL/min using isocratic elution, with 0.01M H₂SO₄ as a mobile
232 phase.

233 The ethanol yield, $Y_{E/S}$, (g of ethanol/g of utilized glucose equivalent) was determined
234 considering the amount of glucose consumed during the fermentation and compared to the
235 maximum theoretical yield of 0.51 g of ethanol/g of consumed glucose equivalent [Cripwell et al.,
236 2019]. The volumetric productivity (Q) was calculated on grams of ethanol produced per litre of
237 culture medium per hour (g/L/h) and the maximum volumetric productivity (Q_{max}) was determined
238 as the highest volumetric productivity displayed by the *S. cerevisiae* strains [Myburgh et al., 2019].

239 Statistical analyses were assessed using the Graphpad Prism 5 package (Graphpad Software,
240 Inc., San Diego, California). Descriptive statistics, mean values and standard deviations were
241 calculated. Data were analyzed also by two ways factorial ANOVA (Analysis Of Variance) with
242 Duncan test.

243

244 3. Results

245 3.1 Pyrolysis, production of bio-oil aqueous phase (BOAP) and hydrolysis

246 Three pyrolysis replicates were conducted with a capacity of 3 kg/h for two h (6 kg of fir pellet each
247 test). The yield of bio-oil, biochar and pyrolysis gas were respectively 48±2%, 41±6%, and (by
248 difference) 9.1±6% (calculated as 100% minus bio-oil and biochar yield). Bio-oil was formed by
249 45% water and 65% organic constituents and spontaneously separated into two phases. Pyrolysis
250 product distribution and water content of bio-oil were in general agreement with the yields related
251 to intermediate pyrolysis performed in the 400-550 °C range with auger pyrolyzers (Table 3). The
252 composition of the whole bio-oil was comparable to that obtained by similar auger intermediate
253 pyrolysis systems with woody biomass. Phase separation generated 85±5% w/w_{bio-oil} bio-oil
254 aqueous phase (BOAP) and 15±5% w/w_{bio-oil} water-insoluble phase (WIP). WIP, being a tarry
255 viscous liquid, contained minimum amounts of water (8% g/g_{WIP}) and acetic acid (2% g/g_{WIP}),
256 being mostly formed by heavy water-insoluble organics (mostly pyrolytic lignin) and minor
257 amounts of extractives (abietic acid derivatives). BOAP, which was first used to assess the
258 microbial tolerance of strains reported in Table 1 and 2, was a reddish aqueous liquid with a density
259 slightly higher than that of water (1.0 kg/L) with pH of 2.7. It consists of almost entirely water-
260 soluble compounds with a negligible content of suspended solids, namely 0.5±0.2%.

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Reactor	Rate	RT (min)	T° (C)	Yields (% w/w)				% (w/w) in pyrolysis oil							Reference		
				Liquid	Char	Gas	H ₂ O	PL	WS	AS	TS	AA	C2-C3	PhOH		Furans	
auger	Int	10	500	48.0	28.0	24.0	45.0	14.0	41.0	2.3	30.0	2.6	1.4	5.0	2.4	2.4	This study
auger	Int	1.2	550	53.0	17.0	29.0	39.0	31.0	30.0	1.8	30.0	2.8	2.4	2.6	0.8	0.8	Kim et al., 2014
auger	Int	1.2	500	60.0	23.0	18.0	35.0	28.0	39.0	2.2	39.0	1.2	2.6	3.9	0.9	0.9	Kim et al., 2014
auger	Int	1.0	500	45.0	18.0	37.0	22.0	15.0	62.0	8.9	14.0	5.6	5.6	12	3.7	3.7	Liaw et al., 2012
auger	Int	1.0	500	45.0	18.0	37.0	22.0	15.0	62.0	8.9	36.0	5.6	5.6	12	3.7	3.7	Liaw et al., 2012
auger	Fast	<1.0	450	58.0	14.0	20.0	22.0	17.0	61.0	5.1	22.0	8.3	3.8	4.8	1.4	1.4	Ingram et al., 2008
fluidized bed	Fast	<1.0	500	62.0	15.0	24.0	18.0	20.0	62.0	6.5	42.0	5.7	6.5	6.5	2.9	2.9	Garcia-Perez et al., 2008

264

265 **Table 3.** Yield and composition of the whole bio-oil obtained in this study. For comparison, other bio-oils obtained from wood after intermediate (Int) or fast
266 pyrolysis are reported.

267

268 Liq.: liquid product, RT: residence time, PL: pyrolytic lignin; WS: water-soluble compounds; AS: anhydrosugars; TS: total sugars (including anhydrosugars and sugar
269 oligomers); AA: acetic acid; C2-C3: small oxygenates (e.g. hydroxyacetone, hydroxyacetaldehyde); PhOH: monolignols:

270

271

272

273

274 Chemical composition of BOAP can be summarized as follows: 52.0±4% water, 4.1±1% phenols,
275 2.4±0.5% furans, 35.0±3% sugar derivatives (e.g. levoglucosan and sugar oligomers), 2.7±0.3%
276 acetic acid, 1.7±0.5% C2-C3 small oxygenates (e.g. hydroxyacetone, hydroxyacetaldehyde).
277 Furthermore, in the case of fermentation kinetics, BOAP was hydrolyzed with H₃PO₄ (0.3% w/w)
278 in order to convert levoglucosan into glucose. After 13 h, the BOAP had 7.0% glucose content and
279 95% hydrolysis of levoglucosan was achieved, with a slightly decreasing trend in sugar content.
280 Interestingly, no significant change was detected for non-sugar constituents of hydrolysed BOAP
281 (data not shown), suggesting that mild hydrolysis poorly affects the concentration of other
282 constituents, such as acetic acid and furans.

283

284 3.2 BOAP tolerance of bacteria

285 In the context of a “bio-based economy”, the present work was aimed at the isolation and/or
286 selection of microbial strains converting pyrolysis BOAP into value-added products and, at the
287 same time, able to tolerate or detoxify the large amounts of toxic compounds resulting from the
288 pyrolytic process. To this objective, possible options were (i) the screening of collection strains
289 previously selected for other interesting properties to test their possible resistance to and utilization
290 of BOAP, (ii) the isolation of microorganisms able to tolerate and possibly grow on BOAP, and,
291 successively, the evaluation of their ability to convert it into added-value products.

292 Firstly, the tolerance to BOAP of 134 bacterial strains, previously selected by the Authors on the
293 basis of interesting industrial characters (ie, biopolymers, biofuels, bacteriocins, and enzymes
294 production), was investigated. With this purpose, a diffusion plate test was employed (see Material
295 and Methods) and the presence/absence of the growth inhibition halos was used to select BOAP
296 tolerating strains.

297 These collection bacteria were found to be rather sensitive since merely 45% of the tested strains
298 were able to grow without any inhibition halo on the plate at the higher BOAP dilution (1:50 v/v),
299 less than 3% tolerated 1:30, while dilution 1:10 was already sufficient to inhibit at various extents
300 the growth of all the tested bacteria (Table 1). However, the group of bacteria here examined were
301 originally isolated not for their possible resistance to BOAP, but for quite different purposes (see
302 above option “i”). As shown in Table 1, four out of seven tested PHA-producers were resistant only
303 to the higher dilution level of BOAP (1:50) and three of them, belonging to *A. temperans* and
304 *Pseudomonas* sp. species, were resistant to dilution 1:30. Among the *E. faecium* bacteriocins
305 producers five out of six were resistant to 1:50, while only one, to 1:30. In the case of bio-hydrogen
306 producer strains, 50 out of 121 were found to grow at the higher dilution, but no one tolerated
307 greater concentrations.

308 These results indicate the absence of any plausible correlation between BOAP resistance and
309 other distinctive properties of the collection bacteria examined and do not provide valid information
310 on the weight of BOAP resistant strains in natural communities.

311 Therefore, with the aim to increase the probabilities to isolate such a phenotype, a BOAP
312 enrichment isolation was carried out by making use of urban compost from domestic organic waste
313 as a special source of biodiversity. Thirteen Gram-positive and four Gram-negative new strains,
314 isolated as resistant and able to use diluted BOAP as carbon source, were identified at species level
315 by 16S rDNA sequencing. By looking at Table 4, it becomes evident that this isolation strategy
316 enabled to increase the probability to obtain resistant strains. Indeed, among the 17 strains resistant
317 to 1:50 BOAP dilution, there are almost 65% of them also resistant at the 1:30 dilution level. In
318 addition, at least one strain (*E. profundum*) was tolerant to 1:10 dilution level, never reached by any
319 of the bacterial collection strains reported above.

320

321 3.3 BOAP tolerance of yeast and fungi

322 Few scientific papers report that few yeast and fungal strains demonstrated some ability to grow in
323 the presence of BOAP (Prosen et al.,1993; Jarboe et al., 2011). Fungi and yeast are important in
324 many biotechnological processes, such as the production of secondary metabolites, enzymes,
325 vitamins or bioethanol, and have a remarkable economic impact. Moreover, fungi are particularly
326 beneficial in carrying out biotransformation processes. Thus, an approach similar to that used for
327 bacteria was adopted for a general survey on 70 collection fungi and yeast strains capable of
328 producing wine, bioethanol, and/or enzymes (Table 2).

329 All the fungal isolates tolerated BOAP until dilution level 1:5, three of them were even tolerant
330 to pure BOAP (*G. applanatum*, *P. ostreatus* and *T. versicolor*) and their possible involvement in
331 added-value products production from BOAP is under investigation. In any case, their possible use
332 for BOAP decontamination represents a real option.

333 Concerning the yeast strains, all of them proved to tolerate the highest dilution (1:50). As the
334 concentration increased, this percentage was reduced (more than 96% at 1:30, about 48% at 1:10,
335 less than 10% at 1:5) and no one was found to grow at the two higher BOAP concentrations (1: 2.5
336 and pure BOAP). However, six yeast strains proved particularly resistant to high BOAP
337 concentrations (four strains belonging to *C. glabrata*, one to *C. zemplinina* and one to *S. cerevisiae*)
338 being able to grow up to the dilution 1:5 (v/v).

339

340

Isolate	Genus/species	(%)	Accession Number	Undiluted	1:2.5	1:5	1:10	1:30	1:50
F1	<i>Micrococcus lutens</i> 0310ARD7G_6	99	FR848405.1	-	-	-	-	+	+
F2	<i>Micrococcus</i> sp. A2-984	99	KF441624.1	-	-	-	-	+	+
F3	<i>Kocuria rhizophila</i> XFB-BG	99	KC429605.1	-	-	-	-	+	+
F4	<i>Pseudomonas</i> sp. Fse30	99	KJ733882.1	-	-	-	-	+	+
F5	<i>Bacillus</i> sp. SGD-V-25	99	KF413433.1	-	-	-	-	-	+
F6	<i>Micrococcus lutens</i> CC27	99	KJ016267.1	-	-	-	-	+	+
F7	<i>Bacillus subtilis</i> ceppo SRF1.14	99	JX232372.1	-	-	-	-	-	+
F8	<i>Micrococcus</i> sp. F16(2014)	99	KJ6051333.1	-	-	-	-	+	+
F9	<i>Exiguobacterium profundum</i> UMTAL01	99	KJ6721938.1	-	-	-	+	+	+
F10	<i>Achromobacter insuavis</i> LMG 26845	99	NR_117706.1	-	-	-	-	+	+
F11	<i>Agrobacterium tumefaciens</i> A75	99	KC196486.1	-	-	-	-	+	+
F12	<i>Brevundimonas diminuta</i> KSW68	99	LK391673.1	-	-	-	-	+	+
F13	<i>Micrococcus lutens</i> SC1204	99	KF938934.1	-	-	-	-	-	+
F14	<i>Kytococcus</i> sp. YB227	99	KJ534254.1	-	-	-	-	-	+
F15	<i>Kytococcus sedentarius</i> DSM 20547	99	CP001686.1	-	-	-	-	-	+
F16	<i>Kytococcus</i> sp. CUA-901	99	KJ732957.1	-	-	-	-	-	+
F17	<i>Micrococcus lutens</i> NCTC 2665	99	NR_075062.2	-	-	-	-	+	+
				-	-	-	1	11	17

Table 4. Bacterial strains newly isolated from a bio-oil enriched compost and identified by 16S rDNA sequencing. Growth in the presence of different dilution levels of bio-oil is reported as “+”.

346 3.4 Production of bio-ethanol from pre-treated BOAP by selected tolerant yeast

347 From all the above results, the most promising microbe for immediate development and/or
348 application activities resulted to be a yeast isolate belonging to *S. cerevisiae* species (Table 2).
349 Indeed, the knowledge on the use of yeast for industrial purposes, the high levels extent of BOAP
350 resistance and the context of bioethanol production under which this strain was originally selected
351 (Favaro et al., 2014b), made this yeast, now named L13, as the best candidate for subsequent
352 studies tailored to process this by-product into biofuel. Noteworthy, as reported in Table 2, *S.*
353 *cerevisiae* L13 was much more resistant than *S. cerevisiae* Ethanol Red™, the most used yeast in
354 both first and second-generation ethanol plants [Favaro et al., 2019a; Walker and Walker, 2018].
355 First of all, the L13's performance as bioethanol producer was again tested in comparison with a
356 known and previously used strain *S. cerevisiae* DSM 70449, resistant up to 1:10 BOAP dilution and
357 employed here as benchmark strain, considering its application in many works concerning ethanol
358 production from different lignocellulosic feedstocks [Almeida et al., 2007, Favaro et al., 2013b, Liu
359 et al., 2004].

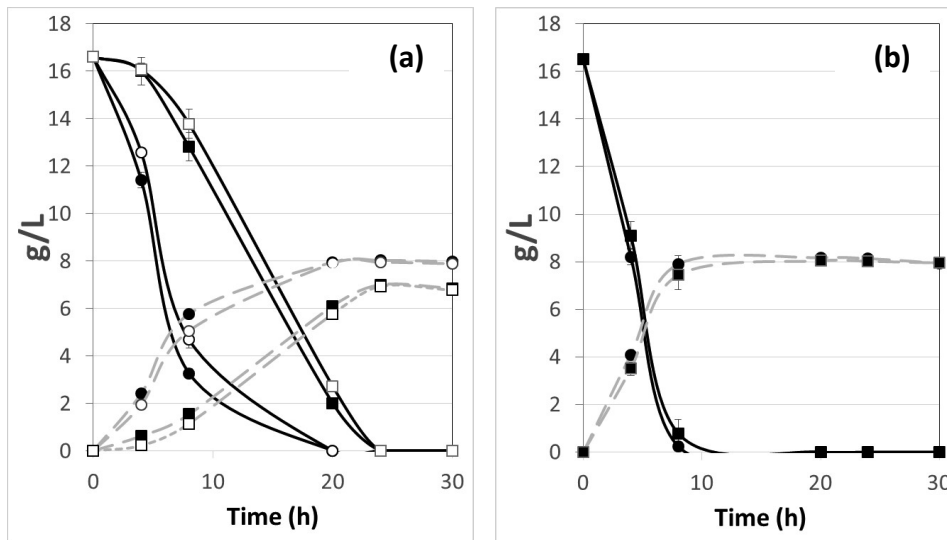
360 Pyrolysis oil is a good source of fermentable sugars (mainly levoglucosan) and acids (mostly
361 acetic acid), that could be biologically converted into ethanol. Levoglucosan is not very abundant in
362 nature. Although many microbes can directly metabolize levoglucosan with various efficiencies
363 [Islam et al., 2015; Lü et al., 2018; Jang et al., 2019], *S. cerevisiae* is not able to efficiently ferment
364 such sugar and *S. cerevisiae* L13 did not grow in the presence of pure or diluted BOAP indicating
365 that was not able to use the available oligomers as carbon source. Therefore, as described in
366 Materials and Methods, BOAP has been pre-treated with H₃PO₄ (0.3% w/w) in order to hydrolyse
367 levoglucosan into glucose before entering the fermentation experiments.

368 Ethanol kinetics obtained by both *S. cerevisiae* strains from 1:5 (v/v) dilution of pre-treated
369 BOAP are plotted in Figure 1a. Reference fermentations, obtained in SC medium supplemented
370 with an equivalent amount of glucose (16.5 g/L), were also reported (Figure 1b). Both strains were
371 able to utilize glucose available in BOAP fermentations, with the newly selected yeast L13
372 exhibiting a higher rate in glucose uptake and, then, ethanol production (Figure 1a). Ethanol levels
373 and yields were again greater in the case of *S. cerevisiae* L13, with up to 8 g/L ethanol,
374 corresponding to 95% of the theoretical (Table 5). Interestingly, both strains fermented glucose
375 even without SC medium supplementation, and the selected yeast displayed again better Q_{max}
376 values (Table 5), thus further supporting the higher BOAP tolerance (Table 2). In the reference
377 medium, SC broth supplemented with 16.5 g/L glucose, the two strains produced statistically
378 similar ethanol values and performances (Figure 1b and Table 5). This is in accordance with the

379 high glucose-to-ethanol yield already described for both strains [Almeida et al., 2007, Favaro et al.,
380 2013b, Favaro et al., 2014b; Liu et al., 2004].

381

382



383

384 **Figure 1.** Ethanol production of *S. cerevisiae* L13 (circle) and the benchmark strain *S. cerevisiae* DSM70449
385 (square) from H₃PO₄-pre-treated BOAP (a) and reference SC medium supplemented with 16.5 g/L glucose
386 (b). In the case of H₃PO₄-pre-treated BOAP, fermentation was performed also without SC broth (empty
387 symbols). Glucose (black lines) and ethanol (gray lines) concentrations (g/L) are represented as a function of
388 time. Data shown are means of three replicates and standard deviations are indicated.

389

390 4. Discussion

391 In view of the potential application of pyrolysis-based biotechnologies, it is crucial to look for, and
392 further improve, novel microorganisms able to convert pyrolysis-derived products into valuable
393 compounds. For the first time, this paper proposed a survey on a collection of microbial strains with
394 well known industrial applications as well as new isolates in order to select microbes able to
395 tolerate the concentration of inhibitors and to convert the bio-oil carbon fractions into valuable
396 products. Furthermore, since the eco-toxicity of BOAPs have been studied so far only on crustacean
397 or algal organisms [Oasmaa et al., 2012; Campisi et al., 2016], such survey was useful towards the
398 assessment of eco-toxicological impact of fast pyrolysis BOAP on different microbial groups,
399 revealing that this product could be metabolized as pure by fungal strains (Table 2) whereas several
400 dilutions are needed to preserve cell viability of many bacterial and yeast isolates (Tables 1, 2 and
401 4).

Yeast strain	Detoxification	Highest ethanol concentration (g/L)	$Y_{E/S}$ (g/g)	Q (g/L/h)	Q_{max} (g/L/h)	Reference
Reference medium (SC with glucose 16.5 g/L)						
<i>S. cerevisiae</i> L13	-	8.17	0.50 (97%)	0.41	1.02	This study
<i>S. cerevisiae</i> DSM 70449	-	8.04	0.49 (96%)	0.40	0.93	This study
Bio-oil hydrolysate (glucose 16.5 g/L)						
<i>S. cerevisiae</i> L13	-	7.94	0.48 (94%)	0.33	0.63	This study
<i>S. cerevisiae</i> DSM 70449	-	6.93	0.42 (82%)	0.29	0.30	This study
Bio-oil hydrolysate (SC with glucose 16.5 g/L)						
<i>S. cerevisiae</i> L13	-	8.02	0.48 (95%)	0.33	0.72	This study
<i>S. cerevisiae</i> DSM 70449	-	6.99	0.42 (83%)	0.29	0.33	This study
<i>S. cerevisiae</i> 2.399	Ca(OH) ₂ neutralization	15.10	0.48 (94%)	0.63	na	Yu and Zhang, 2003
<i>S. cerevisiae</i> T2	water extraction, Na(OH) neutralization and hydrolysate dilution	13.60	0.46 (90%)	0.55	na	Bennett et al., 2009
<i>S. cerevisiae</i> ATCC 200062	Ca(OH) ₂ neutralization and activated carbon	32.00	0.47 (93%)	0.60	na	Lian et al., 2010
<i>S. pastorianus</i> ATCC 2345	n-butanol extraction, Na(OH) and CaCO ₃ neutralization	12.20	0.47 (97%)	0.34	na	Sukhbaatar et al., 2014

Table 5. Conversion of glucose to ethanol from BOAP hydrolysate by the *S. cerevisiae* L13 and DSM70449 yeast applied in this work. For comparison, other *Saccharomyces* sp. yeast performances are reported.

$Y_{E/S}$, ethanol yield per gram of consumed substrate calculated on the highest ethanol production and % of theoretical maximum indicated in brackets; na, not available

408 The results reported in Table 2 indicate that, for bacteria, there is no correlation between interesting
409 phenotypic characters and BOAP degradation and, therefore, the option (i), ie screening of
410 collection strains for tolerance, was not appearing as the most suitable, at least on a preliminary
411 evaluation of the data.

412 Indeed, the isolation from complex environments such as compost proved to increase the
413 probability to find strains resistant to higher concentrations of BOAP, especially if an enrichment
414 medium procedure was followed (Table 4). However, in order to be considered as the most
415 appropriate, this strategy needs to be supported by a much more complex investigation on the
416 ability of the new resistant isolates to convert BOAP into added-value products. Overall, most of
417 the newly isolated strains belong to genera commonly detected in different ecological niches. For
418 instance, the most resistant species here isolated *E. profundum* belongs to the genus
419 *Exiguobacterium*, described as a non sporulating, Gram +, facultative anaerobe, frequently isolated
420 from permafrost, hot springs, rhizosphere and in food processing plants [Crapart et al., 2007;
421 Vishnivetskaya et al., 2009]. *Exiguobacterium*, together with *Kocuria rhizophila* and other
422 Micrococci, are considered catabolically versatile and able to utilize a wide range of unusual
423 substrates, such as aromatic compounds, herbicides, chlorinated biphenyls, and oil [Sims and
424 O'Loughlin, 1992; Doddamani et al., 2001]. That is why they have been widely evaluated for
425 biotechnological purposes, thus characterizing a number of enzyme producers; some of them have
426 been proposed for the degradation of toxic substances or as plant growth promoting bacteria and are
427 currently explored for increasing agricultural production [Kasana et al., 2018], detoxification or
428 biodegradation of other environmental pollutants [Zhuang et al., 2003], and production of useful
429 compounds such as long-chain (C21-C34) aliphatic hydrocarbons for lubricating oils.

430 Although taking a long time, further similar surveys are required for all the new BOAP resistant
431 isolates and are currently in progress. Nevertheless, this work, for the first time, exploited microbial
432 diversity to look for strains with superior ability to withstand and potentially convert BOAP
433 inhibitors opening a new and promising research avenue for the future development of pyrolysis-
434 based biotechnologies.

435 If taken all together, the data reported in Tables 1,2 and 4 indicate that, in terms of resistance,
436 fungi clearly exhibit strains able to grow at all the tested concentrations, including undiluted BOAP.
437 This is another very interesting observation, at least in view of effective degradation/utilization of
438 this pyrolytic product. Indeed, fungi are potentially usable for many purposes as food or feed,
439 biofertilizers, source of metabolites [Kavanagh, 2017; Archer, 2000]. As an example, *Trichoderma*
440 *reesei* is extensively used for the industrial production of cellulolytic enzyme cocktails since it has a
441 very high protein secretion capacity and the ability to synthesize a variety of hydrolytic enzymes

442 [Schuster and Schmoll, 2010]. Fungi and yeast are also widely used as host strains and as microbial
443 cell factories for the production of homologous and heterologous proteins or other metabolites
444 [Nevalainen et al., 2005].

445 In conclusion, the best combination of BOAP resistance and interesting production of added value
446 products seems to be provided by yeast. Indeed, the most burgeoning yeast, *S. cerevisiae* L13, was
447 finally selected on the basis of its resistance (1:5 dilution level, v/v) and its previously proved
448 ability to produce ethanol through sugar fermentation.

449 The challenge of fermenting pyrolytic sugars obtained from BOAP is the presence of various
450 inhibitory compounds that severely inhibit microbial fermentation [Islam et al., 2015; Jang et al.,
451 2019]. A cluster of strategies has been developed in order to remove the toxic inhibitors from
452 hydrolysates, such as over-liming, solvent extraction, adsorption on adsorbents (activated carbon,
453 bentonite, zeolites and diatomite), distillation [Islam et al., 2015; Luque et al., 2014; Jang et al.,
454 2019]. Another approach is to develop microorganisms that can grow well even in the presence of
455 inhibitors and can resist toxic compounds present in this substrate [Luque et al., 2014; Jang et al.,
456 2019]. Table 5 shows a summary of the previous researches on ethanol production from pyrolysis
457 oil and the strategies used to improve the fermentation of pyrolytic sugars. Ethanol levels so far
458 described in the literature are similar or higher than those reported in this work. However, such
459 concentrations have been achieved from higher glucose concentrations and, above all, after
460 complex detoxification approaches which can hamper the overall feasibility of the process.
461 Furthermore, the yeast strains applied were not specifically selected for their resistance towards the
462 inhibitors. On the contrary, this paper made use of a *S. cerevisiae* strain creamed off after a
463 screening procedure on BOAP tolerance. As such, no detoxification procedure has been
464 implemented. Dilution of BOAP hydrolysate was indeed sufficient to achieve high ethanol yields
465 (Table 5) suggesting that *S. cerevisiae* L13's promise as BOAP fermenter is high and likely to be
466 improved upon by repeated fermentations and further optimization of inoculum size and higher
467 concentrations. Noteworthy, as reported in Table 5, both *S. cerevisiae* L13 and DSM70449 applied
468 in this study were able to process BOAP's glucose into ethanol also in the absence of any nutrients
469 supplementation. This is a significant advantage to consider for economical industrial fermentations
470 that should operate without additional nutrients [Cripwell et al., 2020; Favaro et al., 2019a; Walker
471 and Walker, 2018]. *S. cerevisiae* L13 can be considered a great platform for future metabolic
472 engineering and adaptive evolution strategies to develop extremely BOAP tolerant yeast strains
473 potentially able to metabolize levoglucosan as carbon source.

474 Overall, the results of this study encourage to consider BOAP as a potential substrate for microbial
475 conversion into added-value products, although further research is needed to i) scale-up pyrolysis

476 processes, ii) reduce the formation of inhibitors, iii) develop novel and cost-effective detoxification
477 strategies, and, finally, iv) screen for other suitable microorganisms to establish pyrolysis oil as a
478 platform for industrial biotechnology.

479

480 **Credit Author Statement**

481

482 **Marina Basaglia:** Writing- Original draft preparation, Funding acquisition. **Lorenzo Favaro:**
483 Conceptualization, Methodology, Investigation, Data curation, Writing- Original draft preparation
484 and editing, Visualization, Supervision, Funding acquisition. **Cristian Torri:** Methodology,
485 Investigation and Writing- Original draft preparation on pyrolysis activities. **Sergio Casella:**
486 Writing- Original draft preparation, Visualization, Funding acquisition.

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493

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