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

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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;

1. Introduction

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

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

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

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

2. Materials and methods

2.1 Microbial strains

Two hundred and three bacterial, and fungal strains, previously isolated and/or characterized for their promising industrial phenotypes (wine, H_2, bioethanol, biopolymers, bacteriocins, enzymes), were used in this study (Table 1 and 2). Bacterial, yeast, and fungal isolates were grown on NA (Nutrient Agar, Oxoid), PDA (Potato Dextrose Agar, Oxoid) and YPD (Yeast Peptone Dextrose, Sigma), respectively.

2.2 Biomass feedstock and pyrolysis

Bio-oil was obtained by intermediate pyrolysis of fir wood pellet. Pyrolysis equipment consisted of an auger pyrolyzer with 1-10 kg/h capacity, as described elsewhere [Torri et al., 2020]. Briefly, it consists of a pyrolysis reactor with an external diameter of 114 mm, 6 mm thickness, and a length of 1350 mm. The central part of the system was equipped with a single 65 mm screw and 4 electric jackets (total power 4 kW) that maintained the external temperature of the heated zone measured at the top of the pyrolysis chamber at the set value of 400 °C for a length equal to 600 mm. By considering that the electric jackets heated up from the bottom, this corresponded to a maximum measured temperature of about 550 °C at the bottom of the reactor, with an average residence time equal to 30 min. For safety reasons, a flow of N_2 at 0.1 L/min was provided nearby the airlock shaft coupling. The reactor was coaxially attached to a U-tube heat exchanger (stainless steel, AISI 304) with a bio-oil collection tank in the bottom part, and biomass/biochar flowed by means of two opposite radial openings for entrance of biomass from airlock feed, and biochar discharge opposed to shaft coupling.

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

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".

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

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

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

2.3 Antimicrobial activity of BOAP

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

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

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

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

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

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

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

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

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

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

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

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

2.5 Small-scale fermentation studies

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

Before entering fermentation experiments, BOAP has been pre-treated with H_3PO_4 (0.3% w/v in water) to yield glucose from levoglucosan and oligosaccharides. Hydrolysis was performed as follows: the BOAP was 1:5 diluted with 0.3% w/w H_3PO_4, then placed in a closed pyrex vessel at 95°C. Levoglucosan hydrolysis to glucose was monitored over time by silylation and GC-MS of aliquots of hydrolysate (neutralized with $CaCO_3$ and dried). The reaction was then stopped, the solution neutralized with ammonia and vacuum filtrated onto a Buckner filter.

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

Pre-cultures of yeast strains grown to early stationary phase in SC broth containing 20 g/L glucose were used as inoculum. Cells were collected by centrifugation for 5 min at 4000 g, washed twice in sterile distilled water, and used to inoculate 50 mL medium to an initial OD_{600} of 0.3 in triplicate experiments using 55 mL glass serum bottles. The small-scale fermentations were carried out under oxygen-limited conditions. The bottles were sealed with rubber stoppers, incubated at 30°C and mixed on a magnetic stirrer (300 rpm). Syringe needles pierced through the bottle stopper served for sampling purposes and carbon dioxide removal. Samples obtained before and during fermentation were analyzed for glucose, ethanol, and glycerol content using HPLC.

2.6 Analytical methods, calculations, and statistical analysis

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

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

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

system, with a RID-10A refractive index detector (Shimadzu, Kyoto, Japan) and a Phenomenex Rezex ROA-Organic Acid H^+ (8%) column (300mm×7.8mm). The column temperature was set at 65 °C and the flow rate was 0.6 mL/min using isocratic elution, with 0.01M H_2SO_4 as a mobile phase.

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

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

3. Results

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

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

				Yields (%, w/w)			% (w/w) in pyrolysis oil									
Reactor	**Rate**	**RT (min)**	**T° (C)**	**Liquid**	**Char**	**Gas**	**H_2O**	**PL**	**WS**	**AS**	**TS**	**AA**	**C2-C3**	**PhOH**	**Furans**	**Reference**
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	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	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	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	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	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	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	Garcia-Perez al., 2008

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 pyrolysis are reported.

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

Chemical composition of BOAP can be summarized as follows: 52.0±4% water, 4.1±1% phenols, 2.4±0.5% furans, 35.0±3% sugar derivatives (e.g. levoglucosan and sugar oligomers), 2.7±0.3% acetic acid, 1.7±0.5% C2-C3 small oxygenates (e.g. hydroxyacetone, hydroxyacetaldehyde). Furthermore, in the case of fermentation kinetics, BOAP was hydrolyzed with H_3PO_4 (0.3% w/w) in order to convert levoglucosan into glucose. After 13 h, the BOAP had 7.0% glucose content and 95% hydrolysis of levoglucosan was achieved, with a slightly decreasing trend in sugar content. Interestingly, no significant change was detected for non-sugar constituents of hydrolysed BOAP (data not shown), suggesting that mild hydrolysis poorly affects the concentration of other constituents, such as acetic acid and furans.

3.2 BOAP tolerance of bacteria

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

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

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

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

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

3.3 BOAP tolerance of yeast and fungi

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

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

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

Isolate	Genus/species	(%)	Accession Number	Undiluted	1:2.5	1:5	1:10	1:30	1:50
F1	*Micrococcus lutens* 0310ARD7G_6	99	FR848405.1	-	-	-	-	+	+
F2	*Micrococcus sp.* A2-984	99	KF441624.1	-	-	-	-	+	+
F3	*Kocuria rhizophila* XFB-BG	99	KC429605.1	-	-	-	-	+	+
F4	*Pseudomonas sp.* Fse30	99	KJ733882.1	-	-	-	-	+	+
F5	*Bacillus sp.* SGD-V-25	99	KF413433.1	-	-	-	-	-	+
F6	*Micrococcus lutens* CC27	99	KJ016267.1	-	-	-	-	+	+
F7	*Bacillus subtilis* ceppo SRF1.14	99	JX232372.1	-	-	-	-	-	+
F8	*Micrococcus sp.* F16(2014)	99	KJ6051333.1	-	-	-	-	+	+
F9	*Exiguobacterium profundum* UMTAL01	99	KJ6721938.1	-	-	-	+	+	+
F10	*Achromobacter insuavis* LMG 26845	99	NR_117706.1	-	-	-	-	+	+
F11	*Agrobacterium tumefaciens* A75	99	KC196486.1	-	-	-	-	+	+
F12	*Brevundimonas diminuta* KSW68	99	LK391673.1	-	-	-	-	+	+
F13	*Micrococcus lutens* SC1204	99	KF938934.1	-	-	-	-	-	+
F14	*Kytococcus sp.* YB227	99	KJ534254.1	-	-	-	-	-	+
F15	*Kytococcus sedentarius* DSM 20547	99	CP001686.1	-	-	-	-	-	+
F16	*Kytococcus sp.* CUA-901	99	KJ732957.1	-	-	-	-	-	+
F17	*Micrococcus lutens* 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 indicting
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 H_3PO_4 (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

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

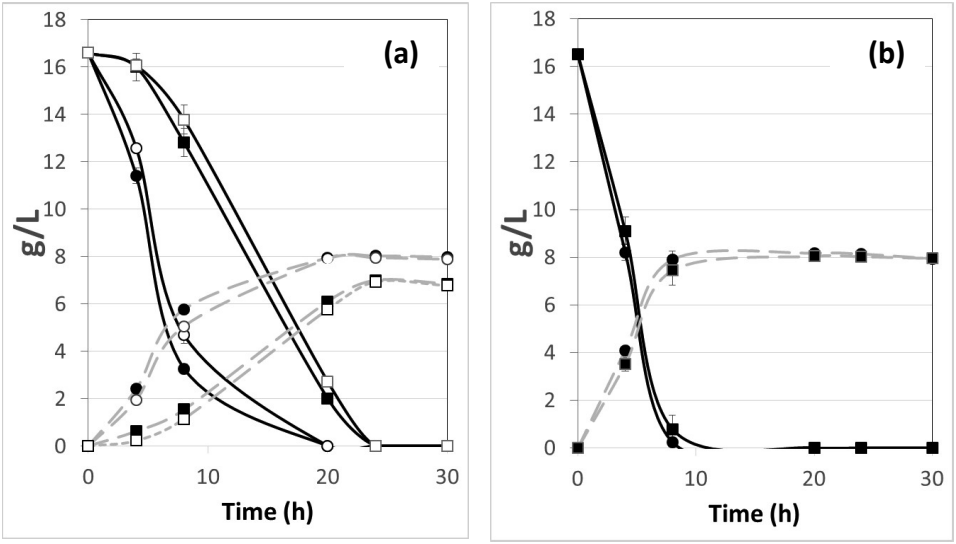

Figure 1. Ethanol production of *S. cerevisiae* L13 (circle) and the benchmark strain *S. cerevisiae* DSM70449 (square) from H_3PO_4-pre-treated BOAP (a) and reference SC medium supplemented with 16.5 g/L glucose (b). In the case of H_3PO_4-pre-treated BOAP, fermentation was performed also without SC broth (empty symbols). Glucose (black lines) and ethanol (gray lines) concentrations (g/L) are represented as a function of time. Data shown are means of three replicates and standard deviations are indicated.

4. Discussion

In view of the potential application of pyrolysis-based biotechnologies, it is crucial to look for, and further improve, novel microorganisms able to convert pyrolysis-derived products into valuable compounds. For the first time, this paper proposed a survey on a collection of microbial strains with well known industrial applications as well as new isolates in order to select microbes able to tolerate the concentration of inhibitors and to convert the bio-oil carbon fractions into valuable products. Furthermore, since the eco-toxicity of BOAPs have been studied so far only on crustacean or algal organisms [Oasmaa et al., 2012; Campisi et al., 2016], such survey was useful towards the assessment of eco-toxicological impact of fast pyrolysis BOAP on different microbial groups, revealing that this product could be metabolized as pure by fungal strains (Table 2) whereas several dilutions are needed to preserve cell viability of many bacterial and yeast isolates (Tables 1, 2 and 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)						
S. cerevisiae L13	-	8.17	0.50 (97%)	0.41	1.02	This study
S. cerevisiae DSM 70449	-	8.04	0.49 (96%)	0.40	0.93	This study
Bio-oil hydrolysate (glucose 16.5 g/L)						
S. cerevisiae L13	-	7.94	0.48 (94%)	0.33	0.63	This study
S. cerevisiae DSM 70449	-	6.93	0.42 (82%)	0.29	0.30	This study
Bio-oil hydrolysate (SC with glucose 16.5 g/L)						
S. cerevisiae L13	-	8.02	0.48 (95%)	0.33	0.72	This study
S. cerevisiae DSM 70449	-	6.99	0.42 (83%)	0.29	0.33	This study
S. cerevisiae 2.399	$Ca(OH)_2$ neutralization	15.10	0.48 (94%)	0.63	na	Yu and Zhang, 2003
S. cerevisiae T2	water extraction, Na(OH) neutralization and hydrolysate dilution	13.60	0.46 (90%)	0.55	na	Bennett et al., 2009
S. cerevisiae ATCC 200062	$Ca(OH)_2$ neutralization and activated carbon	32.00	0.47 (93%)	0.60	na	Lian et al., 2010
S. pastorianus 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

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

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

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

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

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

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

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

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

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

Credit Author Statement

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

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