Screening and evaluation of phenols and furans degrading fungi for the biological pretreatment of lignocellulosic biomass

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ABSTRACT

Physicochemical pretreatments are used to improve the bioconversion of recalcitrant lignocellulosic biomass, but they could generate toxic by-products, as furan and phenols. In this study, 40 fungal strains were analyzed for their capability to grow with different concentrations of furfural, vanillin, 4-hydroxybenzaldehyde, and syringaldehyde. *Byssochlamys nivea* MUT 6321 showed promising growth performance when the inhibitors were used as single molecules and it was the only fungus that could grow when the four molecules were simultaneously present in the culture media. Further trials demonstrated that *B. nivea* was able to completely degrade furfural in 24 h and the phenolic aldehydes in less than 11 days. In the presence of the three phenolic aldehydes, the fungus was able to transform them. However, when furfural was present in the mix, faster and preferential consumption of furfural instead of phenolic aldehydes was observed. This study provides important information for the use of this fungus to remove toxic compounds present in pretreated lignocellulosic biomass that could potentially lead to the enhancement of biofuels and chemicals production.

Keywords:

Byssochlamys nivea; Furfural; Inhibitors; Lignocellulosic biomass; Phenolic aldehydes

1. Introduction

Lignocellulosic biomasses, such as agricultural and forest residues, represent abundant, renewable, and low-cost resources to produce biofuels, chemicals, and polymers (Baruah et al., 2018). Lignocellulose is made up of cellulose, hemicellulose, and lignin fractions, linked by strong hydrogen and covalent bonds, that provide the structure its peculiar resistance to chemical and biological breakdown (Sun et al., 2016). The recalcitrant nature of lignocellulosic biomass is the major issue for its exploitation in biotechnological processes (Baruah et al., 2018). To overcome this drawback and obtain a valuable utilization of lignocellulose-rich biomasses, pretreatment processes can be applied (Sun et al., 2016). Physical and chemical processes that involve the use of high pressure and temperature, strong acids, and bases, are the most commonly employed methods to improve lignocellulosic bioconversion (Chen and Liu, 2015). Unfortunately, they could be associated with the generation of undesired by-products, including furan derivatives (e.g. furfural and 5-hydromethylfurfural), organic acids (e.g. acetic acid, formic acid, and levulinic acid), and phenolic compounds (e.g. vanillin, syringaldehyde, 4-hydroxybenzaldehyde, coniferyl aldehyde, ferulic acid, and cinnamic acid) (Kumar et al., 2019a; Palmqvist and Hahn-Hägerdal, 2000). Vegetal biomasses also have a plethora of phenolic compounds, which are secondary metabolites with different activities in plants, and their chemical structure and abundance vary among species (Kumar et al., 2019b). These compounds have been previously reported to inhibit various bioprocesses, such as the production of methane, ethanol, biohydrogen, xylitol, butanol, and lipids (Monlau et al., 2014). The removal of these inhibitors could enhance the performance of several biotechnological processes (Baruah et al., 2018). Different physical and chemical methods that use membranes, ion-exchange resins, neutralization, liquid–liquid extraction, have been developed for elimination or abatement of the effects of the inhibitory compounds (Kumar et al., 2020). Biological approaches could also be used for lignocellulose detoxification in which intervein microorganisms (either single or in co-culture) or enzymes (pure or cocktails) (Zabed et al., 2019).Transformation of inhibitors into less toxic compounds using biological methods provides several advantages over the physical and chemical methods, such as low costs, mild operative conditions, high degradation efficiency, and low water and energy consumption (He et al., 2016; Jönsson and Martín, 2016). Various microorganisms have already been assessed to degrade the lignocelluloses-derived inhibitors (Ran et al., 2014). Most of the research on biodegradation of phenolic compounds has focused on bacteria, especially *Pseudomonas* genus, and fungi, i.e., Basidiomycota (Al-Khalid and El-Naas, 2012). White-rot fungi possess a nonspecific lignocellulolytic enzyme system that allows them to oxidize a wide range of substrates, including lignin, phenols, and other aromatic compounds (Martínková et al., 2016; Elisashvili et al., 2018). Fungi belonging to other taxonomic groups have been scarcely investigated, even though Ascomycota such as *Aureobasidium*, *Candida, Penicillium, Aspergillus, Fusarium,* and *Graphium* are capable of mineralizing aromatic compounds (dos Santos et al., 2009; Al-Khalid and El-Naas, 2012). Likewise, the biodegradation of furans has been extensively studied on bacteria such as *Cupriavidus basilensis* and *Pseudomonas putida,* which have been characterized at physiological and genetic levels (Koopman et al., 2010; Guarnieri et al., 2017). Furans degradation has also been observed in fungi, including *Amorphotheca resinae* (He et al., 2016; Jönsson and Martín, 2016; Ran et al., 2014; Wang et al., 2015; Yi et al., 2019), *Coniochaeta ligniaria* (López et al., 2004; Nichols et al., 2008; Cao et al., 2015), *Aspergillus niger* (Rumbold et al., 2009), *Trichoderma reesei* (He et al., 2020), *Aspergillus nidulans* (Yu et al., 2011), *Paecilomyces* sp. (Nakasaki et al., 2015), *Chaetomium globosum, Cunninghamella elegans, Mucor plumbeus, Mortierella isabellina*

(Zheng et al., 2012; Ruan et al., 2015), *Pleurotus ostreatus* (Feldman et al., 2015), and *Trametes versicolor* (Kudahettige Nilsson et al., 2016). Even though many fungal species can degrade furan and phenolic compounds separately, it is less likely to find a single organism harboring the whole variety of enzymes needed for the total elimination of these inhibitors. It is necessary to screen a wide diversity of fungal strains to enhance the probability of finding a fungus with an outstanding performance in growing and degrading lignocelluloses-derived inhibitors. For this reason, this study aims to investigate the potential of fungi for detoxification of phenolic aldehydes and furfural, as single molecules and combinations of them. A miniaturized screening was carried out to evaluate the capability of 40 fungal strains to grow in the presence of inhibitors such as furfural, vanillin, syringaldehyde, and 4-hydroxybenzaldehyde. Based on the growth performance, *Byssochlamys nivea* MUT 6321 was selected to analyze its degradation ability against these toxic compounds.

2. Materials and Methods

2.1. Chemicals

Furfural, vanillin, syringaldehyde, 4-hydroxybenzaldehyde, gallic acid, and guaiacol were 98-99 % pure and purchased from Sigma-Aldrich (St. Louis, MO, U.S.A.). Stock solutions were prepared in ethanol. Ultrapure water, ethanol, methanol, and acetic acid were High-Performance Liquid Chromatography (HPLC) grade (Biopack, Argentina). HPLC solvents were filtered through 0.45 µm nylon membrane and degassed in an ultrasonic bath before use.

2.2. Fungal strains

Forty fungal strains from the culture collection of *Mycotheca Universitatis Taurinensis* (Department of Life Sciences and Systems Biology, University of Turin, Italy) were selected to be studied in the high-throughput microplate screening. They included 26 Ascomycota, 11 Basidiomycota, and 3 Mucoromycota, and the selection was based on different criteria (e.g., taxonomy and phylogenetic relationships, adaptability to different and adverse environmental conditions, the substrate of isolation, and potential of producing lignocellulolytic enzymes).

2.3. High-throughput microplate screening

Fungi were pre-grown on Petri dishes (9 cm diameter) with Malt Extract Agar (MEA: 20 g L^{-1} malt extract, 20 g L^{-1} glucose, 2 g L^{-1} peptone, 18 g L^{-1} agar) and incubated at 25 °C for 7-14 days. For Ascomycota and Mucoromycota, a conidia suspension was prepared. For Basidiomycota, mycelia homogenate was prepared using a sterile mixer (Spina et al., 2018). The fungus was suspended in a mineral medium (2 g L^{-1} $NaNO_3$, 1 g L^{-1} NH_4Cl, 0.01 g L^{-1} $ZnSO_4.7H_2O$, 0.005 g L^{-1} $CuSO_4.5H_2O$, 0.5 g L^{-1} KCl, 0.5 g L^{-1} $MgSO_4.7H_2O$, 0.01 g L^{-1} $FeSO_4.7H_2O$, and 0.01 g L^{-1} agar). A turbidimeter (Biolog Inc., Hayward, USA) was used to standardize the concentration of the fungal inoculum: the optical density was set at 60-70 % and 40-60 % transmittance for conidia suspension and mycelium homogenate, respectively.

Fungi were screened in 96-wells microplates (Sarstedt, Nümbrecht, Germany) to evaluate their tolerance towards furfural (F), vanillin (V), syringaldehyde (S), 4-hydroxybenzaldehyde (H), and a mix of the four molecules MPF (Mix Phenols Furfural). Single-molecule trials were run at different concentrations, namely 1 and 2 g L^{-1} for F, 0.5 and 2 g L^{-1} for V, and 0.25 and 2 g L^{-1} for H and S. MPF included 1 g L^{-1} F, 0.5 g L^{-1} V, 0.25 g L^{-1} H and 0.25 g L^{-1} S. Target molecules were used as the sole carbon source or in combination with 2 g L^{-1} glucose as co-substrate. Two biotic controls were performed to evaluate the growth capacity of each isolate in

132 the presence of glucose (0.25, 0.5, 1, 2 g L^{-1}) or without the addition of an external carbon source.
133 Abiotic control was carried out as blank using the mineral medium supplemented with the
134 molecules. Microplates were stored in sealant boxes and incubated at 25 °C in the dark for 3
135 weeks. Every 2 days, the mycelial growth was spectrophotometrically measured at 750 nm
136 (Infinite M2000 with Magellan V 6.5 software, TECAN).
137
138 **2.4. Degradation assays of inhibitor compounds**
139 *Byssochlamys nivea* MUT 6321 (GenBank Accession Number: MT151630) conidia
140 suspension was prepared and adjusted to a concentration of 1×10^6 spores mL^{-1}. The suspension
141 was used to inoculate 150 mL flasks containing 60 mL sterile mineral medium supplemented
142 with 2 g L^{-1} glucose, chloramphenicol, and gentamicin to a final concentration of 1 µg mL^{-1}.
143 Flasks were incubated at 25 °C in agitation at 120 rpm. After 4 days, the molecules were added as
144 i) single compounds (1 g L^{-1} F; 0.5 g L^{-1} V; 0.25 g L^{-1} S; 0.25 g L^{-1} H), ii) MPF mix, iii) MP (Mix
145 Phenols): MPF mix without the addition of 1 g L^{-1} F. Each treatment was performed in triplicate.
146 An aliquot of each culture broth and abiotic control were harvested every day and centrifuged at
147 10.000 x g, 10 min, 4 °C. Supernatants and standard calibration solutions of F, V, H, and S were
148 filtered through 0.22 µm nylon membrane and used for HPLC determinations. Glucose
149 concentration was measured in supernatants using an enzymatic assay kit (Wiener lab.,
150 Argentina). After 23 days, mycelia were filtered and lyophilized to determine the dry weight.
151 HPLC analyses on cell-free supernatants were performed according to Canas et al., (2011),
152 with modifications. The HPLC system Dionex Ultimate 3000 (Thermo Fisher Scientific,
153 Massachusetts, USA) and the software Chromeleon Chromatography Data System (Thermo
154 Fisher Scientific, Massachusetts, USA) were used for the acquisition and management of data.
155 The ProntoSIL Spheribond ODS C18 5 µm column (150 x 4.6 mm) (Bischoff Chromatography,
156 Leonberg, Germany) was used as the stationary phase. The following chromatographic conditions
157 were selected: column temperature of 25 °C, a flow rate of 1 mL min^{-1}, and an injection volume
158 of 20 µL. Concerning the elution program, a binary gradient was selected, using solvent A
159 water/acetic acid (98:2 v/v) and solvent B methanol/water/acetic acid (70:28:2 v/v/v). Separations
160 of single molecules were carried out isocratically at 50 % B. For MP and MPF mixes, the
161 following elution program was used: 0 % B isocratic in 3 min, linear gradient from 0 to 40 % B
162 in 22 min, from 40 to 60 % B in 18 min, 60 % B isocratic in 12 min, linear gradient from 60 to 80
163 % B in 5 min, 80 % B isocratic in 5 min. Simultaneous detection was done at wavelengths of 232
164 (for furoic acid and furfuryl alcohol), 278 (for F, V, and H), and 309 (for S) nm. The
165 identification of compounds was made by comparing the retention time and UV-Vis spectra of
166 the peaks with those obtained by injection of standards solutions. Quantification was carried out
167 at their maximum absorption by referring to a calibration curve in the range of 0-2 g L^{-1} of the
168 standards.
169
170 **2.5. Data and statistical analyses**
171 Four-way Venn diagram analyses were used to display all the 15 possible overlaps among
172 datasets corresponding to the growth percentage of fungi in the presence of the inhibitors in
173 comparison with their respective control growing with glucose as the sole carbon source (Table
174 S1; Oliveros, 2007). Ellipses intersections show those fungi that were inhibited by the four
175 compounds (one overlapping area composed by four ellipses), three compounds (four
176 overlapping areas composed by three ellipses), two compounds (six overlapping areas composed
177 by two ellipses), and those that were inhibited by one compound (the four areas where ellipses
178 are not intersected).

179 Experimental data were subjected to analysis of variance (ANOVA) and Dunnett´s or
180 Tukey's post hoc test to detect significant differences. A p-value of less than 0.05 was considered
181 statistically significant. All experiments were done at least three times.
182

183 ## 3. Results

184 ### 3.1. High-throughput screening for fungal strains tolerant to furfural and phenolic
185 aldehydes

186 A wide fungal biodiversity (40 strains-species) from the *Mycotheca Universitatis*
187 *Taurinensis* collection was analyzed for its potential to grow on F (derived from dehydration of
188 pentoses) and the phenolic aldehydes H, V, and S (lignin derivatives of p-hydroxyphenyl,
189 guaiacyl, and syringyl groups) (Palmqvist and Hahn-Hägerdal, 2000). Two different
190 concentrations of these compounds were assayed, as single molecules or combinations of them,
191 and in the presence or not of glucose as co-substrate (Fig. 1, Table S1). A different degree of
192 growth inhibition was observed for the 40 analyzed fungi when exposed to 2 g L^{-1} F, V, S, or H
193 used as single molecules (Fig. 1). Four-way Venn diagrams were used to perform comparison
194 analyses among datasets of those fungi that displayed a growth inhibition higher than 90 % (Fig.
195 2). In the absence of glucose, the analysis showed that 37 species had an inhibition on biomass
196 production higher than 90 % in all tested conditions, while when glucose was present in the
197 media, 34 species showed this behavior (Fig. 2). *B. nivea*, *Paecilomyces variotii*, and *Aspergillus*
198 *niger* growth were inhibited above 90 % in the presence of V or H (Fig. 2). *B. nivea* growth was
199 inhibited around 50 % by F or S, irrespectively of the presence of glucose in the media (Table
200 S1). *P. variotii* had the best growth performance on F, showing inhibitions of 1 and 18 % in the
201 presence or absence of glucose, respectively (Table S1). As regards S, *P. variotii* had an
202 inhibition on biomass production of 42 and 83 % in the presence or absence of glucose,
203 respectively (Table S1). *A. niger* had a growth inhibition of 62 % by F and 73 % by S when
204 glucose was also present in the media (Table S1). *Ganoderma lucidum* showed an inhibition
205 higher than 90 % when grown in the presence of the three phenolic aldehydes as single molecules
206 but showed a better growth performance in the presence of F (inhibition around 75 %),
207 independently of the presence or not of glucose (Fig. 2; Table S1). In the presence of glucose, 90
208 % of growth inhibition was observed in *Aspergillus terreus* when F, H, or V were in the media,
209 and in the case of *Trametes versicolor* in the presence of F, H, or S (Fig. 2).

210 Growth performance of fungal strains was also analyzed at lower concentrations: 1 g L^{-1} F,
211 0.5 g L^{-1} V, 0.25 g L^{-1} H, and 0.25 g L^{-1} S. Fig. 3 shows those fungi that displayed a growth
212 inhibition lower than 50 % in the absence or presence of glucose. *B. nivea* and *P. varioti* were
213 poorly affected by the four compounds, irrespectively of the presence of glucose as co-substrate.
214 Moreover, *P. varioti* was the least affected by F, followed by *B. nivea* (Fig. 3B, Tukey's test
215 $p<0.05$). *A. niger*, *A. terreus*, and *G. lucidum* were inhibited less than 50 % in the presence of the
216 phenolic aldehydes used as single molecules, regardless of these molecules were the only carbon
217 source or with the addition of glucose. Furthermore, *A. niger* showed the best growth
218 performance when V, H, or S were in the culture media, irrespective of the presence of glucose
219 (Fig. 3B, Tukey´s test $p<0.05$). As regards F, growth inhibition of 26 % was observed in *A. niger*
220 in the presence of glucose whereas this inhibitor deeply affected *A. terreus* and *G. lucidum*
221 growth (Fig. 3B). *Fusarium fujikuroi* showed the worst growth performance in most of the
222 conditions, excepting when it was grown with S in the culture media (Fig. 3B, Tukey´s test
223 $p<0.05$).

224 Fig. 4 shows the growth curves of those fungi that had the best growth performance at the
225 highest (Fig. 2), and the lowest (Fig. 3) inhibitors concentrations. At a concentration of 2 g L^{-1} of
226 each inhibitor, most of the analyzed fungal strains showed a clear growth inhibition, which was
227 reflected by longer lag phases and lower OD values, in comparison to their respective controls
228 grown with glucose as the sole carbon source (Fig. 4A and C). Some of the growth curves
229 obtained with fungi growing with the inhibitors at the lowest concentrations reached a stationary
230 phase as in the case of their controls (Fig. 4B and C). In general, the maximum OD values were
231 higher in the presence than in the absence of glucose (Fig. 4B). *P. variotii* showed a similar
232 growth in the presence of F (2 and 1 g L^{-1}) and with glucose as the sole carbon source, reaching
233 similar maximum OD values and with similar length of the lag phases (Fig.4). In the case of the
234 phenolic aldehydes, *P. variotii* could not grow in the presence of V and H at the maximum
235 concentration, although it could do so with S, and the inhibition was less notable in the presence
236 of glucose as a co-substrate (Fig 4A). In the case of *B. nivea*, although it was able to grow with F
237 and S at both concentrations, it could be observed that growth curves were shifted to the right
238 with increasing the inhibitor concentration (Fig. 4A and B).
239 Since lignocellulosic hydrolysate generally contains multiple inhibitors, a mix with the four
240 molecules at the lowest concentrations was tested (MPF mix). Using the MPF mix, most of the
241 fungi (39 out of 40 strains) almost did not grow (inhibition above 90 %). *B. nivea* was the only
242 exception and the inhibition was even halved in the presence of glucose (from 78 % to 35 %
243 inhibition) (Fig. 1, Table S1).
244
245 **3.2. Degrative Ability of *Byssochlamys nivea* MUT 6321**
246 Among the screened fungal strains, only *B. nivea* was able to grow in the presence of 1 g L^{-}
247 ^{1} F; 0.5 g L^{-1} V; 0.25 g L^{-1} H, and 0.25 g L^{-1} S separately and when exposed simultaneously to the
248 four compounds (Fig. 1, 3 and Table S1). Thus, it was selected to perform quantitative studies
249 and the experiments were carried out using the inhibitory compounds as single molecules or
250 mixes of them at the concentrations stated above. Glucose was added to the culture media
251 considering that during physicochemical lignocellulose biomass pretreatment, partial hydrolysis
252 of cellulose into glucose takes place in addition to the formation of toxic compounds (Ran et al.,
253 2014).
254 The capacity of *B. nivea* to degrade F and the phenolic aldehydes was analyzed by
255 monitoring the residual concentration of each compound in the media. None of the compounds
256 suffered abiotic degradation (i.e. in negative controls without the fungus; Fig. S1). *B. nivea*
257 showed a great efficiency degrading F, despite the high concentration tested (1 g L^{-1}). A
258 reduction of 99 % was observed after 24 h that was due to the active metabolism of *B. nivea* (Fig.
259 5). As regards the phenolic aldehydes, results showed that 99 % of H, S, and V were transformed
260 after 4, 9, and 11 days, respectively (Fig. 5). Even though the concentrations used for S and H
261 were the same (0.25 g L^{-1}), S degradation was slower than H (Fig. 5). V was more recalcitrant
262 since the transformation rate was slower than the other phenolic molecules (Fig. 5).
263 To analyze the combined effect of the toxic molecules, two different mixes were tested: i) a
264 mix of the four compounds at the same concentration as single molecules (MPF), ii) a mix with
265 only the three phenolic aldehydes (MP). MP mix was included in the analysis to study the effect
266 of F in V, H, and S degradation. Phenolic aldehydes were almost completely metabolized in the
267 MP mix. H and V concentrations decreased faster than S, being these two molecules undetectable
268 in the culture media after 16 days of treatment (Fig. 6A). On the other hand, S was the least
269 biodegradable compound, remaining 2 % of the molecule at the end of the experiment (Fig. 6A).
270 In the presence of MPF, the fungus first degraded F, being completely removed after 4 days of

271 exposure to the four molecules (Fig. 6B). At the end of the experiment, V and H concentrations
272 were reduced up to 80 %, but S was unaltered (Fig. 6B).
273 In the bioconversion experiments performed with *B. nivea*, it was observed that when F
274 was completely degraded, 87 % of the initial glucose was still present in the culture media (Table
275 1). In the case of phenolic aldehydes, by the time H or S was completely converted, the
276 percentage of the initial glucose that remained in the culture media were 35 % and 11 %,
277 respectively (Table 1). After 23 days of *B. nivea* growing in the MPF mix, it was observed that F
278 and the phenolic aldehydes V and H were almost undetectable while 15 % of glucose remained in
279 the supernatant (Table 1). Regarding the effect of inhibitors on *B. nivea* biomass, there were no
280 statistical differences in mycelium dry weight among F, V, S, and the control sample grown in
281 the absence of inhibitors (Dunnett's test, $p>0.05$, Table 1). As expected, the collected biomass
282 was significantly lower in MP and MPF samples than the control (Dunnett's test, $p<0.05$, Table
283 1).
284 HPLC analysis showed the appearance of different peaks corresponding to V, H, and S
285 derivatives (Fig. S2). Two peaks that were detected in H and S but not in V samples showed
286 increased areas as the experiment proceeded. One of these peaks had a retention time of 1.6 min
287 and a spectrum with a maximum at 270 nm that coincided with retention time and UV-Vis
288 spectral characteristics of the gallic acid standard. A peak with a longer retention time (2.2 min)
289 and a different spectrum (maximums 232 and 280 nm) was detected in V chromatograms but did
290 not appear in H and S samples (Fig. S2). This peak does not correspond to guaicol standard
291 (retention time 5.5 min; maximum 276 nm), which could be a possible derivative of V (Mäkelä et
292 al., 2015 and references therein). On the other hand, no F derivatives (e.g. furoic acid and furfuryl
293 alcohol) were detected in HPLC chromatograms (data not shown).
294

295 ## 4. Discussion

296 The concentration and the composition of lignocellulose-derivative compounds formed
297 during physicochemical pretreatments usually differ according to the lignocellulosic biomass
298 used and the applied treatment. For instance, He et al., (2016) reported that corn stover
299 hydrolysate contained 0.75 g L^{-1} F, 0.57 g L^{-1} V, 0.25 g L^{-1} S, and 0.06 g L^{-1} H. Srilekha Yadav et
300 al., (2011) showed that in rice straw hydrolysate, F concentration was 0.21 g L^{-1} and the total
301 phenolic concentration reached up to 1.58 g L^{-1}. It should be noted that many studies on the
302 inhibitory effects of phenolic and furanic compounds have been performed using higher
303 concentrations than those present in the lignocellulosic hydrolysates (e.g. 5 g L^{-1}) (Palmqvist and
304 Hahn-Hägerdal, 2000). For this reason, the concentrations used in this study for F, V, H, and S
305 were in the range of the levels found in physicochemical pretreated corn stover and rice straw
306 (Srilekha Yadav et al., 2011; He et al., 2016).
307 The high-throughput screening performed with the 40 fungal strains confirmed the strong
308 toxicity of phenolic aldehydes and furanic compounds, even at low concentrations. Furthermore,
309 results showed that in the presence of single molecule solutions, the growth inhibition depends
310 not only on the nature and concentration of the assayed compounds but also on the presence of
311 glucose as co-substrate (Fig. 1, Table S1). *P. variotii*, *B. nivea, A. niger, A. terreus*, and *G.*
312 *lucidum* have shown high tolerance in most of the tested conditions, even when the inhibitors
313 were used as the sole source of nourishment (Fig. 2 and 3). Growth curves of these fungi showed
314 different shapes according to the fungal strain but also was dependent on the presence of glucose
315 as co-substrate, and the type and concentration of the inhibitors (Fig. 4). Many phenotypic,
316 physiological, and molecular changes occur at the beginning of the growth curve to adapt the cell

317 to stressful conditions which are reflected in variations in the lag phase length, the slope of the
318 growth curve, or both (Hamill et al., 2020). No data are available on these strains about growth
319 inhibition using the assayed molecules and literature reports just a few examples. For instance, *A.*
320 *resinae* ZN_1 could grow with F as the sole carbon source but its degradation was significantly
321 accelerated in the presence of glucose (Ran et al., 2014). *Candida tropicalis* demonstrated the
322 ability to use phenol as the sole carbon source up to a concentration of 2 g L^{-1} (Komarkova et al.,
323 2003). On the other hand, other studies showed that many fungi metabolize lignin-related
324 phenolic compounds only in the presence of a co-substrate (Kirk and Farrell, 1987; Kowalczyk et
325 al., 2019). According to the screening datasets, *P. variotii*, *B. nivea, A. niger, A. terreus*, and *G.*
326 *lucidum* presented in all the tested conditions lower percentages of growth inhibition when
327 glucose was present (Fig. 3B, Fig. 4, Table S1). These results confirmed that glucose could
328 promote better fungal growth, favoring the survival or activating a co-metabolism suitable for the
329 degradation.
330 Some fungal species tested in this study were previously reported for their ability to tolerate
331 furans and phenols but were highly inhibited under the screening conditions used in the present
332 work. For instance, the white-rot fungus *T. versicolor* CBS 109428 metabolized phenols and
333 furans when used as single molecules or a mixture of them, and at a range of concentrations
334 between 0.2-0.6 g L^{-1} (Kudahettige Nilsson et al., 2016). However, the *T. versicolor* strain used in
335 this study was very sensitive to F, S, H, and V presence (inhibition ranging from 70 to 90 %)
336 (Table S1). Similarly, *M. isabellina* ATCC 42613 can tolerate 1 g L^{-1} F (Ruan et al., 2015) while
337 the strain used in this study did not grow (97 % inhibition). These findings underline that the
338 ability to tolerate and degrade compounds is a strain-specific feature. Considering the limited
339 research on fungi in comparison to bacteria (Al-Khalid and El-Naas, 2012), this study emphasizes
340 the importance of investigating fungal biodiversity to identify new species and strains for the
341 development of improved bioprocesses.
342 An efficient detoxification step of lignocellulosic material subjected to physicochemical
343 pretreatments requires microorganisms that are capable of transforming inhibitors into less toxic
344 compounds in the shortest time possible and that the consumption of sugars in this period be as
345 low as possible since they would be used in the synthesis of the target biotechnological product.
346 Fig. 5 shows that *B. nivea* was able to biodegrade 1 g L^{-1} F in 24 h, showing a better performance
347 than the kerosene fungus strain *A. resinae* ZN_1 that completely degrade the same concentration of
348 F only after 60-70 h (Ran et al., 2014). As regards the phenolic aldehydes, when these molecules
349 were assayed individually, the least biodegradable compound was V, followed by S and H (Fig.
350 5). Even though H and S were tested at the same concentration, S appeared to be less toxic than H
351 since the dry cell mass obtained at the end of the experiment was higher for the former than for
352 the latter (Table 1).
353 *B. nivea* completely degraded V, H, and S present in MP mix (Fig. 6A). However, when F
354 was present in the mix, faster and preferential consumption of F instead of phenolic aldehydes
355 was observed. Furthermore, V, H, and S in the MPF mix showed a delay in their transformation
356 in comparison to MP mix (Fig. 6A and B). This finding could be explained considering that F
357 and the phenolic aldehydes are molecules with different chemical structures and may then require
358 the activation of different catabolic mechanisms, demanding time and resources to accomplish it
359 (Yi et al., 2019). The preference in these toxic molecules transformation is a promising feature of
360 *B. nivea*, considering that phenolic compounds, such as S and V, were reported to have less
361 impact than F on biotechnological processes (e.g. biohydrogen production) (Monlau et al., 2014).
362 Furthermore, the differences in the time course profiles of fungal degradation of single molecules

363 compared to the mixes could be explained by the increase of the total concentration of
364 compounds and a possible synergistic toxic effect (Monlau et al., 2014).
365 Transcriptional analysis performed with *A. resinae* ZN_1 exposed to the toxic effects of F, V,
366 or S showed that these molecules inhibited the expression of genes encoding glucose transporter
367 and some enzymes involved in sugar metabolism that could explain the fact that these molecules
368 are consumed before glucose (Yi et al., 2019). In the case of *B. nivea*, the toxic effect of F and the
369 phenolic aldehydes could also affect the metabolization of glucose since a percentage of this
370 sugar remained in the media after the inhibitors were degraded (Table 1).
371 Even though information about furan metabolization in fungi is scarce, two pathways for F
372 degradation have been proposed based on RNA-Seq data from *A. resinae* (Yi et al., 2019) and
373 studies performed with the bacteria *C. basilensis* HMF14 (Koopman et al., 2010). F could be first
374 reduced to the less toxic furfuryl alcohol and then, one or the other molecule is oxidized to the
375 intermediate furoic acid. This molecule is transformed after several enzymatic steps into 2-
376 oxoglutarate that enters the tricarboxylic acid (TCA) cycle. In the present study, furoic acid and
377 furfuryl alcohol were not detected in HPLC analysis (data not shown). This could be due to the
378 fast rate of F transformation that may have prevented these derivatives to be detected. As regards
379 the biotransformation of phenolic compounds, literature data about the degradation pathway
380 involved are sometimes contrasting. For instance, *A. resinae* transformed V, H, and S into the
381 less toxic phenolic alcohols and then they were oxidized to phenolic acids before being
382 metabolized through the TCA cycle. Based mainly on transcriptomic data, it was predicted that S
383 could be converted to gallic acid before entering the TCA cycle whereas H and V may be
384 converted to protocatechuic acid and then catabolized using similar enzymes (Yi et al., 2019). In
385 the present study, results suggest that H and S were metabolized through the gallate pathway
386 since gallic acid was formed during degradation assays (Fig. S2). On the other hand, it was
387 reported that filamentous fungi may catabolize V via different pathways: (1) non-oxidative
388 decarboxylation to guaiacol, (2) oxidation of V to protocatechuate which is followed by aromatic
389 ring opening, and (3) oxidative decarboxylation to methoxy-p-hydroquinone (Mäkelä et al., 2015
390 and references therein). The non-oxidative degradation route of V is an infrequent pathway
391 because it has only been described in a limited number of ascomycetes species such as
392 *Sporotrichum thermophile* (Topakas et al., 2003), *P. variotii* (Rahouti et al., 1989), some
393 Aspergilli and yeasts (Guiraud et al., 1992; Huang et al., 1993). Guaiacol did not appear in HPLC
394 chromatograms of V degradation by *B. nivea* (Fig. S2). Besides, when the fungus was grown
395 with V, an increment in brown discoloration of the culture media was observed while the
396 concentration of V diminished (data not shown). Conversion of V to methoxyhydroquinone by
397 decarboxylating vanillate hydroxylase and further to hydroxyquinol has been reported in several
398 white-rot fungi (Buswell et al., 1981). These quinones could be the dark-colored metabolite
399 present in those cultures where V was present and degraded. For these reasons, it could be
400 hypothesized that in *B. nivea* the catabolism of V may proceed mainly through an oxidative
401 pathway different from that used by H and S.
402 To the best of the authors' knowledge, this is the first report on the degradation of
403 lignocellulose-derivative inhibitors by *B. nivea* strains. Members of the genus *Byssochlamys*
404 (Family Trichocomaceae) are extremotolerant fungi, adaptable to adverse environmental
405 conditions, widespread in soils, and with a high capability to survive across a broad range of pH
406 and temperatures, and under low-oxygen conditions (Stamps et al., 2020). Because of these
407 features, *Byssochlamys* spp. are often implicated in spoilage of thermally processed or
408 pasteurized foods, as packaged and canned fruit products (i.e. juices) (Tournas, 1994). The ability
409 to exploit these matrices could suggest that these fungi may be tolerant towards molecules

derived from thermal pretreatment of plant material (i.e. fruit), such as furans, which are formed at high temperatures and pressure due to dehydration reactions of hexose and pentose sugars (Palmqvist and Hahn-Hägerdal, 2000). Recently, the genome of *Byssochlamys* isolate BYSS01 adapted to kerosene fuel was sequenced, and several genes and proteins involved in carbon and energy metabolism, transport of molecules, and degradation of alkanes and aromatic hydrocarbons were identified. The genome sequence reflects the ability of the BYSS01 isolate to grow in the presence of fuel and this information could help to understand the adaptive mechanisms employed by *Byssochlamys* to survive in the presence of these toxic compounds (Radwan et al., 2018). *B. nivea* MUT 6321 was isolated from digestate obtained from maize-silage based biogas production. Digestate is the by-product of the anaerobic digestion process, which occurs at relatively high temperatures (approx. 40 °C) and it may contain several compounds resulting from the hydrolysis of plant cell wall polymers. Panuccio et al., (2016) reported that the liquid and solid fractions of digestate contained total phenol concentrations of 395 ± 12 mg L^{-1} and 325 ± 9 mg L^{-1}, respectively. All these findings could further explain the adaptability and ability of *B. nivea* MUT 6321 to grow and degrade F and phenolic aldehydes.

5. Conclusions

The present study highlights the importance to explore fungal biodiversity to discover new strains for future biotechnological applications. Among the 40 fungal strains analyzed, *B. nivea* MUT 6321 was able to grow on F, V, H, or S as the sole carbon source and it was the only fungus that could grow with the four inhibitors. Furthermore, this fungus has a high performance in degrading F and phenolic aldehydes, as single molecules or mixes. In conclusion, *B. nivea* has a great potential to remove furan and phenols from lignocellulosic biomasses subjected to physicochemical pretreatments to improve bioprocesses in which they are used.

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Appendix A. Supplementary data

The following is the Supplementary data to this article:

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601

602 **Data Availability**

603 The nucleotide sequences of the Internal Transcribed Spacer (ITS1-ITS2) of *Byssochlamys*
604 *nivea* MUT 6321 can be found in Genbank https://www.ncbi.nlm.nih.gov/genbank/, hosted at the
605 National Center for Biotechnology Information (NCBI) database, under the Accession Number
606 MT151630.
607
608

609 ## Figure and Table Captions

610 **Figure 1.** Number of species-strains and respective percentage of growth inhibition (I %)
611 after 21 days of growth in the presence of furfural (F), vanillin (V), 4-hydroxybenzaldehyde (H),
612 and syringaldehyde (S) in comparison with the respective control growing with glucose as the
613 sole carbon source. The molecules tested as inhibitors were used at the different concentrations
614 shown in the table and in the presence or not of 2 g L^{-1} glucose (Glu) as co-substrate.
615
616 **Figure 2.** Four-way Venn diagrams showing the relationship among fungi with a
617 percentage of growth inhibition (I %) higher than 90 % in the presence of furfural (F), vanillin
618 (V), 4-hydroxybenzaldehyde (H), and syringaldehyde (S). Inhibitors were used at 2 g L^{-1} as the
619 sole carbon source or in the presence of 2 g L^{-1} glucose as co-substrate.
620
621 **Figure 3.** (A) Four-way Venn diagrams showing the relationship among fungi with a
622 percentage of growth inhibition (I %) lower than 50 % in the following conditions: furfural (F) 1
623 g L^{-1}, vanillin (V) 0.5 g L^{-1}, 4-hydroxybenzaldehyde (H) 0.25 g L^{-1}, and syringaldehyde (S) 0.25
624 g L^{-1}. Inhibitors were used as the sole carbon source or in the presence of 2 g L^{-1} glucose (Glu) as
625 co-substrate. (B) Average fungal growth percentage ± SE (*n=6*) of fungi that appear in the Venn
626 diagrams in comparison with their respective control. Negative values indicate inhibition, while
627 values equal to or higher than 0 indicate growth comparable or higher than controls. In bold,
628 fungi inhibited less than 50 %. Different capital letters (A, B, C, D, or E) show significant
629 differences among fungi within each treatment (Tukey´s test, $p<0.05$).
630
631 **Figure 4**. Growth curves of *A. niger*, *A. terreus*, *B. nivea*, *G. lucidum*, and *P. variotii* in the
632 presence of 2 or 1 g L^{-1} furfural (F), 2 or 0.5 g L^{-1} vanillin (V), 2 or 0.25 g L^{-1} 4-
633 hydroxybenzaldehyde (H), and 2 or 0.25 g L^{-1} syringaldehyde (S). Each concentration was
634 performed in the presence or not of 2 g L^{-1} glucose. Controls correspond to the five fungi grown
635 with 0.25, 0.5, 1, and 2 g L^{-1} of glucose as the sole carbon source. The data represent the mean of
636 the OD values measured at 750 nm at each time point. The error bar indicates the SE (*n*=6).
637
638 **Figure 5.** Time course profiles of fungal degradation of furfural (F), vanillin (V), 4-
639 hydroxybenzaldehyde (H), and syringaldehyde (S). *B. nivea* MUT 6321 was grown in the
640 presence of the inhibitors as single molecules, at the following concentrations: 1 g L^{-1} F, 0.5 g L^{-1}
641 V, 0.25 g L^{-1} H, and 0.25 g L^{-1} S. The data represent the mean of the relative concentrations of
642 the molecules at each time point in comparison to their initial concentrations. The error bar
643 indicates the SE (*n*=3).
644
645 **Figure 6.** Time course profiles of fungal degradation of (A) MP mix: vanillin (V), 4-
646 hydroxybenzaldehyde (H), and syringaldehyde (S) and (B) MPF mix: MP with the addition of
647 furfural (F). *B. nivea* MUT 6321 was grown in the presence of the three phenolic compounds,
648 with the addition or not of 1 g L^{-1} F, at the following concentrations: 0.5 g L^{-1} V, 0.25 g L^{-1} H,
649 and 0.25 g L^{-1} S. The data represent the mean of molecules concentrations at each time point. The
650 error bar indicates the SE (*n*=3).
651
652 **Table 1.** Effect of furfural (F), vanillin (V), 4-hydroxybenzaldehyde (H), and
653 syringaldehyde (S), as single molecules or mixes (MP and MPF), on glucose consumption during
654 *B. nivea* MUT 6321 growth, and the fungal dry biomass at day 23 day. Percentage of glucose

remaining in the culture media was calculated at the time inhibitors were completely degraded or at the end of the experiment (day 23) in relation to the initial glucose concentration. Control corresponds to *B. nivea* grown under the same conditions, except that inhibitors were not present in the culture media. Values are expressed as mean values ± SE (*n=3*). The asterisks indicate a significant difference between the control and treatment samples (Dunnett's test, $p<0.05$).

Supplementary Figure and Table Captions

Figure S1. Time course profiles of abiotic degradation of furfural (F), vanillin (V), 4-hydroxybenzaldehyde (H), and syringaldehyde (S). This control was performed in parallel and under the same conditions as biotic degradation experiments, except that *B. nivea* was not present in the culture media. Inhibitors were added to the culture media as single molecules, at the following concentrations: 1 g L^{-1} F, 0.5 g L^{-1} V, 0.25 g L^{-1} H, and 0.25 g L^{-1} S. The data represent the mean of the relative concentrations of the molecules at each time point in comparison to their initial concentrations. The error bar indicates the SE (*n*=3).

Figure S2. HPLC chromatograms showing the peaks profile detected at different time points in the supernatant of *B. nivea* MUT 6321 cultures growing in the presence of the phenolic aldehydes as single molecules. UV-Vis spectra corresponding to the peaks marked with an arrow are shown in the insets. A wavelength of 278 nm was used to detect vanillin (V) and 4-hydroxybenzaldehyde (H), and 309 nm was used to detect syringaldehyde (S).

Table S1. Average growth percentage ± SE (*n=6*) of fungi in comparison with their respective control growing with glucose for 21 days (end of the experiment). Negative values indicate inhibition, while values equal to or higher than 0 indicate growth comparable or higher than controls. Fungi are listed in alphabetical order. F: furfural (2 and 1 g L^{-1}); V: vanillin (2 and 0.5 g L^{-1}); H: 4-hydroxybenzaldehyde (2 and 0.25 g L^{-1}); S: syringaldehyde (2 and 0.25 g L^{-1}); MPF: mix with 1 g L^{-1} F; 0.5 g L^{-1} V; 0.25 g L^{-1} H; 0.25 g L^{-1} S; Glu: glucose 2 g L^{-1}.

686 **Figures and Tables**

687 **Figure 1**

688

	Molecules	F		Glu + F		V		Glu + V		S		Glu + S		H		Glu + H		MPF	Glu + MPF
	Concentrations (g L^{-1})	2	1	2	1	2	0.5	2	0.5	2	0.25	2	0.25	2	0.25	2	0.25	1 F + 0.5 V + 0.25 S + 0.25 H	1 F + 0.5 V + 0.25 S + 0.25 H
Percentage of growth inhibition (I %)	I % ≥ 90 %	37	37	36	32	40	33	39	27	38	27	36	22	40	28	40	23	39	39
	90 % < I % < 50 %	2	1	3	5		2	1	8	2	7	3	12		7		11	1	
	I % < 50%	1	2	1	3		5		5		6	1	6		5		6		1

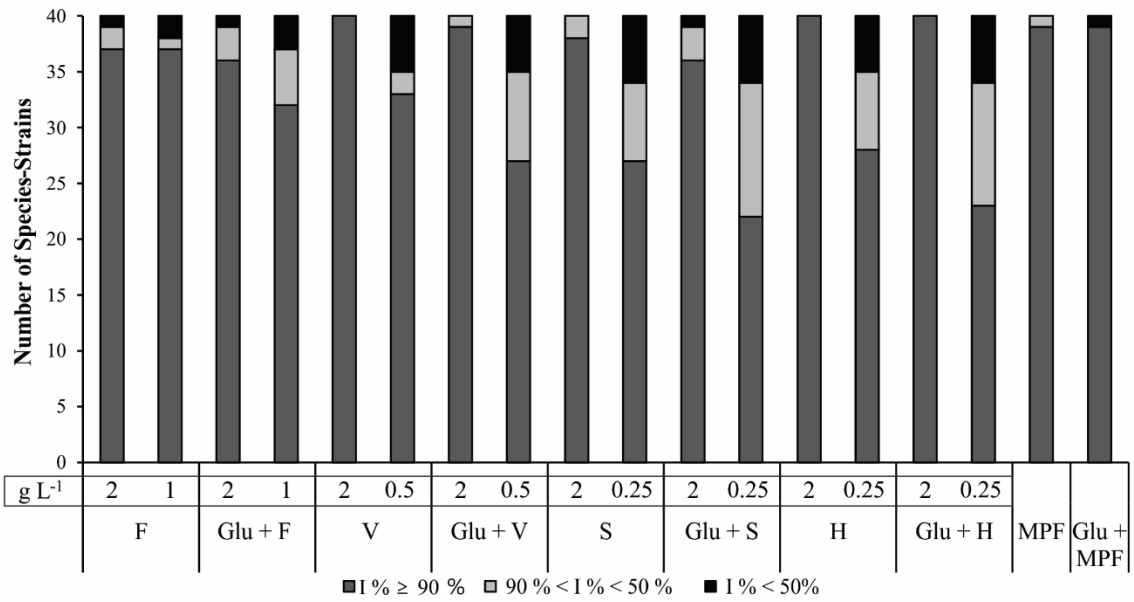

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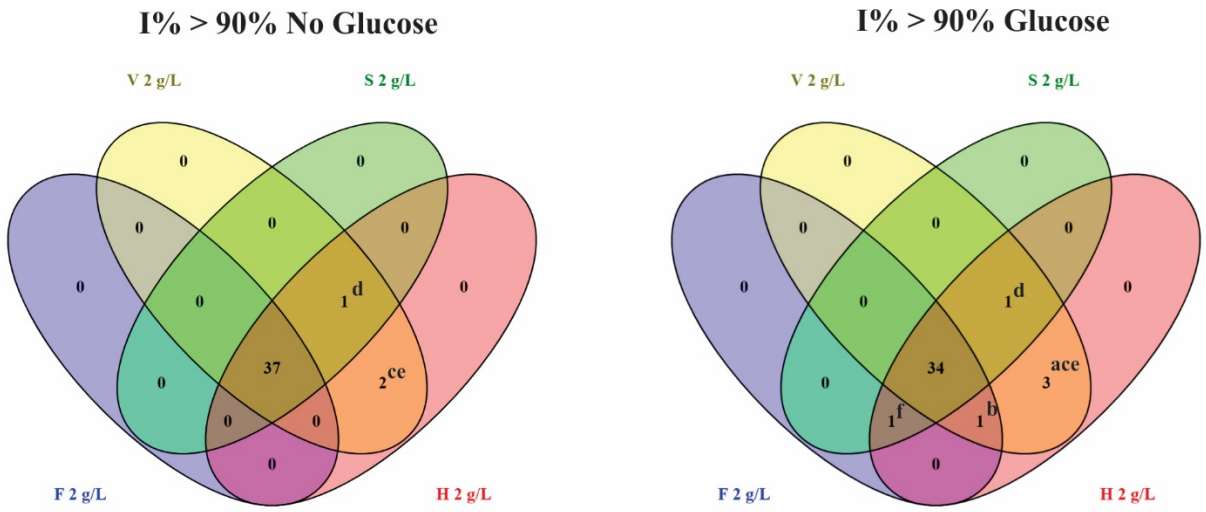
Figure 2



a	*Aspergillus niger*
b	*Aspergillus terreus*
c	*Byssochlamys nivea*
d	*Ganoderma lucidum*
e	*Paecilomyces variotii*
f	*Trametes versicolor*

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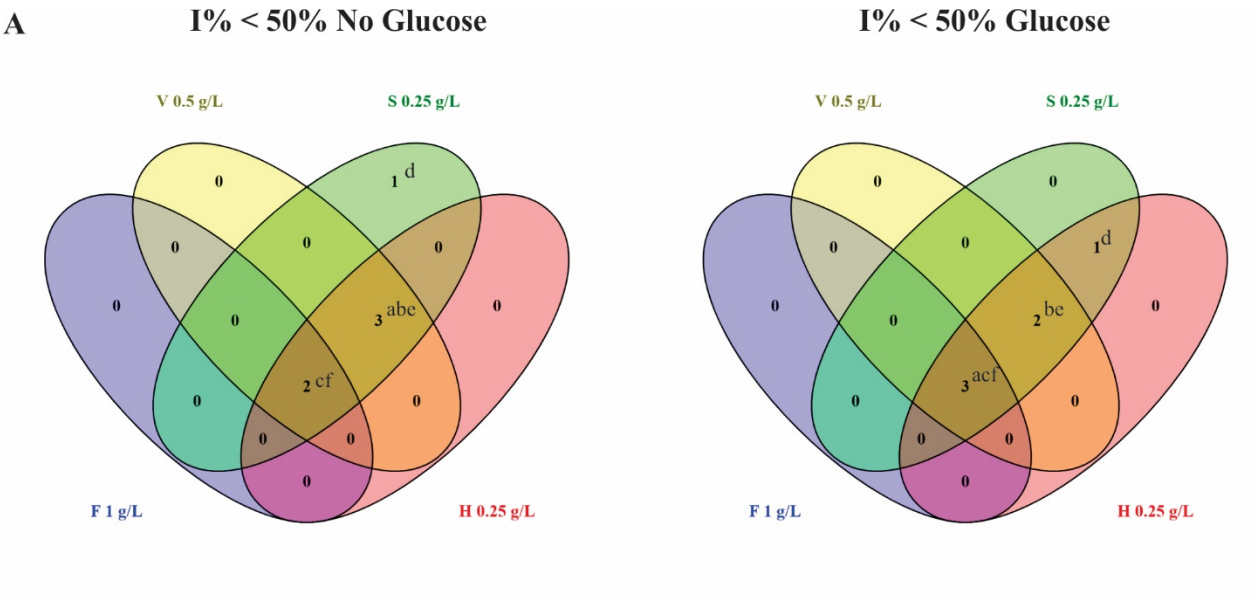
Figure 3

A



B

	Fungal Species	Fungal Growth (%)							
		F 1 g L^{-1}		V 0.5 g L^{-1}		S 0.25 g L^{-1}		H 0.25 g L^{-1}	
		No Glu	Glu	No Glu	Glu	No Glu	Glu	No Glu	Glu
a	*Aspergillus niger*	-99±0^{D}	**-26±3^{B}**	**49±8^{A}**	**117±6^{A}**	**32±5^{A}**	**91±8^{A}**	**53±7^{A}**	**117±6^{A}**
b	*Aspergillus terreus*	-98±0^{D}	-85±0^{D}	**-3±1^{B}**	**2±1B^{B}**	**-21%±1^{B}**	**-15±1^{CD}**	**-2±1^{C}**	**1±1^{CD}**
c	*Byssochlamys nivea*	**-41±4^{B}**	**-37±3^{B}**	**-29±5^{BC}**	**-12±3^{BC}**	**-20%±11^{B}**	**17±10^{BC}**	**16±3^{B}**	**40±16^{B}**
d	*Fusarium fujikuroi*	-95±2^{D}	-57±2^{C}	-98±0^{D}	-98±0^{D}	**-33±1^{B}**	**-33±1^{D}**	-98±1^{E}	**-40±2^{E}**
e	*Ganoderma lucidum*	-67±2^{C}	-52±3^{C}	**-38±12^{C}**	**-24±5^{C}**	**-35±2^{B}**	**-34±4^{D}**	**-25±2^{D}**	**-19±2DE**
f	*Paecilomyces variotii*	**7±2^{A}**	**23±2^{A}**	**-21±4^{BC}**	**11±2^{B}**	**28±2^{A}**	**28±2^{B}**	**-19±4^{CD}**	**33±7^{BC}**

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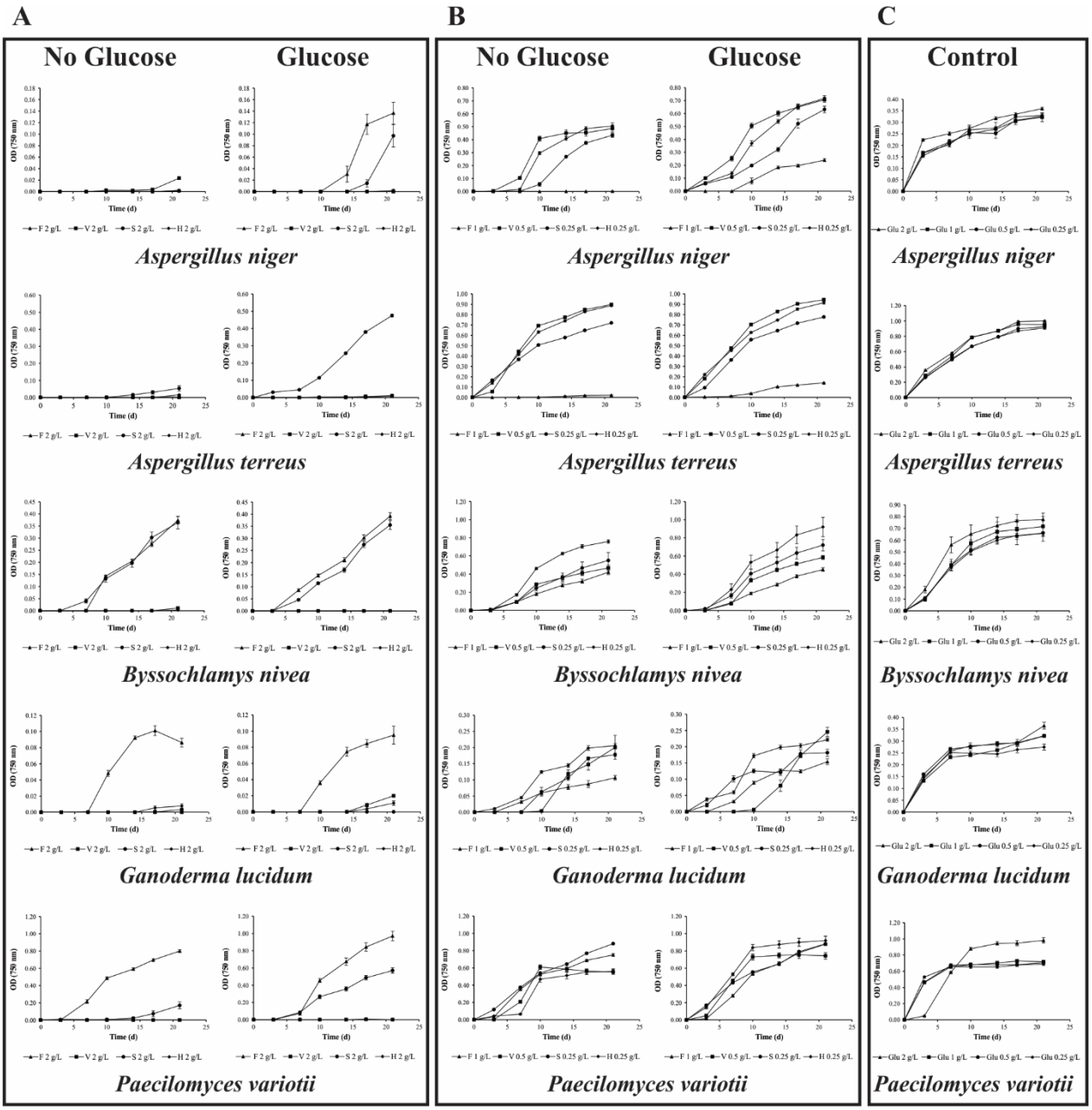
Figure 4



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703 **Figure 5**
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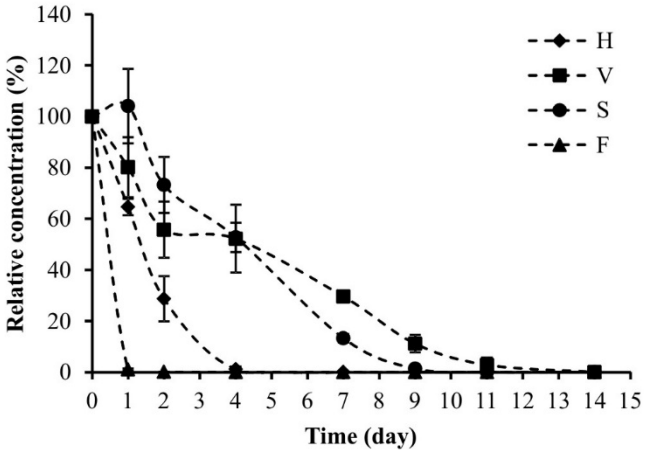

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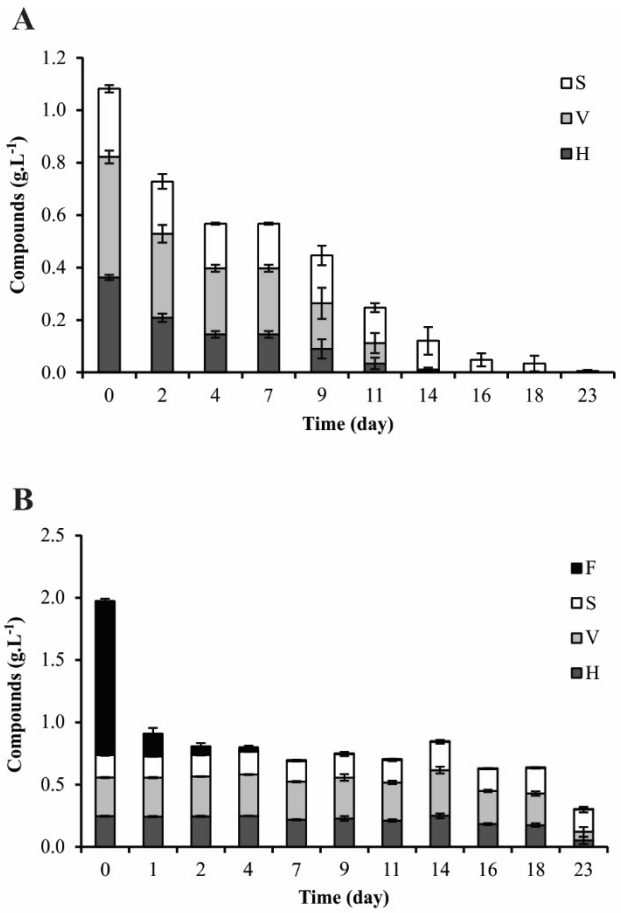
Figure 6



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711 **Table 1**
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Samples	Inhibitors concentration ($g\ L^{-1}$)	Degradation time (d)	Remaining Glucose (%)	Dry Fungal Biomass (mg)
F	1	1	86.6 ± 7.4	130±13
V	0.5	11	0.7 ± 0.2	104±7
H	0.25	4	35.2 ± 3.6	91±1*
S	0.25	9	11.0 ± 3.8	123±6
MP	1	23	0.3 ± 0.1	85±12*
MPF	2	>23	15.1 ± 6.9	37±4*
Control	0	-	-	139±6

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715 *Significant difference with respect to control (Dunnett's test, $p<0.05$).