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Alice Romagnolo[a], Federica Spina[a], Elisabetta Brenna[b], Michele Crotti,[b] Fabio Parmeggiani[b] and Giovanna Cristina Varese[a*]

[a] Department of Life Science and Systems Biology, University of Turin, viale P. A. Mattioli 25, 10125 Turin, Italy

[b] Department of Chemistry, Materials and Chemical Engineering "G. Natta", Politecnico di Milano, via L. Mancinelli 7, 20131 Milan, Italy

*Corresponding author (G.C. Varese)

Tel.: +39 0116705984;

Fax: +39 0116705962

E-mail address: cristina.varese@unito.it

Postal address: viale P.A. Mattioli 25, 10125 Turin, Italy

E-mail: alice.romagnolo@unito.it; federica.spina@unito.it; elisabetta.brenna@polimi.it; michele.crotti@chem.polimi.it; cristina.varese@unito.it

Abstract

Bioeconomy stresses the need of green processes promoting the development of new methods for biocatalyzed alkene reductions.

A functional screening of 28 fungi belonging to Ascomycota, Basidiomycota and Zygomycota isolated from different habitats was performed. Their capability to reduce C=C double bonds was evaluated towards three substrates (cyclohexenone, α-methylnitrostyrene and α-methylcinnamaldehyde) with different electron-withdrawing groups, i.e., ketone, nitro and aldehyde, respectively.

Almost all the fungi showed this reducing activity. Noteworthy *Gliomastix masseei, Mucor circinelloides* and *Mucor plumbeus* resulted very versatile and effective, being able to reduce all the model substrates quickly and with high yields.

Keywords

Biocatalysis, filamentous fungi, ene-reductases, α,β-unsaturated compounds, bioreduction.

1. Introduction

Nowadays, the synthesis of molecules with biotechnological exploitations is mainly done by traditional chemical processes, which generally have high costs and environmental impact. The growing awareness about safety problems brought to restrain the use of chemical catalysts as heavy metals or unsafe gasses that require harsh working conditions in terms of temperature and pressure (Faber, 2011).

On the other hand, biocatalysts seem to be a viable alternative to traditional methods for the minimal environmental impact due to the low energy demand, waste and by-products formations and for the reduced process costs. Moreover, biocatalysis is a powerful tool to obtain chiral molecules in enantiomerically pure form, which are highly valued for instance in the pharmaceutical field (Soartet and Vandamme, 2010).

The reduction of C=C double bonds conjugated with different electron-withdrawing groups (EWG) such as carbonyl, nitro and ester can be catalyzed by Ene-Reductases (E.C. 1.6.99.1, ERs). Most of the known ERs are flavin-dependent oxidoreductases belonging to the Old Yellow Enzyme family, which require NAD(P)H as cofactor (Stuermer et al., 2007). They were first described in *Saccharomyces pastorianus* (Stott et al., 1993) and *S. cerevisiae* (Karplus et al., 1995); in the following years, many ERs were described in other yeasts, bacteria, plants and animals, but still little is known about their occurrence in filamentous fungi (Stuermer et al., 2007). To date, these enzymes were poorly investigated at molecular and structural level in these microorganisms. These studies are complex because only a few genomes are completely sequenced and hence it is very difficult to investigate the presence of genes coding for ERs in filamentous fungi. Moreover, no information is available relating to the structure

of these proteins in these microorganisms and to date no ERs have been purified and characterized from this source. Otherwise, for some yeasts, bacteria and plants the presence of ERs at molecular level, their structure and their characterization have been extensively investigated. Their biological role is still unknown although some authors suggested their involvement in the stress response (Brigè et al., 2006).

Several authors described the capability of filamentous fungi to reduce the C=C double bonds of a single substrate or of a set of compounds belonging to the same structural class (Arnone et al., 1990; Fuganti et al., 1998 a; Hall et al., 2006; Skrobiszewski et al., 2013). To date, the main functional screening of filamentous fungi was performed by Carballeira et al. (2004). Among the 241 fungi, only 3 were capable to reduce the C=C double bond of carvone.

Considering the natural biodiversity and the broad heterogeneous enzymatic pattern, filamentous fungi are indeed excellent biocatalysis agents. Actually, some strains or their enzymes are formerly used in the production of building blocks of pharmaceuticals, agrochemicals or fragrances (Colwell, 2002; Gavrilescu and Chisti, 2005). On the whole, there is a strong need to identify potential biocatalysts to enlarge the portfolio of microorganisms and enzymes to be used for synthetic applications.

Regarding ERs, a validated enzymatic assay has not yet been developed, to date. So far, the only available method is related to the oxidation of the NAD(P)H by purified enzymes (Gao et al., 2012). Its applicability is limited because many unsaturated substrates commonly used in biocatalysis absorb at the same wavelength of the cofactor. Since it is not possible to verify directly the presence of these enzymes in fungi, either with molecular methods or enzymatic assays, a screening to identify the products of the reaction that probably involved ERs is a convenient approach. In addition, this method also allows to analyze a wide biodiversity of microorganisms.

Data comparison to literature is difficult due to the few model substrates used to investigate ERs activity, since this enzymatic activity has been poorly deepened. Few studies take into consideration more than one compound. For example, Goretti et al. (2011) described a whole-cell system of non-conventional yeasts in the bioconversion of α,β-unsaturated ketones and aldehydes.

The present study aims to identify filamentous fungi showing ER activity. A functional screening was set up using 28 fungi belonging to Ascomycota, Basidiomycota and Zygomycota, isolated from different habitats. Three representative model substrates characterized by different EWGs (ketone, nitro and aldehyde) conjugated with the C=C double bond were selected. The reduction of the C=C double bonds was followed by GC/MS analysis.

2. Materials and Methods

2.1 Fungi

The fungi belong to different physiological and taxonomical groups and were isolated from different habitats (Table 1). They are preserved at the *Mycotheca Universitatis Taurinensis* (MUT, Department of Life Sciences and Systems Biology, University of Turin).

2.2 Chemicals

Cyclohexenone (CE) and α-methylcinnamaldehyde (MCA) were purchased from Sigma-Aldrich (Italy). (*E*)-α-methylnitrostyrene (MNS) was synthesized according to the literature (Kawai et al., 2001).

Stock solutions (500 mM) of each substrate were prepared by dissolving them in dimethyl sulfoxide (DMSO).

2.3 Biotransformation experiments

Fungal strains were pre-grown in Petri dishes containing malt extract solid medium (MEA: 20 g/l glucose, 20 g/l malt extract, 20 g/l agar, 2 g/l peptone) from

99 which the fungal inoculum for liquid cultures was set up. When possible, a conidia
100 suspension was prepared ($1 \cdot 10^6$ conidia final concentration in flask). Otherwise, the
101 inoculum was made by homogenizing agar squares derived from the margins of an
102 overgrown colony together with sterile water (1 cm^2/ml). Fungi were inoculated in 50
103 ml flasks containing 30 ml of malt extract liquid medium. Flasks were incubated at 25
104 °C and were maintained in agitation (110 rpm) in the dark.
105 After two days of pre-growth, the substrates were separately added (5 mM final
106 concentration). For each substrate, three biological replicates were run.
107 The experiment was run for 7 days: 1 ml of cultural broth was collected after 2,
108 4 and 7 days and extracted by two-phase separation using 0,5 ml of methyl *t*-butyl ether
109 (MTBE) as solvent. The organic phases were dried over anhydrous Na_2SO_4 and
110 analyzed by means of GC/MS.
111 After two days, one flask for each fungus was sacrificed to measure the initial
112 biomass and pH. Those parameters were also evaluated for all the flasks at the end of
113 the experiment. The liquid was separated from the biomass by filtration and was used
114 for pH measurement. The mycelia were dried at 60 °C for 24 h to measure the
115 biomasses dry weight.
116 2.4 GC/MS analysis
117 GC/MS analyses were performed on an Agilent HP 6890 gas chromatograph
118 equipped with a 5973 mass detector and an HP-5-MS column (30 m × 0.25 mm × 0.25
119 μm, Agilent), employing the following temperature program: 60 °C (1 min) / 6 °C min^{-1}
120 / 150 °C (1 min) / 12 °C min^{-1} / 280 °C (5 min). GC retention times: cyclohexenone
121 (CE) 5.40 min, cyclohexanone (CO) 4.65 min, cyclohexanol (COH) 4.45 min, (*E*)-α-
122 methylnitrostyrene (MNS) 17.7 min, (*Z*)-α-methylnitrostyrene (MNS) 15.6 min, 2-
123 nitropropylbenzene (NPB) 14.8 min, α-methylcinnamaldehyde (MCA) 14.7 min, α-
124 methylcinnamyl alcohol (MCOH) 15.5 min, α-methyldihydrocinnamyl alcohol

(MDHCOH) 13.6 min. The enantiomeric excess (ee) values of MDHCOH was determined by GC analysis, performed using a Chirasil Dex CB column (0.25 μm × 0.25 mm × 25 m, Varian), according to the following conditions: 60 °C / 5 °C min^{-1} / 95 °C (25 min) / 50 °C min^{-1} / 220 °C (10 min). GC retention times: (*R*)-enantiomer 26.6 min, (*S*)-enantiomer 27.9 min.

3. Results and Discussion

The results of the biotransformation of the three substrates by the 28 fungi are shown in Table 2, the maximal percentage of C=C double bonds reduction and the timing of the reaction are reported. Four groups were established on account of the rate of substrates transformation by means of a putative ER activity: group A 100-75 %; group B 74-50 %; group C 49-25 % and group D 24-0 %.

Regarding pH measurements, the cultural broth of each fungus remained unchanged during the experiments. The pH values ranged between 3 and 6 and seemed to depend on the metabolism of each fungus; variations due to the addition of substrates were not detected.

3.1 CE biotransformation

Almost all the fungi (96,4 %) were able to reduce the C=C double bond of CE among which 75 % could be listed in group A. The other fungi were 3,6 % in group B, 11 % in group C and 11 % in group D. As it can be seen in Table 2, 11 out of 28 fungi were able to completely transform this substrate within two days (*C. herbarum*, *G. masseei, P. citrinum*, *S. fimicola*, *T. viride*, *A. cylindracea*, *A. splendida*, *Coprinellus* sp., *T. pubescens*, *M. circinelloides*, *M. plumbeus*). On the whole, the majority of the fungi were not only able to reduce the C=C double bond of this substrate but also reached the complete biotransformation of this molecule.

CE is a well-accepted substrate; in this study only one fungus out of 28 was ineffective towards this compound. Ketonic substrates have been frequently considered

151 in the literature; for example, Gatti et al. (2014) discussed several ketone substrates such
152 as carvone or ketoisophorone used in bioconversions that involved ERs.

153 The results obtained in this study may be compared with literature. In particular,
154 two fungi belonging to the genus *Mucor* were very effective toward CE confirming the
155 results obtained by Fuganti and Zucchi (1998 b).

156 Other authors analyzed the biotransformation of molecules with ketonic EWG
157 with a basic scaffold similar to CE. Skrobiszewski et al. (2013) described a strain of *P.*
158 *ostreatus* effective towards C=C double bonds reduction; this data was confirmed also
159 by the strain used in this study. *Absidia glauca* and *Beauveria bassiana* were poorly
160 effective towards C=C double bonds reduction of CE while the strains used by
161 Carballeira et al. (2004) and Fuganti and Zucchi (1998 b) reduce ketonic substrate with
162 high yield in benzalacetones derivatives.

163 CE biotransformation led to the identification of two products (Figure 1):
164 cyclohexanone (CO) in which ER activity is involved in the reduction of C=C double
165 bond, and cyclohexanol (COH) in which an alcohol dehydrogenase (ADH) reduces the
166 C=O bond. Most of the fungi (67 %) convert CE into COH showing the action of ERs
167 and ADHs, while 26 % preferentially reduced the C=C double bond, producing only
168 CO. When the reduction was slow, it was possible to define a putative reaction profile
169 in which the two enzymes act in cascade (Figure 2). In most cases, the reaction was
170 very fast and only the formation of COH was detected.

171 The same reaction profile was hypothesized by other authors in the reduction of
172 analogous substrates of CE (Fuganti et al., 1998 a; Fuganti and Zucchi, 1998 b;
173 Carballeira et al., 2004; Hall et al., 2006; Stuermer et al., 2007; Skrobiszewski et al.,
174 2013).

175 3.2 MNS biotransformation

176 The C=C double bond of this substrate was reduced by 82 % of the fungi (Table
177 2), among which 14 % could be listed in group A. The other fungi were 7,1 % in group
178 B, 25 % in group C, and the majority (53,6 %) in group D. As it can be seen in Table 2,
179 two fungi out of 28 (*A. niger* and *M. circinelloides*) were very active and reached an
180 almost complete conversion of the substrate within 2 days.
181 MNS biotransformation led to the identification of 2-nitropropylbenzene (NPB)
182 as the sole product through the reduction of a C=C double bond by ER activity (Figure
183 3). Since the stereogenic center of the reduced product is too labile under the reaction
184 conditions no reliable ee values could be obtained.
185 Although nitrostyrene derivatives are good substrates for ERs (Toogood et al.,
186 2008; Gatti et al., 2014), to our knowledge, this is the first report of the reduction of the
187 C=C double bonds of nitroalkenes by filamentous fungi. A whole-cell system using
188 *Saccharomyces cerevisiae* in the reduction of the C=C double bond of MNS was
189 reported by Kawai et al. (2001). These authors obtained yields comparable to the fungi
190 gathered in group A.
191 3.3 MCA biotransformation
192 The C=C double bond of this substrate was reduced by 35,7 % of the fungi
193 (Table 2) among which only 7,1 % in group A. The other fungi were 3,6 % in group B
194 and 89,3 % in group D. Two fungi, *M. circinelloides* and *M. plumbeus,* completely
195 converted MCA within 2 days into the (*S*)-enantiomer of the corresponding saturated
196 alcohol MDHCOH, showing an ee value of 80 %. This result is promising compared to
197 literature: Fronza et al. (2009) reported a conversion rate of 12 % and ee value of 70 %
198 ((*S*)- enantiomer) in the reduction of MCA with *S. cerevisiae* whole-cell.
199 The difficulty to reduce MCA has been also found by other authors. For example
200 Goretti et al. (2011) screened non-conventional yeasts but only *Kazachstania*
201 *spencerorum* out of 23 microorganisms was able to convert this substrate (60 %).

202 The MCA biotransformation led to the identification of two products, probably
203 involving two enzymes (Figure 4). The reduction of C=C and C=O double bonds led to
204 the formation of α-methyldihydrocinnamyl alcohol (MDHCOH) by means of ERs and
205 ADHs. The reduction of the aldehyde EWG by ADHs before the C=C reduction could
206 take place, led to the formation of α-methylcinnamyl alcohol (MCOH). Since the
207 MCOH lacks the necessary EWG, it is not a substrate for ERs and consequently
208 accumulates in the medium. In our experiments, two fungi (*A. glauca* and *E. nigrum*)
209 formed MCOH showing only ADH activity. By contrast, 8 fungi formed a mixture of
210 MCOH and MDHCOH, preferentially reducing the aldehydic group. This reaction
211 profile was noticed also by other authors for *S. cerevisiae* (Gatti et al. 2014).
212 3.4 General considerations
213 The screening clearly showed that the EWG on the C=C double bonds strongly
214 influenced the reaction rate of the various strains tested. Probably the catalytic activity
215 of the enzymes was also affected by the steric hindrance of the other substituents and by
216 electronic effects, as widely discussed by Stuermer et al. (2007) and Gatti et al. (2014).
217 In detail, the substrates were transformed following this outline: CE>MNS>MCA. CE
218 was the most easily converted substrate, due to the presence of a strong EWG (ketone)
219 and only two substituents on the double bond, both with a modest steric hindrance.
220 MNS and MCA share the same basic scaffold, with higher steric hindrance due to the
221 aromatic ring, but they differ in the EWGs (nitro and aldehyde, respectively): the higher
222 conversion of MNS can be justified by the much higher electron-withdrawing power of
223 the nitro group.
224 It has to be noticed that this enzymatic activity appears to be genus specific. For
225 instance, the strains of *Mucor* reached the same conversion yields in the
226 biotransformation of all the substrates. Whereas, the strains of *Penicillium* behave
227 differently towards the substrates analyzed (Table 2). This consideration makes the

228 screening for the selection of strains for definite reactions a required step for the
229 analysis of the intraspecific variability.
230 The fungi afforded very different yields in the conversion of these substrates. This
231 may not only be due to the different affinity of the enzyme for the substrate but also to
232 an activation of the secondary metabolism of the fungus that would lead to the
233 production of putative ERs in the presence of different substrates.
234 3.5 Biomasses
235 On the whole, all fungi were able to grow with the substrates. In some cases
236 weight differences have been reported in the three biological replicates despite the
237 biomass seems to be very similar morphologically. For this reason, some standard
238 deviations were high. In particular, as it can be seen in Figure 5 each substrate had a
239 different effect on the biomass production. It was not possible to measure the biomass
240 of the yeast *G. cucujoidarum*. The growth of this fungus was measured by cell counting
241 using a Burker chamber and the growth was about $3{,}6 \cdot 10^8$ cells/ml in each of the
242 replicates.
243 Interestingly, CE seems to stimulate the production of biomass in almost all the
244 fungi. For almost all the fungi, the presence of MNS seems to cause a scarce
245 development of the biomass. In fact, at the end of the experiment they were lower than
246 the initial growth. For example, *P. citrinum* and *C. funicola* displayed a biomass loss of
247 80 % suggesting that this compound may inhibit the biomass growth. Despite this
248 strong biomass decrease, many fungi reached very high conversion yields.
249 The addition of MCA partially inhibited the development of biomass in almost all
250 the fungi. The fungus that displayed the highest biomass loss (– 91 %) was *C. funicola*.
251 In general, the biomass growth is an important parameter that most often is not
252 taken into account by other authors, reason for which it is not easy to make comparisons
253 with other studies. Regarding CE, a correlation between biomass and ER activity is not

254 possible because the majority of the fungi increased the biomass independently of the
255 biotransformation yield. For example, *A. glauca* displayed a biomass loss of 40 % but
256 reached the total biotransformation of CE. On the other hand, *B. bassiana* displayed a
257 growth of over 80 %, although it was not able to convert this substrate.

258 Even in the case of MNS and MCA a proper correlation between the biomass
259 growth and the biotransformation yields was not possible. In particular, for these
260 compounds the majority of the fungi displayed a decrease in biomass. Nonetheless, the
261 fungi that reached the highest conversion rate had an important biomass loss. For
262 example *M. circinelloides* almost totally converted MNS but during the trial this fungus
263 had a weight loss of 40 %. Similarly, *M. plumbeus* almost totally converted MCA and
264 had a biomass decrease of 45 %. The few fungi that increased the biomass showed two
265 behaviors: either partial conversion of the two substrates with low yields or
266 ineffectiveness towards these compounds.

267 Since the biomass development implicates the primary metabolism, and a
268 correlation between the biomass development and the biotransformation yields cannot
269 be drawn, the involvement of the secondary metabolism in the conversion of CE, MNS
270 and MCA may be hypothesized.

271 **4. Conclusions**

272 The screening highlighted that ER activity is widespread in filamentous fungi. In
273 fact, 27 out of 28 microorganisms reduced at least one substrate. Consequently,
274 although the biological role of ER is still unknown, this activity may be involved in the
275 secondary metabolism of the microorganisms analyzed.

276 *M. circinelloides, M. plumbeus* and *G. masseei* resulted the most versatile strains
277 converting all the substrates analyzed, with the highest yields. Moreover, this study also
278 highlighted problems related to substrate selection: by now, several chemical classes

279 have been investigated, but these activities are difficult to compare due to the lack of
280 validated model compounds.

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Table 1: list of the strains analyzed during the screening and their isolation site (MUT: accession number).

Fungi	MUT	Species	Isolation site
Asco-	3874	*Aspergillus niger*	air
	1720	*Beauveria bassiana*	air
	1087	*Botrytis cinerea*	fresco of Botticelli
	3726	*Chaetomium funicola*	dried *Boletus* fungi from Europe
	3856	*Cladosporium herbarum*	air
	3848	*Epicoccum nigrum*	air
	4824	*Geotrichum cucujoidarum*	wastewater of a tanning industry
	4855	*Gliomastix masseei*	*Flabelia petiolata* (marine algae)
	281	*Mesobotrys simplex*	cultivated soil
	1749	*Myxotrichum deflexum*	air
	1381	*Oidiodendron maius*	roots of *Vaccinium myrtillus* (black raspberry)
	4862	*Penicillium citrinum*	*Flabelia petiolata* (marine algae)
	4831	*Penicillium purpurogenum*	wastewater of a tanning industry
	4892	*Penicillium vinaceum*	*Padina pavonica* (marine algae)
	4833	*Scopulariopsis* sp.	wastewater of a tanning industry
	1148	*Sordaria fimicola*	*Picea abies* (norway spruce)
	1166	*Trichoderma viride*	tallus of *Parmelia taractica* (lichen)
	3788	*Trichurus spiralis*	book pages
Basidio-	2753	*Agrocybe cylindracea*	carpophore
	2755	*Agrocybe farinacea*	carpophore
	3696	*Agrocybe splendida*	carpophore
	4897	*Coprinellus* sp.	*Padina pavonica* (marine algae)
	2976	*Pleurotus ostreatus*	carpophore on *Populus* sp. (poplar)
	2400	*Trametes pubescens*	carpophore on *Populus* sp. (poplar)
Zygo-	1157	*Absidia glauca*	tallus of *Peltigera praetextata* (lichen)
	2769	*Mucor plumbeus*	air
	44	*Mucor circinelloides*	-
	2770	*Syncephalastrum racemosum*	air

Table 2: maximal percentage of C=C double bond reduction. According to the conversion of the substrates, four groups were defined: group A: 100-75 %; group B: 74-50 %; group C: 49-25 %; group D: 24-0 %. The table shows also the timing of the reactions.

	Fungi	Conversion %			Group			Days		
		CE	MNS	MCA	CE	MNS	MCA	CE	MNS	MCA
Asco-	*A. niger*	57	97	0	B	A	D	2	2	7
	B. bassiana	0	0	0	D	D	D	7	7	7
	B. cinerea	10	0	0	D	D	D	2	2	2
	C. funicola	89	30	0	A	C	D	2	7	7
	C. herbarum	100	24	3	A	D	D	2	7	2
	E. nigrum	30	32	0	C	C	D	7	7	7
	G. cucujoidarum	32	26	0	C	C	D	7	7	7
	G. masseei	100	72	50	A	B	B	2	2	4
	M. simplex	100	18	0	A	D	D	7	7	7
	M. deflexum	100	44	0	A	C	D	7	7	7
	O. maius	100	20	12	A	D	D	4	2	2
	P. citrinum	100	98	3	A	A	D	2	7	2
	P. purpurogenum	85	0	0	A	D	D	7	7	7
	P. vinaceum	23	11	0	D	D	D	7	7	7
	Scopulariopsis sp.	100	17	0	A	D	D	7	7	7
	S. fimicola	100	32	18	A	C	D	2	2	7
	T. viride	100	30	10	A	C	D	2	7	7
	T. spiralis	100	0	0	A	D	D	7	7	7
Basidio-	*A. cylindracea*	100	0	0	A	D	D	2	7	7
	A. farinacea	100	13	0	A	D	D	4	7	7
	A. splendida	100	34	0	A	C	D	2	7	7
	Coprinellus sp.	100	11	5	A	D	D	2	7	7
	P. ostreatus	100	19	0	A	D	D	7	7	7
	T. pubescens	100	52	14	A	B	D	2	4	4
Zygo-	*A. glauca*	35	22	0	C	D	D	2	7	7
	M. circinelloides	100	82	100	A	A	A	2	2	2
	M. plumbeus	100	79	98	A	A	A	2	7	2
	S. racemosum	100	16	0	A	D	D	7	7	7

Figure 1: putative CE reaction profile. ER: ene-reductase, ADH: alcohol dehydrogenase, CE: cyclohenanone, CO: cyclohexanone, COH: cyclohexanol.

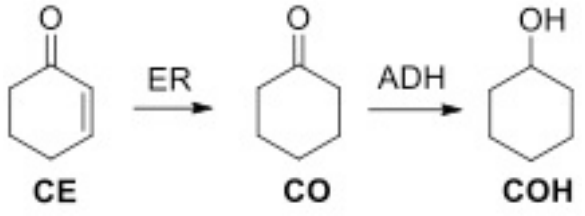


Figure 2: products formation profile of *P. citrinum* during the experiment (2, 4 and 7 days). CE: cyclohenanone, CO: cyclohexanone, COH: cyclohexanol.

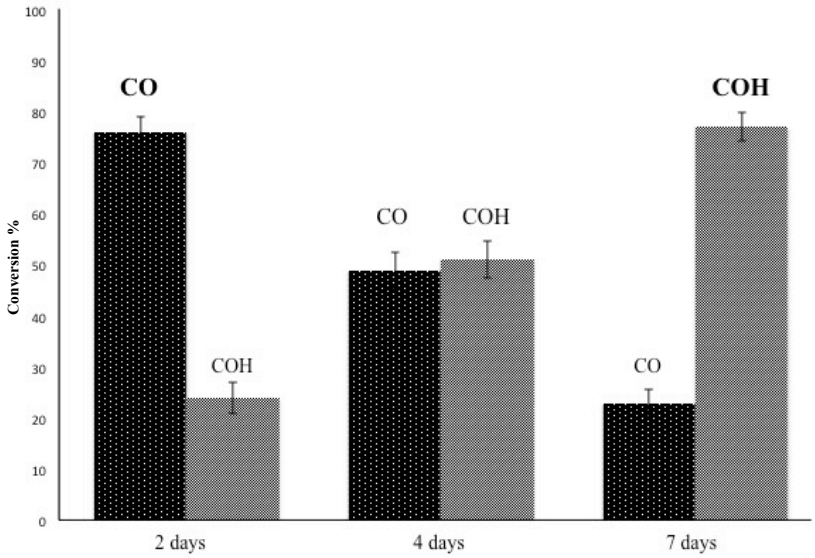


Figure 3: putative MNS reaction profile. ER: ene-reductase, MNS: (*E*)-α-methylnitrostyrene, NPB: 2-nitropropylbenzene.

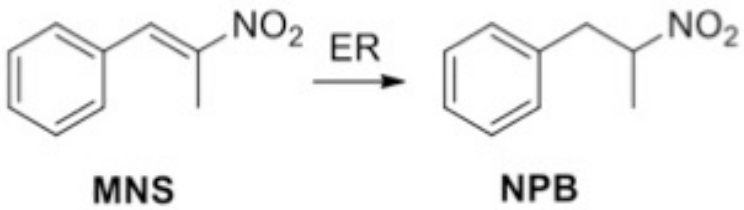
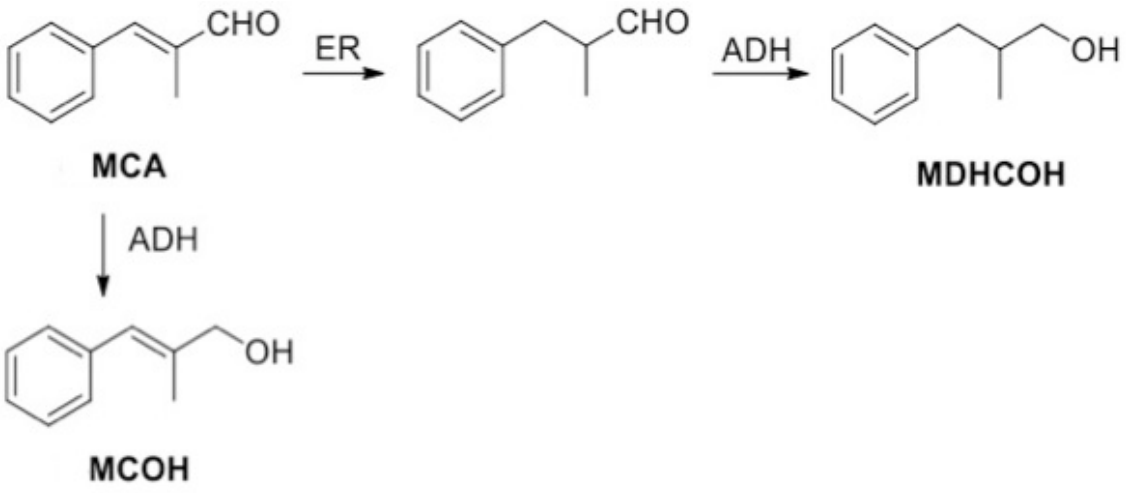


Figure 4: putative MCA reaction profile. ER: ene-reductase, ADH: alcohol dehydrogenase, MCA: α-methylcinnamaldehyde, MCOH: α-methylcinnamyl alcohol, MSHCOH: α-methyldihydrocinnamyl alcohol.



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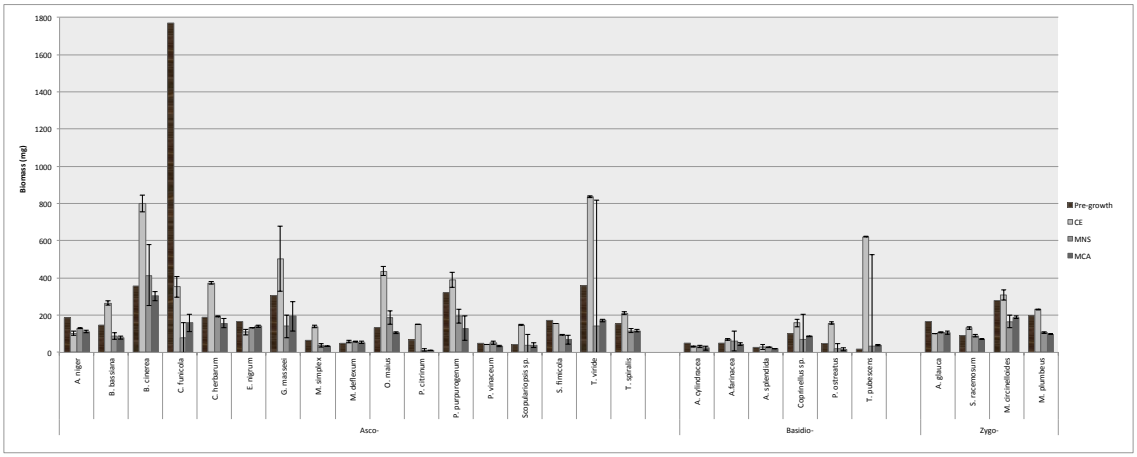
382 **Figure 5**: biomass dry weight measurement. Comparison between the pre-growth and
383 the end of the trial for each substrate. The y axis represent the biomass weight (mg) and
384 the x axis represent the fungi used in this study. Since it was not possible to measure the
385 biomass (mg) of the yeast *G. cucujoidarum*, this fungus was not included in the figure.



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