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Identification of fungal ene-reductase activity by means of a functional screening.

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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 maseei*, *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

which the fungal inoculum for liquid cultures was set up. When possible, a conidia suspension was prepared ($1 \cdot 10^6$ conidia final concentration in flask). Otherwise, the inoculum was made by homogenizing agar squares derived from the margins of an overgrown colony together with sterile water ($1 \text{ cm}^2/\text{ml}$). Fungi were inoculated in 50 ml flasks containing 30 ml of malt extract liquid medium. Flasks were incubated at 25 °C and were maintained in agitation (110 rpm) in the dark.

After two days of pre-growth, the substrates were separately added (5 mM final concentration). For each substrate, three biological replicates were run.

The experiment was run for 7 days: 1 ml of cultural broth was collected after 2, 4 and 7 days and extracted by two-phase separation using 0,5 ml of methyl *t*-butyl ether (MTBE) as solvent. The organic phases were dried over anhydrous Na_2SO_4 and analyzed by means of GC/MS.

After two days, one flask for each fungus was sacrificed to measure the initial biomass and pH. Those parameters were also evaluated for all the flasks at the end of the experiment. The liquid was separated from the biomass by filtration and was used for pH measurement. The mycelia were dried at 60 °C for 24 h to measure the biomasses dry weight.

2.4 GC/MS analysis

GC/MS analyses were performed on an Agilent HP 6890 gas chromatograph equipped with a 5973 mass detector and an HP-5-MS column ($30 \text{ m} \times 0.25 \text{ mm} \times 0.25 \mu\text{m}$, Agilent), employing the following temperature program: 60 °C (1 min) / 6 °C min^{-1} / 150 °C (1 min) / 12 °C min^{-1} / 280 °C (5 min). GC retention times: cyclohexenone (CE) 5.40 min, cyclohexanone (CO) 4.65 min, cyclohexanol (COH) 4.45 min, (*E*)- α -methylnitrostyrene (MNS) 17.7 min, (*Z*)- α -methylnitrostyrene (MNS) 15.6 min, 2-nitropropylbenzene (NPB) 14.8 min, α -methylcinnamaldehyde (MCA) 14.7 min, α -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 $\mu\text{m} \times$ 0.25 mm $\times$ 25 m, Varian), according to the following conditions: 60 $^{\circ}\text{C}$ / 5 $^{\circ}\text{C min}^{-1}$ / 95 $^{\circ}\text{C}$ (25 min) / 50 $^{\circ}\text{C min}^{-1}$ / 220 $^{\circ}\text{C}$ (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

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

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

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

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

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

3.2 MNS biotransformation

The C=C double bond of this substrate was reduced by 82 % of the fungi (Table 2), among which 14 % could be listed in group A. The other fungi were 7,1 % in group B, 25 % in group C, and the majority (53,6 %) in group D. As it can be seen in Table 2, two fungi out of 28 (*A. niger* and *M. circinelloides*) were very active and reached an almost complete conversion of the substrate within 2 days.

MNS biotransformation led to the identification of 2-nitropropylbenzene (NPB) as the sole product through the reduction of a C=C double bond by ER activity (Figure 3). Since the stereogenic center of the reduced product is too labile under the reaction conditions no reliable ee values could be obtained.

Although nitrostyrene derivatives are good substrates for ERs (Toogood et al., 2008; Gatti et al., 2014), to our knowledge, this is the first report of the reduction of the C=C double bonds of nitroalkenes by filamentous fungi. A whole-cell system using *Saccharomyces cerevisiae* in the reduction of the C=C double bond of MNS was reported by Kawai et al. (2001). These authors obtained yields comparable to the fungi gathered in group A.

3.3 MCA biotransformation

The C=C double bond of this substrate was reduced by 35,7 % of the fungi (Table 2) among which only 7,1 % in group A. The other fungi were 3,6 % in group B and 89,3 % in group D. Two fungi, *M. circinelloides* and *M. plumbeus*, completely converted MCA within 2 days into the (*S*)-enantiomer of the corresponding saturated alcohol MDHCOH, showing an ee value of 80 %. This result is promising compared to literature: Fronza et al. (2009) reported a conversion rate of 12 % and ee value of 70 % ((*S*)- enantiomer) in the reduction of MCA with *S. cerevisiae* whole-cell.

The difficulty to reduce MCA has been also found by other authors. For example Goretti et al. (2011) screened non-conventional yeasts but only *Kazachstania spencerorum* out of 23 microorganisms was able to convert this substrate (60 %).

The MCA biotransformation led to the identification of two products, probably involving two enzymes (Figure 4). The reduction of C=C and C=O double bonds led to the formation of α -methyldihydrocinnamyl alcohol (MDHCOH) by means of ERs and ADHs. The reduction of the aldehyde EWG by ADHs before the C=C reduction could take place, led to the formation of α -methylcinnamyl alcohol (MCOH). Since the MCOH lacks the necessary EWG, it is not a substrate for ERs and consequently accumulates in the medium. In our experiments, two fungi (*A. glauca* and *E. nigrum*) formed MCOH showing only ADH activity. By contrast, 8 fungi formed a mixture of MCOH and MDHCOH, preferentially reducing the aldehydic group. This reaction profile was noticed also by other authors for *S. cerevisiae* (Gatti et al. 2014).

3.4 General considerations

The screening clearly showed that the EWG on the C=C double bonds strongly influenced the reaction rate of the various strains tested. Probably the catalytic activity of the enzymes was also affected by the steric hindrance of the other substituents and by electronic effects, as widely discussed by Stuermer et al. (2007) and Gatti et al. (2014). In detail, the substrates were transformed following this outline: CE>MNS>MCA. CE was the most easily converted substrate, due to the presence of a strong EWG (ketone) and only two substituents on the double bond, both with a modest steric hindrance. MNS and MCA share the same basic scaffold, with higher steric hindrance due to the aromatic ring, but they differ in the EWGs (nitro and aldehyde, respectively): the higher conversion of MNS can be justified by the much higher electron-withdrawing power of the nitro group.

It has to be noticed that this enzymatic activity appears to be genus specific. For instance, the strains of *Mucor* reached the same conversion yields in the biotransformation of all the substrates. Whereas, the strains of *Penicillium* behave differently towards the substrates analyzed (Table 2). This consideration makes the

screening for the selection of strains for definite reactions a required step for the analysis of the intraspecific variability.

The fungi afforded very different yields in the conversion of these substrates. This may not only be due to the different affinity of the enzyme for the substrate but also to an activation of the secondary metabolism of the fungus that would lead to the production of putative ERs in the presence of different substrates.

3.5 Biomasses

On the whole, all fungi were able to grow with the substrates. In some cases weight differences have been reported in the three biological replicates despite the biomass seems to be very similar morphologically. For this reason, some standard deviations were high. In particular, as it can be seen in Figure 5 each substrate had a different effect on the biomass production. It was not possible to measure the biomass of the yeast *G. cucujoidarum*. The growth of this fungus was measured by cell counting using a Burkner chamber and the growth was about $3,6 \cdot 10^8$ cells/ml in each of the replicates.

Interestingly, CE seems to stimulate the production of biomass in almost all the fungi. For almost all the fungi, the presence of MNS seems to cause a scarce development of the biomass. In fact, at the end of the experiment they were lower than the initial growth. For example, *P. citrinum* and *C. funicola* displayed a biomass loss of 80 % suggesting that this compound may inhibit the biomass growth. Despite this strong biomass decrease, many fungi reached very high conversion yields.

The addition of MCA partially inhibited the development of biomass in almost all the fungi. The fungus that displayed the highest biomass loss (– 91 %) was *C. funicola*.

In general, the biomass growth is an important parameter that most often is not taken into account by other authors, reason for which it is not easy to make comparisons with other studies. Regarding CE, a correlation between biomass and ER activity is not

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

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

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

4. Conclusions

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

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

have been investigated, but these activities are difficult to compare due to the lack of 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	<i>Aspergillus niger</i>	air
	1720	<i>Beauveria bassiana</i>	air
	1087	<i>Botrytis cinerea</i>	fresco of Botticelli
	3726	<i>Chaetomium funicola</i>	dried <i>Boletus</i> fungi from Europe
	3856	<i>Cladosporium herbarum</i>	air
	3848	<i>Epicoccum nigrum</i>	air
	4824	<i>Geotrichum cucujoidarum</i>	wastewater of a tanning industry
	4855	<i>Gliomastix masseei</i>	<i>Flabelia petiolata</i> (marine algae)
	281	<i>Mesobotrys simplex</i>	cultivated soil
	1749	<i>Myxotrichum deflexum</i>	air
	1381	<i>Oidiodendron maius</i>	roots of <i>Vaccinium myrtillus</i> (black raspberry)
	4862	<i>Penicillium citrinum</i>	<i>Flabelia petiolata</i> (marine algae)
	4831	<i>Penicillium purpurogenum</i>	wastewater of a tanning industry
	4892	<i>Penicillium vinaceum</i>	<i>Padina pavonica</i> (marine algae)
	4833	<i>Scopulariopsis</i> sp.	wastewater of a tanning industry
	1148	<i>Sordaria fimicola</i>	<i>Picea abies</i> (norway spruce)
	1166	<i>Trichoderma viride</i>	tallus of <i>Parmelia taractica</i> (lichen)
	3788	<i>Trichurus spiralis</i>	book pages
Basidio-	2753	<i>Agrocybe cylindracea</i>	carpophore
	2755	<i>Agrocybe farinacea</i>	carpophore
	3696	<i>Agrocybe splendida</i>	carpophore
	4897	<i>Coprinellus</i> sp.	<i>Padina pavonica</i> (marine algae)
	2976	<i>Pleurotus ostreatus</i>	carpophore on <i>Populus</i> sp. (poplar)
	2400	<i>Trametes pubescens</i>	carpophore on <i>Populus</i> sp. (poplar)
Zygo-	1157	<i>Absidia glauca</i>	tallus of <i>Peltigera praetextata</i> (lichen)
	2769	<i>Mucor plumbeus</i>	air
	44	<i>Mucor circinelloides</i>	-
	2770	<i>Syncephalastrum racemosum</i>	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
<i>A. niger</i>	57	97	0	B	A	D	2	2	7
<i>B. bassiana</i>	0	0	0	D	D	D	7	7	7
<i>B. cinerea</i>	10	0	0	D	D	D	2	2	2
<i>C. funicola</i>	89	30	0	A	C	D	2	7	7
<i>C. herbarum</i>	100	24	3	A	D	D	2	7	2
<i>E. nigrum</i>	30	32	0	C	C	D	7	7	7
<i>G. cucujoidarum</i>	32	26	0	C	C	D	7	7	7
<i>G. masseei</i>	100	72	50	A	B	B	2	2	4
<i>M. simplex</i>	100	18	0	A	D	D	7	7	7
<i>M. deflexum</i>	100	44	0	A	C	D	7	7	7
<i>O. maius</i>	100	20	12	A	D	D	4	2	2
<i>P. citrinum</i>	100	98	3	A	A	D	2	7	2
<i>P. purpurogenum</i>	85	0	0	A	D	D	7	7	7
<i>P. vinaceum</i>	23	11	0	D	D	D	7	7	7
<i>Scopulariopsis</i> sp.	100	17	0	A	D	D	7	7	7
<i>S. fimicola</i>	100	32	18	A	C	D	2	2	7
<i>T. viride</i>	100	30	10	A	C	D	2	7	7
<i>T. spiralis</i>	100	0	0	A	D	D	7	7	7
<i>A. cylindracea</i>	100	0	0	A	D	D	2	7	7
<i>A. farinacea</i>	100	13	0	A	D	D	4	7	7
<i>A. splendida</i>	100	34	0	A	C	D	2	7	7
<i>Coprinellus</i> sp.	100	11	5	A	D	D	2	7	7
<i>P. ostreatus</i>	100	19	0	A	D	D	7	7	7
<i>T. pubescens</i>	100	52	14	A	B	D	2	4	4
<i>A. glauca</i>	35	22	0	C	D	D	2	7	7
<i>M. circinelloides</i>	100	82	100	A	A	A	2	2	2
<i>M. plumbeus</i>	100	79	98	A	A	A	2	7	2
<i>S. racemosum</i>	100	16	0	A	D	D	7	7	7

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

371
 372 **Figure 2:** products formation profile of *P. citrinum* during the experiment (2, 4 and 7
 373 days). CE: cyclohexenone, CO: cyclohexanone, COH: cyclohexanol.

374
 375 **Figure 3:** putative MNS reaction profile. ER: ene-reductase, MNS: (*E*)- α -
 376 methylnitrostyrene, NPB: 2-nitropropylbenzene.

377
 378 **Figure 4:** putative MCA reaction profile. ER: ene-reductase, ADH: alcohol
 379 dehydrogenase, MCA: α -methylcinnamaldehyde, MCOH: α -methylcinnamyl alcohol,
 380 MSHCOH: α -methyldihydrocinnamyl alcohol.

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