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

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; +39 0116705964

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 to analyze their capability to reduce C=C double bonds 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 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 important 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, biocatalyst seems to be a viable alternative to traditional methods because it may minimize the environmental impact due to the low energy demand, waste and by-products formations and reduce the 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 discovered in 1933 in *Saccharomyces pastorianus* (Stott et al., 1993), then they were found in *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), as well the biological role although some authors suggested ERs involvement in the stress response pathways (Brigè et al., 2006). The C=C double bond reduction is a crucial intermediate step for the production of bulk chemicals (Gatti et al.,

2014). The identification of new biocatalysts is an actual need in order to enlarge the portfolio of microorganisms and enzymes to be used for biosynthesis processes. Considering the ecological biodiversity and the potentially expressed heterogeneous enzymatic pattern, filamentous fungi offer a great portfolio of biocatalyst already involved in the production of pharmaceuticals, agrochemicals or fragrances (Colwell, 2002; Gavrilescu and Chisti, 2005).

ERs of yeasts, bacteria and plants have been characterized at genomic, structural and catalytical level (Gatti et al., 2014), but very few information are available for filamentous fungi. The definition of the catalytic cycle allowed developing an enzymatic assay, based on the co-factor consumption (Gao et al., 2012). Indeed its use is limited because many unsaturated substrates commonly used in biocatalysis absorb at the same wavelength of the cofactor. Moreover since several enzymes use NAD(P)H as cofactor, this method is elective of ER activity only in presence of purified enzymes.

Since the purification of fungal ERs is not a common practice, a whole-cell system is still the method of choice for biocatalysis experiments. 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; Hall et al., 2006; Skrobiszewski et al., 2013). To date, the main functional screening of filamentous fungi was performed by Carballeira et al. (2004) that takes into account 241 fungi of which only 3 were capable to reduce the C=C double bond of carvone. Nevertheless, data comparison with literature is difficult due to the few model substrates used to investigate ER activity; actually, few studies take into account more than one compound. For example, Goretti et al. (2011) described a whole-cell system of non-conventional yeasts in the bioconversion of several α,β-unsaturated ketones and aldehydes.

The present study aims to identify filamentous fungi showing ER activity through a functional screening of 28 filamentous 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 used in this study belong to different physiological and taxonomical groups and were isolated from several 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 cm^2/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.

Biomass weight was obtained by drying in oven at 60 °C for 24 h. Since it was not possible to collect *G. cucujoidarum* mycelium due to its yeast-like growth, a Burker chamber was used to count the cells number in each ml of culture solution.

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 m × 0.25 mm × 0.25 μm, 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, 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 the following tables, the maximum percentage of C=C double bonds reduction and the percentage of product yield 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 biotransformation of this molecule in its corresponding alcohol.

CE is a well-accepted substrate, actually 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 effective toward CE confirming the results obtained by Fuganti and Zucchi (1998).

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) reduce ketonic substrate with high yield in benzalacetones derivatives.

CE biotransformation led to the identification of two products: first ERs reduce the C=C double bond of CE leading to the formation of CO, then ADHs reduce the keto group of CO leading to the formation of COH, the putative reaction profile is shown in Figure 1. Most of the fungi (67 %) convert CE into COH showing the action of ERs and ADHs, while 26 % showed only ERs activity reducing preferentially the C=C double bond, leading to the production of CO. When the reduction was slow for instance during the biotransformation of CE by the fungus *P. citrinum* (Figure 2), it was possible to define a putative reaction profile in which the two enzymes act in cascade. 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; Fuganti and Zucchi, 1998; 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 3,

two fungi out of 28 (*A. niger* and *M. circinelloides*) were active and reached an almost complete conversion of the substrate within 2 days.

MNS biotransformation led to the identification of NPB as the sole product through the reduction of a C=C double bond by ER activity (Figure 3). It was not possible to analyze ee values because the product of the conversion undergo to a rapid non-enzymatic racemization, which leads to the production of racemates.

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 *S. cerevisiae* in the reduction of the C=C double bond of MNS was reported by Kawai et al. (2001) which 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 4) 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 whole-cell of *S. cerevisiae*.

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 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

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 has recently been reported 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 ER 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, 3, 4). 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 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

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. Data are reported in Table 5. On the whole, all fungi were able to grow in presence of all substrates: no morphological differences have been observed but a slight biological variability among replicates.

A detailed correlation between biomass and ER activity was not outlined because several factors may play single or combinatorial effects (i.e. primary or secondary metabolism, nourishment sources, toxic substrate or product, stress responses, etc.).

Noteworthy, in presence of CE, fungal growth was often higher than in the other cultural lines: the highest biomass development was observed for CE for 19 up to 28 strains. Further analyses are indeed necessary to understand the metabolic effect played by different substrates on fungal growth.

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* were the most versatile strains converting all the analyzed substrates, 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).

Table 2: biotransformation of CE as the maximum percentage of C=C double bond reduction and the percentage of product yield. 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 %.

Table 3: biotransformation of MNS as the maximum percentage of C=C double bond reduction and the percentage of products yield. 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 %.

Table 4: biotransformation of MCA as the maximum percentage of C=C double bond reduction and the percentage of products yield. 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 %.

Table 5: final biomass dry weight measurement (7 days). According to the biomass development, five groups were defined: group 1 < 100 mg; group 2 100-200mg; group 3 200-400 mg; group 4 400-600 mg; group 5 > 600 mg. *: 3.6×10^8 cells/ml

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.

Table 1: list of the strains analysed 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: biotransformation of CE as the maximum percentage of C=C double bond reduction and the percentage of product yield. 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 %.

Group	Fungus	Max % of C=C reduction	CO yield (%)	COH yield (%)
A	*B. cinerea*	100	0	100
	C. funicola	89	89	0
	C. herbarum	100	5	95
	G. masseei	100	14	86
	M. simplex	100	9	91
	O. maius	100	0	100
	P. citrinum	100	23	77
	P. purpurogenum	85	75	10
	Scopulariopsis sp.	100	54	46
	S. fimicola	100	6	94
	T. viride	100	0	100
	T. spiralis	100	0	100
	A. cylindracea	100	30	70
	A. farinacea	100	74	26
	A. splendida	100	2	98
	Coprinellus sp.	100	3	97
	P. ostreatus	100	30	70
	T. pubescens	100	0	100
	M. circinelloides	100	0	100
	M. plumbeus	100	0	100
	S. racemosum	100	7	93
B	*A. niger*	57	53	4
C	*E. nigrum*	30	24	6
	G. cucujoidarum	32	24	8
	A. glauca	35	27	8
D	*B. bassiana*	0	0	0
	M. deflexum	10	10	0
	P. vinaceum	23	23	0

Table 3: biotransformation of MNS as the maximum percentage of C=C double bond reduction and the percentage of products yield. 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 %.

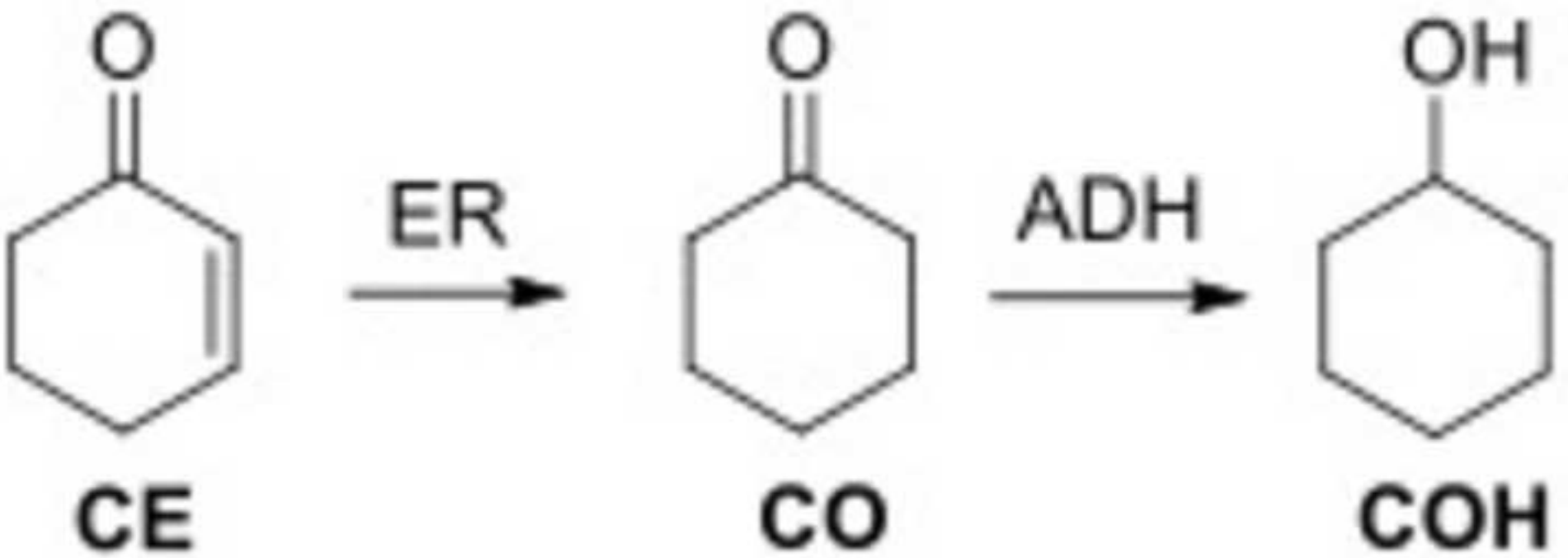
Group	Fungus	Max % of C=C reduction	NPB yield (%)
A	*A. niger*	97	97
	P. citrinum	98	98
	M. circinelloides	82	82
	M. plumbeus	79	79
B	*G. masseei*	72	72
	T. pubescens	52	52
C	*C. funicola*	30	30
	E. nigrum	32	32
	G. cucujoidarum	26	26
	M. deflexum	44	44
	S. fimicola	32	32
	T. viride	30	30
	A. splendida	34	34
D	*B. bassiana*	0	0
	B. cinerea	0	0
	C. herbarum	24	24
	M. simplex	18	18
	O. maius	20	20
	P. purpurogenum	0	0
	P. vinaceum	11	11
	Scopulariopsis sp.	17	17
	T. spiralis	0	0
	A. cylindracea	0	0
	A. farinacea	13	13
	Coprinellus sp.	11	11
	P. ostreatus	19	19
	A. glauca	22	22
	S. racemosum	16	16

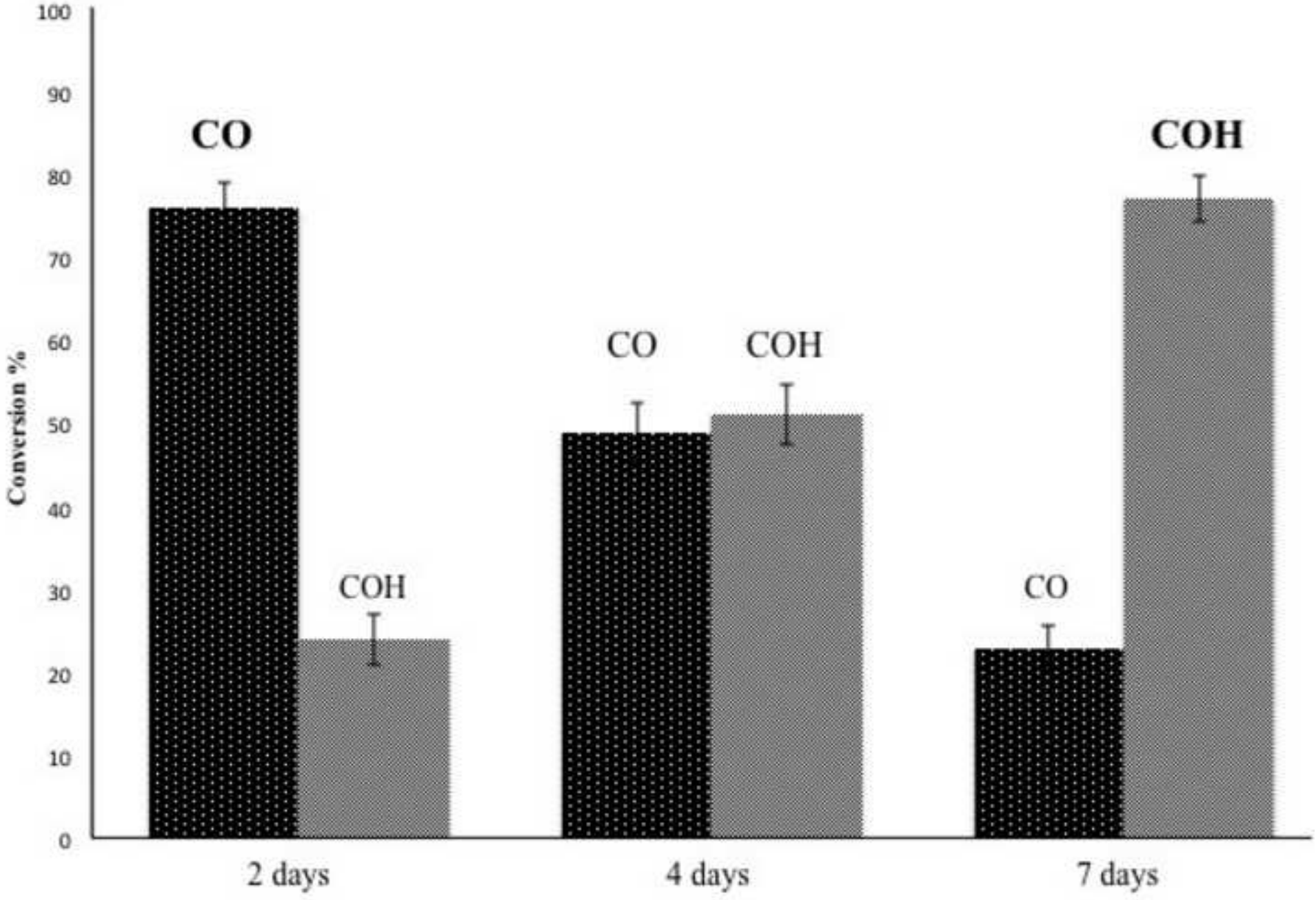
Table 4: biotransformation of MCA as the maximum percentage of C=C double bond reduction and the percentage of products yield. According to the conversion of the substrates, four groups were defined: group A: 100-75 %; group B: 74-50 %; group C: 49-25 %; group D: 24-0 %.

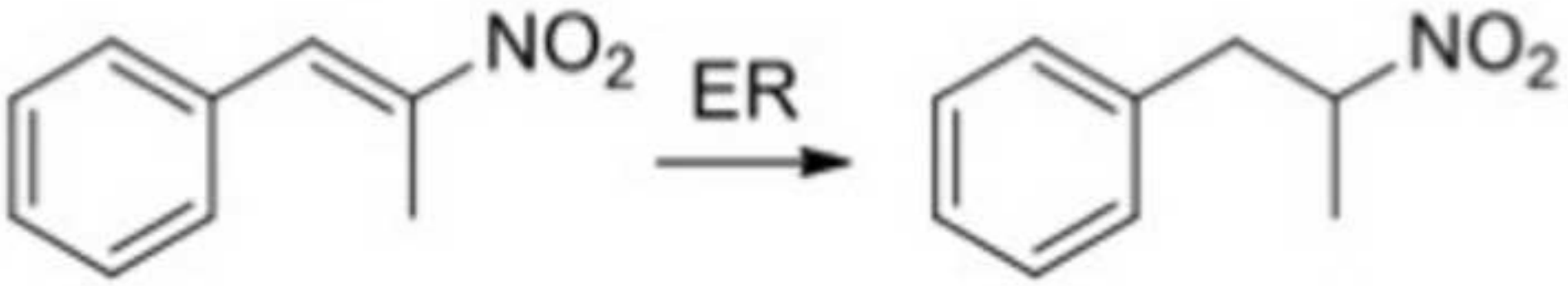
Group	Fungus	Max % of C=C reduction	MCOH yield (%)	MDHCOH yield (%)
A	*M. circinelloides*	100	0	100
	M. plumbeus	98	2	98
B	*G. masseei*	50	23	50
D	*A. niger*	0	0	0
	B. bassiana	0	0	0
	B. cinerea	18	62	18
	C. funicola	0	3	0
	C. herbarum	3	29	3
	E. nigrum	0	66	0
	G. cucujoidarum	0	35	0
	M. simplex	0	0	0
	M. deflexum	0	0	0
	O. maius	10	5	10
	P. citrinum	3	26	3
	P. purpurogenum	0	0	0
	P. vinaceum	0	0	0
	Scopulariopsis sp.	0	0	0
	S. fimicola	0	8	0
	T. viridae	10	88	10
	T. spiralis	0	0	0
	A. cylindracea	0	0	0
	A. farinacea	0	5	0
	A. splendida	0	0	0
	Coprinellus sp.	5	65	5
	P. ostreatus	0	0	0
	T. pubescens	14	33	14
	A. glauca	0	76	0
	S. racemosum	0	45	0

Table 5: final biomass dry weight measurement (7 days). According to the biomass development, five groups were defined: group 1 < 100 mg; group 2 100-200 mg; group 3 200-400 mg; group 4 400-600 mg; group 5 > 600 mg. *: 3.6 x 10^8 cells/ml.

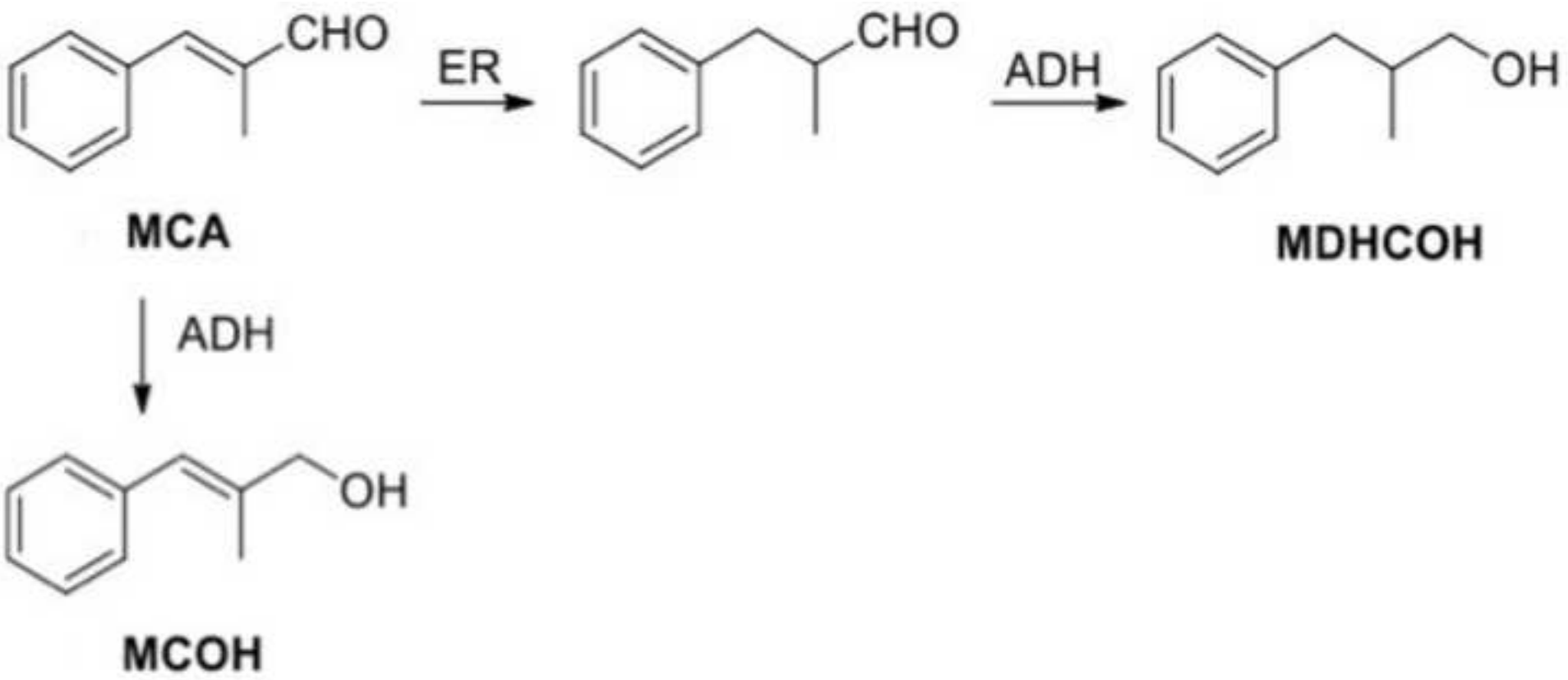
Fungus	Biomass growth			Max development
	CE	MNS	MCA	
A. niger	2	2	2	-
B. bassiana	3	1	1	CE
B. cinerea	5	4	3	CE
C. funicola	3	1	2	CE
C. herbarum	3	2	2	CE
E. nigrum	2	2	2	-
G. cucujoidarum	*	*	*	-
G. masseei	4	2	2	CE
M. simplex	2	1	1	CE
M. deflexum	1	1	1	-
O. maius	4	2	2	CE
P. citrinum	2	1	1	CE
P. purpurogenum	3	2	2	CE
P. vinaceum	1	1	1	-
Scopulariopsis sp.	2	1	1	CE
S. fimicola	2	1	1	CE
T. viridae	5	2	2	CE
T. spiralis	3	2	2	CE
A. cylindracea	1	1	1	-
A. farinacea	1	1	1	-
A. splendida	1	1	1	-
Coprinellus sp.	2	1	1	CE
P. ostreatus	2	1	1	CE
T. pubescens	5	1	1	CE
A. glauca	2	2	2	-
M. plumbeus	3	2	2	CE
M. circinneloides	3	2	2	CE
S. racemosum	2	1	1	CE


O
ER
O
ADH
OH
CE
CO
COH


100
90
80
70
60
50
40
30
20
10
0
Conversion %
CO
COH
CO
COH
CO
COH
2 days
4 days
7 days



MNS **NPB**


CHO
ER
CHO
ADH
OH
MCA
MDHCOH
ADH
OH
MCOH