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Fungal Oxidoreductases as Biocatalysts for Fine Chemicals Transformations

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Fungal oxidoreductases are promising tools for the production of fine chemicals as pharmaceuticals, flavors and fragrances. In this study, 28 filamentous fungi, belonging to 24 genera, were tested in the bioconversion of methyl cinnamate; only *Syncephalastrum racemosum*, *Cunninghamella bertholletiae* and *Mucor plumbeus* reduced the C=C double bond and the carboxylic group showing ene reductase activity and carboxylic acid reductase activity, respectively. Since the latter activity has been poorly investigated in filamentous fungi, further analysis was conducted. Three strains of *C. bertholletiae* and nine strains of *S. racemosum* converted a carboxylic acid (phenoxyacetic acid) and its methyl ester (methyl phenoxyacetate). Interestingly no intraspecific difference could be highlighted. Most of *S. racemosum* strains completely reduced both compounds within two days; the strength of the expressed enzymatic pattern is confirmed by the fact that it was not perturbed by the nature of the original substrate.

1. Introduction

Industrial biotechnology is facing the challenge to reduce both the environmental impact and the process costs to set technical and economic suitable methods. In recent years bio-based processes candidate themselves as a viable alternative to traditional methods offering mild reaction conditions and low energetic requirements, and avoiding the use of toxic concentrated compounds (Gao et al., 2012)
The reduction of C=C double bond and carboxylic groups are key reactions in organic chemistry for the production of bulk and fine chemicals as pharmaceuticals, flavors and fragrances (Stueckler et al., 2010; Toogood and Scrutton, 2014). Today they are performed by traditional chemical synthesis with the use of polluting and expensive compounds (Gao et al., 2012).
The reduction of C=C double bond conjugated with electron withdrawing groups (EWG) may be achieved with ene reductases (ERs, E.C. 1.6.99.1), flavin dependent oxidoreductases that catalyze the asymmetric hydrogenation of activated C=C double bond using NADH as cofactor. These enzymes belong to the “Old Yellow Enzyme“ (OYE) family, found for the first time in 1932 in *Saccharomyces cerevisiae*. Through years, homologues have been discovered in bacteria, plants, animals and recently in filamentous fungi (Stuermer et al., 2007).
The reduction of carboxylic acids and esters to aldehydes may be achieved with carboxylic acid reductases (CARs, E.C.1.2.1.30); they belong to the aldehyde oxidoreductase family and require ATP, Mg^{2+} and NADPH as cofactors. CARs are versatile enzymes acting on a wide range of substrates as ibuprofen, vanillin and ferulic acid; the last two are important precursor for the synthesis of vanilla flavor, one of the most common and costly aromas on the market (Venkitasubramanian et al., 2008). Although CARs are promising biotechnological tools, few information are available about their occurrence among organisms and their physiological role. They have been found in few bacteria (i.e. *Nocardia asteroides*) (Gross, 1972) and fungi (i.e. *Neurospora crassa*) (Kato et al., 1988), but their purification and characterization have been rarely reached. One of the main drawbacks is indeed their sensitivity to O_2 that ultimately hinder the purification of stable enzymes (Napora-Wijata et al., 2014).

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Filamentous fungi have been used in a wide range of biotechnological processes; the heterogeneous enzymatic pattern, more stable and active than bacteria and the high physiological variability makes them strong biocatalysts. Their capability to catalyze stereoselective reactions is of great interest in organic synthesis (Borges et al., 2009). Information about the occurrence of ERs and CARs in the Fungal Kingdom is scarce, claiming for focused researches. The main aim of this study was to assess the biotransformation potential of 28 fungi belonging to Ascomycetes, Basidiomycetes and Zygomycetes. Both C=C double bond and carboxylic group were targeted in order to explore the fungal ER and CAR activity.

2. Materials and methods

2.1 Isolates

Fungal strains are preserved at the *Mycotheca Universitatis Taurinensis* (MUT, Department of Life Science and Systems Biology, University of Turin) (Table 1).

Table 1: List of filamentous fungi and isolation site. MUT: accession number referred to strains deposited in the Mycotheca Universitatis Taurinensis collection

Fungi	Species	MUT	Isolation site
Ascomycota	*Aspergillus niger*	3874	air
	Beauveria bassiana	1720	air
	Botrytis cinerea	1087	fresco of Botticelli
	Chaetomium funicola	3726	dried *Bolutus* fungui from Europe
	Cladosporium herbarum	3856	air
	Epicoccum nigrum	3848	air
	Geotrichum cucujoidarum	4824	wastewater of tanning industry
	Gliomastix masseei	4855	*Flabelia petiolata* (marine algae)
	Mesobotrys simplex	281	air
	Myxotrichum deflexum	1749	air
	Oidiodendron maius	1381	roots of *Vaccinius myrtillus* (black raspberry)
	Penicillium citrinum	4862	*Flabelia petiolata* (marine algae)
	Penicillium purpurogenum	4831	wastewater of a tanning industry
	Penicillium vinaceum	4892	*Padina pavonica* (marine algae)
	Scopulariopsis sp.	4833	wastewater of a tanning industry
	Sordaria fimicola	1148	*Picea abies* (Norway spruce)
	Trichoderma viride	1166	tallus of *Parmelia taractica* (lichen)
	Trichurus spiralis	3788	book pages
Basidiomycota	*Agrocybe cylindracea*	2753	carpophore
	Agrocybe farinacea	2755	carpophore
	Agrocybe splendida	3696	carpophore
	Coprinellus sp.	4897	*Padina pavonica* (marine algae)
	Pleurotus ostreatus	2976	carpophore on *Populus* sp. (poplar)
	Trametes pubescens	2400	carpophore on *Populus* sp. (poplar)
Zygomycota	*Absidia glauca*	1157	tallus of *Peltigera praetextata* (lichen)
	Cunninghamella bertholletiae	2231	aeration pipe in a composting plant
	Cunninghamella bertholletiae	2861	vermicompost
	Cunninghamella bertholletiae	4924	sea grass *Posidonia oceanica* floating leaves
	Mucor plumbeus	2769	air
	Syncephalastrum racemosum	42	hazelnut
	Syncephalastrum racemosum	642	soil under *Pinus halepensis*
	Syncephalastrum racemosum	2217	aeration pipe in a composting plant
	Syncephalastrum racemosum	2486	rhizosphere of *Solanum lycopersicum* wild type
	Syncephalastrum racemosum	2770	air
	Syncephalastrum racemosum	2771	air
	Syncephalastrum racemosum	3117	phyllosphere of Solanum lycopersicum
	Syncephalastrum racemosum	3118	rhizosphere of Solanum lycopersicum
	Syncephalastrum racemosum	3241	air

2.2 Chemicals
Methyl cinnamate (MCI), phenoxyacetic acid (PA) and methyl phenoxyacetate (MP) were purchased from Sigma-Aldrich (Italy). Stock solutions (500 mM) were prepared by dissolving the substrate in dimethyl sulfoxide (DMSO).

2.3 Fungal screening

2.3.1 Evaluation of enzymatic activities occurrence: MCI reduction by 28 fungi
The 28 fungal strains were pre-grown in malt extract solid medium (MEA: 20 g/L glucose, 20 g/L malt extract, 20 g/L agar, 2 g/L peptone). As regard Ascomycota and Zygomycota strains, conidia suspension was prepared ($1x10^6$ final concentration); for Basidiomycota the inoculum was made homogenizing agar squares derived from the margin of an overgrown colony with sterile water (1 cm^2/mL). Flasks of 100 mL containing 40 mL of MEA liquid medium were inoculated and incubated at 25 °C in agitation (110 rpm).
After two days, MCI was added (5 mM final concentration) and three biological replicates were set up. The experiment was run for 7 d: periodically (2, 4 and 7 d) 1 mL of cultural broth was collected and extracted by two-phase separation using 0.4 mL of methyl t-butyl ether (MTBE) as solvent. The organic phases were dried over anhydrous Na_2SO_4 and analyzed by GC/MS.

2.3.2 Enzymatic variability: PA and MP reduction by *C. bertholletiae* and *S. racemosum* strains
On the base of the previous screening using 28 fungi, the two most promising strains were selected to evaluate the intraspecific variability of CAR activity. The reduction of PA and MP was carried out by all the strains preserved at MUT of *C. bertholletiae* (three strains) and of *S. racemosum* (9 strains) (Table 1). Cultural conditions have been described in paragraph 2.3.

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). The GC retention times of cinnamic alcohol, benzyl alcohol, 3-phenylpropanol, 2-phenoxy ethanol have been already described (Brenna et al., 2015).

3. Results

3.1 Bioconversion of MCI
Only 3 fungi were able to reduce the substrate MCI, indicating the high recalcitrance of the unsaturated ester to biological transformation. The substrate was completely reduced by *S. racemosum* MUT 2770 whereas *C. bertholletiae* MUT 2231 and *M. plumbeus* MUT 2769 reached maximum yields of 18 and 9 %, respectively.
S. racemosum MUT 2770, *C. bertholletiae* MUT 2231 and *M. plumbeus* MUT 2769 expressed both ER and CAR activity. *C. bertholletiae* MUT 2231 and *M. plumbeus* MUT 2769 converted no more than 20 % of MCI, producing 3-phenylpropanol as the sole reaction product (Figure 1). The reaction was fast (2 days) but no significant improvements could be seen afterwards. Noteworthy *S. racemosum* MUT 2770 totally reduced MCI after 4 days (Figure 1). Through time, the formation of three products was detected. As can be seen in Figure 1, cinnamic alcohol and 3-phenylpropanol were the major products (> 40 %) at 4^{th} day. Benzyl alcohol concentration was negligible after 4 days but rose during the experiment (from 5 to 47 %). At the end of the experiment, only 3-phenylpropanol and benzyl alcohol were detected.

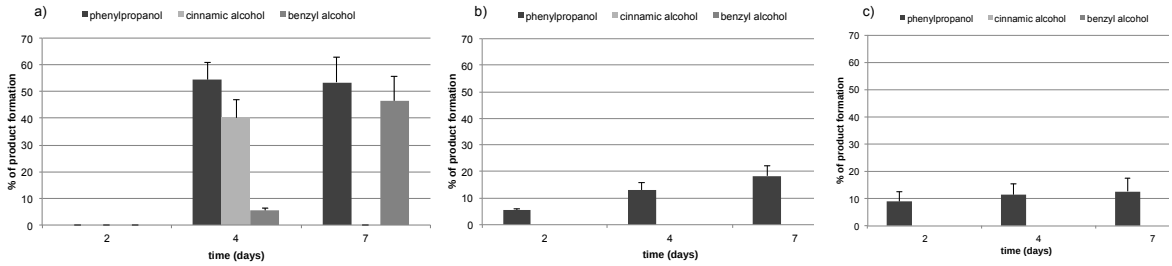

Figure 1: Products profile of MCI bioconversion by: a) S. racemosum MUT 2770; b) C. bertholletiae MUT 2231; c) M. plumbeus MUT 2769.

3.2 Bioconversion of PA and MP
Considering the promising reduction yields obtained by *C. bertholletiae* and *S. racemosum,* the intraspecific variability among these two species has been evaluated taking into consideration a poor reactive ester (MP) and the corresponding acid (PA).

The results of the biotransformation of PA and MP are shown in Table 2. It is interesting to notice that all the fungi were not influenced by the chemical nature of the former substrate; applying PA or MP to the fungal system, the final concentration of 2-phenoxyethanol was comparable. As regard MP, an initial ester hydrolysis was performed producing PA as the first intermediate product; 2-phenoxyethanol outlined the further reduction of the carboxyl group.
For both the substrates, the transformation yields seemed to be species-dependent. *C. bertholletiae* strains were less efficient, producing almost 20 % of the corresponding alcohol. All the tested strains gave coherent results. On the contrary, all the strains of *S. racemosum* totally converted PA and MP to the corresponding alcohol. In most cases, the reaction was very fast (2 days) and this did not allow monitoring any differences among the strains. Even though *S. racemosum* MUT 642 produced 100 % of 2-phenoxyethanol, the reaction was remarkably slower: at least 4-7 days were necessary to observe a complete reduction.

Table 2: Maximal percentage of PA and MP and their corresponding products; the time needed to reach the maximal yield is indicated in parenthesis

		PA conversion %		MP conversion %		
Species	**MUT**	**PA**	**2-phenoxyethanol**	**MP**	**PA**	**2-phenoxyethanol**
C. bertholletiae	2231	76.3 (7 d)	23.7 (7 d)	-	78.3 (7 d)	21.7 (7 d)
C. bertholletiae	2861	75.3 (7 d)	24.7 (7 d)	-	81.3 (7 d)	18.7 (7 d)
C. bertholletiae	4924	78.3 (7 d)	21.7 (7 d)	-	81.5 (7 d)	18.5 (7 d)
S. racemosum	42	-	100 (2 d)	-	-	100 (2 d)
S. racemosum	642	-	100 (7 d)	-	-	100 (4 d)
S. racemosum	2217	-	100 (2 d)	-	-	100 (2 d)
S. racemosum	2486	-	100 (2 d)	-	-	100 (2 d)
S. racemosum	2770	-	100 (2 d)	-	-	100 (2 d)
S. racemosum	2771	-	100 (2 d)	-	-	100 (2 d)
S. racemosum	3117	-	100 (2 d)	-	-	100 (2 d)
S. racemosum	3118	-	100 (4 d)	-	-	100 (2 d)
S. racemosum	3241	-	100 (2 d)	-	-	100 (2 d)

4. Discussion

Traditional chemical-physical methodologies applied in the organic synthesis have been shown many drawbacks, often coupling high energetic requirement to massive use of toxic and concentrated compounds. The overcoming of these limits is one of the first goals of alternative advanced solutions and could be reached by biological-based methods. Bacteria and yeast bioconversion potentials have been already investigated, while filamentous fungi have been left aside. The results achieved in this study allowed enhancing the knowledge of the biotechnological potential of fungi and their enzymatic pattern.
The evaluation of variability at genus and species level is a required step to select the most proper biocatalysts. In detail, this approach led to identify some potential biocatalysts responsible of the reduction of unsaturated esters. This goal was desirable but not obvious, because many evidences previously demonstrated that MCI was a stable and unreactive compound. For instance, Tasnadi et al. (2012) described the reduction of several α,β unsaturated carboxylic esters by means of seven OYE homologues purified by yeast, bacteria and plant: in detail, MCI was not converted (< 1%) by all of the tested enzymes. Gao et al. (2012) reported that a purified ER from *Lactobacillus casei* was not able to reduce this substrate. Among 23 recombinant ERs tested, only four were active on an ester molecule, with very low (9-11%) conversion yields (Reß et al. 2015).
According to this background, the reduction of MCI catalyzed by three out of 28 strains was an important result. In particular, *S. racemosum* MUT 2770 aroused great interest, being able to totally reduced MCI by expressing a various, fast and efficient enzymatic pattern.
The final major product, e.g. 3-phenylpropanol, indicates the mutual presence of two enzymatic classes, leading to the reduction of both the C=C double bond and the carboxylic group. The combined action of ERs and CARs could be described in the following scheme (Figure 2).

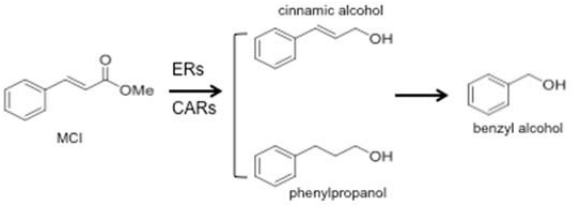

Figure 2: MCI reduction profile involving both ERs and CARs.

Even though the literature is still deficient, the presence of ER activity in fungi should not be a surprise: several studies have indeed described it in strains belonging to different phyla (Romagnolo et al., 2013; Skrobiszewski et al., 2013). Although their biological role is still under debating, ERs seem to be involved in several biological pathways as stress response mechanisms, fatty acid and alkaloids production (Stuermer et al., 2007; Robinson and Panaccione, 2015). Nowadays, many of these natural substrates and/or their metabolites have been targeted by biotechnological researches arising great interest for ERs exploitation. For instance the reduction of C=C double bonds of ergot alkaloids has been recently associated to ER activity which can then be used to produce fine chemicals useful in agriculture and medicine (Robinson and Panaccione, 2015).

On the contrary CAR activity was less described and no fungal homologues have been biochemically characterized (Napora-Wijata et al., 2014). Indeed it was the focus of further insights. Particular attention was given to the possible intraspecific physiological variability because no studies have deal with the diffusion of CAR activity among strains belonging to the same species. Even though this phenomenon is well-known in the fungal kingdom (Johnson et al., 2012), slight differences were monitored among *C. bertholletiae* and *S. racemosum* strains. It should be noticed however that the latter gave such a rapid response that the detection of any difference would need shorter data collection points.

The strength of *S. racemosum* strains was confirmed by the complete reduction of both the carboxylic acid (e.g. phenylacetic acid) and its methyl ester. The formation of the alcohol can be explained by a two-step sequence: i) the reduction of the carboxylic acid to the corresponding aldehyde by a CAR; ii) the reduction of the aldehyde to the alcohol mediated by an alcohol dehydrogenase (ADH) (Figure 3). These data acquired particular emphasis because phenylacetic acid derivatives seemed to be weak substrates of fungal bioreduction. Farmer et al. (1959) reported the conversion of several acid substrates by *Polystictus versicolor:* PA was not reduced. *Coriolus hirsutus* reduced 2-methoxyphenylacetic acid, 2-nitrophenylacetic acid and 3-nitrophenylacetic acid at lower extent, e.g. 25 %, 6.5 % and 1.1 %, respectively (Arfmann and Abraham, 1993).

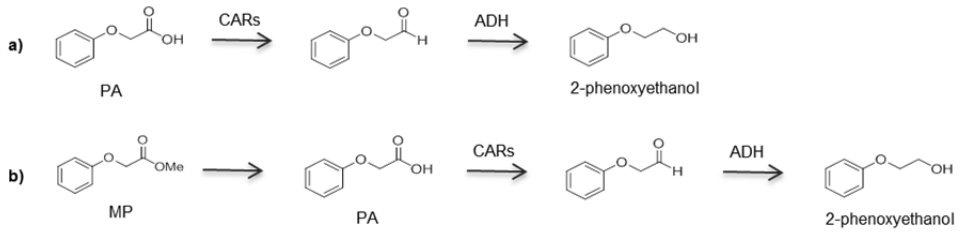

Figure 3: Reduction profile of a) PA and b) MP.

5. Conclusions

In this study, the potential fungi to crucial reactions of the organic synthesis (C=C double bond and carboxylic group reduction) have found important confirmations. Even though the used unsaturated and saturated compounds have rarely reduced by bio-based systems, few fungal strains were instead capable of mediating such reactions. The information about the biochemical features of the expressed ERs and CARs needs to be enlarged but some promising fungal biocatalysts have been defined; among the tested fungi, *S. racemosum* strains showed the most promising activity. The performed screening is an initial but fundamental step aimed to the selection of fungal producers of enzymes of interest for organic synthesis purposes. This would then allow deepening mostly unknown aspects of the fungal metabolism, as the expression of CARs.

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