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# A competitive approach for the reduction of unsaturated compounds based on fungal ene-reductases

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**Abstract**

This work aimed to select potential biocatalysts for the reduction of unsaturated compounds. The capability of *Gliomastix masseei*, *Mucor circinelloides*, *Mucor plumbeus*, *Penicillium citrinum* and *Syncephalastrum racemosum* of converting four model and six non-conventional molecules was tested, taking into consideration the role of substituents linked to the C=C bond on the process efficiency. All the tested fungi expressed ene-reductase activity towards several types of compounds: the ketone derivative was the best substrate followed by the nitroalkene and the unsaturated aldehyde, whereas the ester was the most recalcitrant to the bioreduction.

The results highlighted the potential of *Mucor circinelloides* MUT 44 and *Mucor plumbeus* MUT 2769 as versatile whole-cell systems: fast and efficient reduction was obtained for most of the compounds. Comparative analysis of the substrate spectrum has been performed for three *Mucor circinelloides* strains: reaction rate and timing varied, indicating a strong physiological diversity of ene-reductase activity at intraspecific level. Moreover, a preliminary molecular analysis demonstrated the presence of at least a putative sequence coding for ene-reductase in the genomic DNA of all the fungi tested.

**Key words** – asymmetric bioreduction – ene-reductases – filamentous fungi – *Mucor circinelloides* – unsaturated compounds

## Introduction

In the last decade biocatalysis has been established as a viable alternative to traditional chemical synthesis, coupling economic, technological and environmental sustainability. Microorganisms and their enzymes usually need mild reaction conditions, low energetic consumption and minimal use of chemicals (Torres et al. 2003). Therefore, bio-based systems are perfect candidates to be integrated along the chemicals manufacturing chain, for the sustainable production of existing and novel products (Liese et al. 2006).

The reduction of the C=C bond of unsaturated compounds is a key reaction in organic synthesis and in particular for the production of pharmaceuticals, flavors and biological active compounds (Stueckler et al. 2010, Toogood & Scrutton 2014, Zagozda & Plenkiewicz 2006, Williams et al. 2004). Up to date, industries promote this desired reaction by metal-based catalysts claiming indeed for help by renewable resources (Gao et al. 2012). In the past decades, many reports highlighted the involvement of microorganisms in the reduction of C=C double bond, identifying the major responsible enzymatic activity. Ene-reductases (ERs) are flavin-dependent oxidoreductases belonging to the Old Yellow Enzymes (OYE) family (E.C. 1.6.99.1) capable of acting on C=C double bonds conjugated with electron withdrawing groups (EWG) in presence of

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NAD(P)H as cofactor (Gatti et al. 2013). Their enrollment in the actual industrial scenario also arises from the capability to produce chiral molecules with high stereo-selectivity.

ERs have been first discovered in 1932 in *Saccharomyces pastorianus* and through years have been described in yeasts, bacteria, plants, animals and few filamentous fungi (Stuermer et al. 2007). Even though their physiological role remains mostly unknown (Williams & Bruce 2002), some evidences demonstrated their involvement in detoxification responses by bacteria (Fitzpatrick et al. 2003) and ergot alkaloid synthesis (Chilton et al. 2014). They have been successfully used to reduce several alkenes for research and industrial purposes (Huang et al. 2014, Toogood & Scrutton 2014).

In recent years, many researchers have focused their attention on new potential biocatalysts within the fungal kingdom. Fungi are robust to harsh operative conditions and their physiological versatility is often an indication of a heterogeneous and highly efficient enzymatic pattern (Colwell 2002). Several evidences have shown fungal enantioselective reduction of several unsaturated compounds, e.g. including ketones to their secondary alcohols (Carballeira et al. 2004, Zagozda & Plenkiewicz 2006).

The identification of promising ERs producers could be achieved by genome mining of databases looking for targeted sequences. Even though OYE genes of *S. pastorianus* could be used as reference constructs, the low availability of complete fungal genomes deeply limits this approach (Toogood & Scrutton 2014). On the other hand, a whole-cell system is a good strategy to screen ERs activity expression among fungi. The use of fungal biomass leads also to overcome some drawbacks, commonly associated to purified enzymes: ERs need the regeneration of the cofactor and are often only one step of a multienzyme cascade procedure (Toogood & Scrutton 2014). Whole cells represent instead complete biocatalysts, able to express several enzymes and provide them the required cofactors.

Besides, the biocatalytic potential of fungi is often species and strain dependent, making necessary to enlarge the range of the studied microorganisms. For instance, 3 fungi have been assessed for the reduction of unsaturated ketones showing great catalytic variability: *Mortierella isabellina* and *Geotrichum candidum* reduced the C=O bond affording the corresponding alcohol with high enantiomeric excess (94-99%), whereas *Rhodotorula rubra* was active only towards the C=C double bond with moderate enantioselectivity (Zagozda & Plenkiewicz 2006). *Gongronella butleri, Schizosaccharomyces octosporus* and *Diplogelasinospora grovesii* reduced carvones to dihydrocarvones by the joint action of ERs and alcohol dehydrogenases but the reaction profile and the secondary products deeply varied (Carballeira et al. 2004).

The present study lays its bases on a previous screening, carried out by Romagnolo and collaborators (2015). Most of the 28 tested filamentous fungi belonging to different taxonomical groups reduced the double bond of ketone, nitro derivative and aldehyde compounds. The main aim of this paper is to enlarge the information about alkene reduction by five selected strains by investigating the versatility of the expressed ERs activity by using model and non-conventional substrates (i.e. difficult to convert by purified OYEs from yeasts) and its variability among fungi.

## Materials & Methods

### Fungal strains

The filamentous fungi used are preserved at the *Mycotheca Universitatis Taurinensis* (MUT, Department of Life Sciences and Systems Biology, University of Turin). A first screening included two ascomycetes and three zygomycetes, while the intraspecific variability was assessed among *Mucor circinelloides* strains (Table 1).

**Table 1** Strains, MUT identification number and isolation site.

| Specie | MUT | Isolation site |
|---|---|---|
| *Gliomastix masseei* (Sacc. & Trotter) Matsush. | 4855 | *Flabelia petiolata* |

| | | |
|---|---|---|
| *Mucor circinelloides* Tiegh. | 44 | unknown |
| *Mucor circinelloides* Tiegh. | 724 | unknown |
| *Mucor circinelloides* Tiegh. | 2223 | air from a compost plant |
| *Mucor plumbeus* Bonord. | 2769 | air |
| *Penicillium citrinum* Thom | 4862 | *Flabelia petiolata* |
| *Syncephalastrum racemosum* Cohn | 2770 | air |

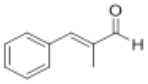
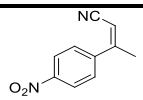
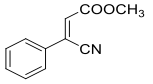
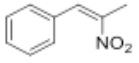
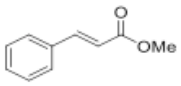
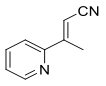
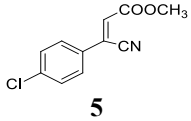
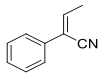
**Substrates**

Cyclohexenone (CE), α-methylcinnamaldehyde (MCA) and α-methyl cinnamate (MCI) were purchased from Sigma-Aldrich (Italy). (E)-α-methylnitrostyrene (MNS) was synthesized according to the literature (Kawai et al. 2001) (Table 2).

(*E*)-3-(4-nitrophenyl)but-2-enenitrile (**1**), (*E*)-3-(pyridin-2-yl)but-2-enenitrile (**2**), (Z)-2-phenylbut-2-enenitrile (**3**), (Z)-methyl 3-cyano-3-phenylacrylate (**4**), (Z)-methyl 3-cyano-3-(4-chlorophenyl)acrylate (**5**) were prepared according to the literature (Brenna et al 2015a, Knowles & Cloke 1932, Yan et al. 2015, Yu et al. 2014). 1-(3,3-Dimethylcyclohex-1-en-1-yl)ethanone (**6**) was a kind gift from Dr. Philip Kraft (Givaudan Scheiwz AG) (Table 2).

Stock solutions (500 mM) of each substrate were dissolved in dimethyl sulfoxide (DMSO).

**Table 2** Structure of conventional and non-conventional substrates.

| Conventional substrates | | Non-conventional substrates | |
|---|---|---|---|
|  CE |  MCA |  **1** |  **4** |
|  MNS |  MCI |  **2** |  **5** |
| | |  **3** |  **6** |

**Biotransformation experiment**

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). For liquid inoculation a conidia suspension was prepared ($1x10^6$ conidia final concentration in flask) and inoculated in 100 ml flasks containing 40 ml of malt extract liquid medium (MEA without agar). Flasks were maintained at 25 °C in agitation (110 rpm). After two days, the substrates were separately added (5 mM final concentration). Three flasks for each fungus were sacrificed to measure the initial biomass and pH. For each substrate, three other biological replicates were set up. The experiment run for 7 days and daily, 1 ml of cultural broth was collected to carry out the chemical analysis.

**GC/MS analysis**

The samples were 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 means of GC/MS. 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 of CE, MCA, MNS, and MCI and of the corresponding reduced compounds have been already described (Brenna et al. 2015b, Romagnolo et al. 2015). GC retention times of compounds **1-3** and **6** and of the reduced products obtained from **3** and **6** are the following ones: **1** 23.35 min , **2** 16.50 min, **3** 14.32 min**,** red-**3** 12.87 min**, 6** 11.04 min, red-**6** 10.35

min, GC retention times of compounds **4-5** and of the corresponding reduced products have been already described (Brenna et al. 2015b).

**pH and biomass dry weight**

After two days of pre-growth and at the end of the experiment, pH was measured.

Biomass dry weight was calculated by separating the biomass from the liquid by filtration which was further dried at 60 °C for 24 h.

**Molecular analysis**

After 24 h of growth in MEA, the mycelium was collected and the total genomic DNA was extracted by CTAB method (Gardes & Bruns 1993).

*ER primer* was designed on an ER putative sequence of *M. circinelloides* obtained by the alignment with the sequence of OYE1 of *Saccharomyces pastorianus*. For most of fungi, ITS was used as positive control (White et al. 1990) whereas β-tubulin was used for *Penicillium citrinum* (Glass & Donaldson 1995). The PCR mixture included water, PCR buffer (10 X), 1 mM deoxynucleotide triphosphates (dNTPs), 10 mM of each primer (Table 3), 0.5 U of DNA polymerase and 100 ng of genomic DNA template.

**Table 3** List of primers.

| Primers | Sequences (5'-3') | Length |
|---|---|---|
| ITS 1 | TCCGTAGGTGAACCTGCGG | 700 |
| ITS 4 | TCCTCCGCTTATTGATATGC | |
| Bt 2a | GGTAACCAAATCGGTGCTGCTTTC | 500 |
| Bt 2b | ACCCTCAGTGTAGTGACCCTTGGC | |
| ER F | TGCCAATGGTTATCTGGTCG | 200 |
| ER R | CTGGAAGCCGTTACCTGGA | |

Amplifications were performed using a T100 Thermal Cycler (BIO RAD). PCR products were loaded in a 1.5 % agarose electrophoresis gel to check the PCR products. Each product was sequenced at Macrogen (The Netherlands). Sequence analyses and contig were carried out with Sequencher 5.4 (Gene Code Corporation). Sequences obtained were used as queries for BLAST analyses (Basic Local Alignment Search Tool, NCBI, USA) against the available *M. circinelloides* sequence (Joint Genome Institute, Mycocosm) to validate the identity of the amplicons.

The yeast strain BY4741 (Y, EUROSCARF, Frankfurt, Germany) was used as positive control for the presence of OYE in the genome (Stott et al. 1993).

**Results**

**Reduction of conventional substrates**

All the tested strains reduced at least three out of four model substrates (Table 4). Even though the timing of the reaction varied among fungi, a common reaction profile of CE reduction was observed. Independently by the fungus, due to ERs presence, chromatographic analysis detected at first a saturated ketone (cyclohexanone) that was further transformed in the corresponding saturated alcohol (cyclohexanol). All the strains catalyzed a complete reduction; the two strains belonging to *Mucor* genera were the fastest ones, reducing 100% of CE within the first 2 days of treatment.

With the only exception of *S. racemosum* MUT 2770, a good level of MNS conversion was observed in all the strains. *P. citrinum* MUT 4862 obtained the highest yields (99%), but the two *Mucor* strains were the fastest (79-86% in 2 days).

Fungal conversion of MCA led to a mixture of unsaturated and saturated alcohol, indicating the reduction of the carbonyl group (CHO) has already occurred. The complete reduction of MCA,

producing saturated alcohol as the final product, was achieved by *M. circinelloides* MUT 44 and *M. plumbeus* MUT 2769.

The transformation of MCI led exclusively to the formation of the saturated alcohol (3-phenylpropanol). *M. circinelloides* MUT 44 (100%) and *S. racemosum* MUT 2770 (99%) were the only strains capable of extensively reducing it.

**Table 4** Maximum percentage of C=C double bond reduction; the timing is reported in brackets.

| | *G. masseei* | *M. circinelloides* | *M. plumbeus* | *P. citrinum* | *S. racemosum* |
|---|---|---|---|---|---|
| **CE** | 100 (7d) | 100 (2d) | 100 (2d) | 100 (7d) | 100 (7d) |
| **MNS** | 61 (7d) | 86 (2d) | 79 (2d) | 99 (7d) | 16 (7d) |
| **MCA** | 51 (7d) | 99 (2d) | 98 (7d) | 3 (7d) | - |
| **MCI** | - | 100 (7d) | 13 (7d) | - | 99 (7d) |

**Reduction of non-conventional substrates**

To explore further the profile of the fungal ER activity, other unsaturated compounds have been used. The substrates were chosen because they were difficult to convert by purified wild type OYE1-3 from yeasts (data not shown). Four out of five fungi converted at least one of the analyzed substrates (Table 5). *M. plumbeus* MUT 2769 and *M. circinelloides* MUT 44 were the most versatile fungi being able to transform four and three substrates, respectively. *G. masseei* MUT 4855 did not reduce any substrate.

The recalcitrance of the compounds for ERs-mediated reduction was highlighted by the scarce or absent conversion observed in most of the cases (Table 5). Substrate **1** and **2** were left unreacted by all the fungi. Moderate transformation (20-30%) of substrate **3** and **4** was obtained by the two fungi belonging to the *Mucor* genus.

Substrate **5** was reduced only by *S. racemosum* MUT 2770 and *M. plumbeus* MUT 2769, the latter with high yields (86%).

Substrates **6** was converted by three fungi, but only *M. circinelloides* MUT 44 reached considerable process yields (69%).

**Table 5** Maximum percentage of C=C double bond reduction; the timing is reported in brackets.

| | *G. masseei* | *M. circinelloides* | *M. plumbeus* | *P. citrinum* | *S. racemosum* |
|---|---|---|---|---|---|
| **1** | - | - | - | - | - |
| **2** | - | - | - | - | - |
| **3** | - | 21 (7d) | 11 (7d) | - | - |
| **4** | - | 5 (7d) | 29 (2d) | - | - |
| **5** | - | - | 86 (2d) | - | 53 (2d) |
| **6** | - | 69 (7d) | 7 (7d) | 10 (7d) | - |

**Evaluation of the intraspecific variability**

*M. circinelloides* MUT 44 was capable of reducing 7 model and non-conventional compounds, five of which more than 60%. Noteworthy it was active toward compounds (e.g. MCI and substrate **6**) which were instead weak substrates for the other tested fungi. Since little is known about the occurrence of ERs within strains of the same species, three strains of *M. circinelloides* have been used to treat the model compounds, since representative of different chemical classes.

*M. circinelloides* MUT 44 and MUT 724 converted all the substrates showing different process yields and substrate spectrum: MUT 44 was more active toward MCI, while MUT 724 toward MCA and MNS. On the contrary, the reduction potential of *M. circinelloides* MUT 2223 was very weak, being partially active (12%) only toward CE (Table 6). The biotransformation always occurred within the first two days and no significant variation could be observed afterwards.

**Table 6** Maximum percentage of C=C double bond reduction of three *M. circinelloides* strains; the timing is reported in brackets.

| | MUT 44 | MUT 724 | MUT 2223 |
|---|---|---|---|
| **CE** | 100 (2d) | 100 (2d) | 12 (2d) |
| **MCA** | 60 (2d) | 100 (2d) | - |
| **MNS** | 75 (2d) | 95 (2d) | - |
| **MCI** | 78 (2d) | 43 (2d) | - |

**Molecular analysis**

The results of the amplification using ER primer pairs are shown in Fig 1. All the strains showed a common band of 200 bp. The same band was observed also for the yeast strain BY4741, known to express ERs.

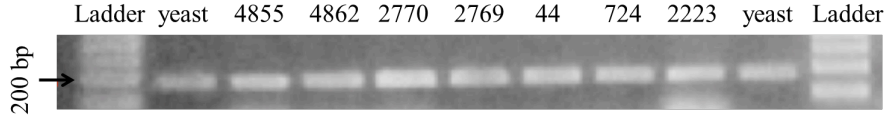



**Figure 1** – PCR products using ER primer pairs (ER1/ER4).

BLAST analyses were performed using PCR products sequences as query on the available genome of *M. circinelloides*. As can be seen in Table 7, all the PCR products showed a good alignment (98-100%) with two proteins, potentially attributable to ERs, i.e. flavin oxidoreductases that use NADH as cofactor.

**Table 7** Data of the BLAST analysis: percentage of identity, protein ID and molecular function of the PCR products.

| PCR product | % of identity | Protein ID | Molecular function |
|---|---|---|---|
| *G. masseei* MUT 4855 | 100/100 | 160302/137297 | NADH:flavin oxidoreductase |
| *P. citrinum* MUT 4862 | 100/99 | 137297/160302 | NADH:flavin oxidoreductase |
| *S. racemosum* MUT 2770 | 100/100 | 160302/137297 | NADH:flavin oxidoreductase |
| *M. plumbeus* MUT 2769 | 100/100 | 160302/137297 | NADH:flavin oxidoreductase |
| *M. circinelloides* MUT 44 | 100/98 | 160302/137297 | NADH:flavin oxidoreductase |
| *M. circinelloides* MUT 724 | 100/98 | 160302/137297 | NADH:flavin oxidoreductase |
| *M. circinelloides* MUT 2223 | 100/98 | 160302/137297 | NADH:flavin oxidoreductase |

**Discussion**

Bioreduction of C=C double bond is an attractive solution for *green* production of building block compounds, but the number of biocatalysts (microorganisms and enzymes) showing strong catalytic potential is still limited. Even though OYEs of yeast and bacteria have already been investigated (Stuermer et al. 2007, Toogood & Scrutton 2014), there is still a need of new bio-based systems capable of performing stereoselective reductions. Since fungi have long been on the fringes of the scientific scenario, little was known about the occurrence and the catalytic potential of filamentous fungi. This study poses its basis on a wider screening of fungal ERs (Romagnolo et al. 2015), from which five filamentous fungi have been selected. Results indicated that a strong ER activity was expressed by all the species but not homogenously diffused among them. Bioreduction rate and timing often resulted strain dependent underlining the importance of the choice of the most proper biocatalyst.

*G. masseei* MUT 4855, *M. circinelloides* MUT 44, *M. plumbeus* MUT 2769, *P. citrinum* MUT 4862 and *S. racemosum* MUT 2770 displayed a versatile ER activity, being capable of reducing unsaturated compounds belonging to at least three different chemical classes.

Even though a number of α,β-unsaturated compounds has been tested, only rarely the used compounds coincided (Toogood & Scrutton 2014). Moreover, the majority of the works have studied the reduction of few molecules belonging to a single chemical class (i.e. ketones) giving

little information about the versatility of the microorganisms (Goretti et al. 2009, Stueckler et al. 2010). Hence the absence of model substrates arises concerns about the possibility to compare data with the literature.

Besides non-activated C=C double bonds are difficult to reduce and the presence of an EWG on the alkene is required (Toogood & Scrutton 2014). A large variety of activating groups (e.g. aldehyde, ketone, imide, nitro, carboxylic acid and ester) have been assessed and correlated to the conversion yields of yeast OYEs and bacterial and plant homologues: reaction rate and enantioselectivity varied according to the substrate and the isoform (Hall et al. 2008a, Hall et al. 2008b, Reß et al. 2015). To the best of the authors' knowledge, a broad range of substrates including ketones, nitro derivatives, aldehydes and esters has never been considered for filamentous fungi. These data indeed may help to enlarge the knowledge about the role of EWGs in reactions catalyzed by OYE homologues among ascomycetes and zygomycetes. In particular, carboxylic acids, nitriles and esters are well-known to be weak EWGs, limiting the reduction efficiency towards these substrates (Stuermer et al. 2007). These evidences perfectly fit with the results of this study: the conversion of four model substrates followed the following profile, ketone (CE) > nitro alkene (MNS) > aldehyde (MCA) > ester (MCI).

Fungal-mediated biocatalysis has found to be very efficient in presence of a ketone group that probably acted as a strong EWG; even highly branched and substituted unsaturated ketones resulted good substrate for fungi, i.e. the ascomycetes *P. citrinum*, *G. candidum* and *Diplogelasinospora grovesii,* the zygomycetes *M. isabellina* and the basidiomycete *Pleurotus ostreatus* (Aquino et al. 2012, Ferreira et al. 2015, Quezada et al. 2012, Skrobiszewski et al. 2012, Zagozda & Plenkiewicz 2006). Among the studied fungi, *P. citrinum* has already been found capable of producing ERs (Aquino et al. 2012, Ferreira et al. 2015); it should not therefore surprise the conversion of CE observed by *P. citrinum* MUT 4862. In the present study all fungi achieved a complete conversion of CE within two days. Few studies have already taken into consideration CE and its analogue, allowing to compare the obtained data with previous evidences: filamentous fungi resulted highly competitive with other organisms (yeasts, bacteria and plants) and their enzymes. For instance, among 146 yeasts, only 11% reduced an unsaturated simple ketone (e.g. carvone), but the conversion was completed only by *Pichia amylophila* and *S. naganishii* (Goretti et al. 2009). OYE1 of *Saccharomyces carlsbengensis* carried out almost complete bioconversions of CE and its derivatives, whereas OYE2 and OYE3 of *S. cerevisiae* were less efficient, i.e. 75% and 7% of CE reduction, respectively (Stueckler et al. 2010). Controversial results are instead associated to bacterial and plant enzymes (Stueckler et al. 2010). Among the tested 5 bacterial OYE homologues and the two enzymes from tomato, only two of them were capable of reducing the C=C double bond of CE: YqjM from *Bacillus subtilis* and *Zymomonas* NCR-reductase converted 85% and 7%, respectively (Stueckler et al. 2010).

Unsaturated aldehydes are usually good substrates for ERs, as demonstrated by the high reactivity of ERs from the yeast *Kluyveromyces lactis* and the bacteria *Pseudomonas putida* and *Yersinia bercovieri* (Chaparro-Riggers et al. 2007). However, contrasting data have been also reported: among 17 bacteria, 20 yeasts and 9 filamentous fungi, only the bacterium *Zymomonas mobilis* reduced the aldehyde citral (Muller et al. 2006). Considering that only *S. racemosum* MUT 2770 did not react toward MCA, the reduction of aldehydes seemed to be a widely spread reaction among filamentous fungi. The highest activity was observed for the two zygomycetes belonging to *Mucor* genus. The unique features of the enzymatic pattern of *M. plumbeus* MUT 2769 were demonstrated by the comparison with literature data, where the overall conversion of the aldehyde citral by *M. plumbeus* strain was instead negligible (Hall et al. 2006).

A common reaction profile could be observed. The transformation of MCA gave a mixture of unsaturated and saturated alcohol, indicating that a carbonyl reductase have already reduced the aldehyde to the corresponding alcohol. This pathway accords with previous observations with recombinant strains of *S. cerevisiae* (Romano et al. 2014) and filamentous fungi (Romagnolo et al. 2015), indicating the presence of a multi-step enzymatic cascade able to reduce both the carbonyl group and the C=C double bond.

Nitroalkenes reduction is a mandatory step in the synthesis of nitroalkanes for pharmaceuticals preparation and, so far, it has been performed by bacteria- and yeast-based methods. In the present study three out of five filamentous fungi achieved percentages of conversion above 80% of MNS, being in accordance with previous finding with whole-cell systems based on *Saccharomyces cerevisiae*, *E. coli* and *Clostridium sporongenes* (Fryszkowska et al. 2008, Jovanovic et al. 2014, Kawai et al. 2001). Since both the typology and the position of each substituent has found to deeply affect the stereoselectivity of bacteria, yeast and plant enzymes (Burda et al. 2013, Fryszkowska et al. 2008, Hall et al. 2008a, Reß et al. 2015), the tested analytes range should be extended also for filamentous fungi.

Since unsaturated esters are usually poorly converted by ERs, the complete reduction of MCI catalyzed by *M. circinelloides* MUT 44 and *S. racemosum* MUT 2770 arouses great interest. For instance, among the 23 recombinant ERs tested, only four were active on an ester molecule, with very low (9-11%) conversion yields (Reß et al. 2015). A putative ER of *Clavispora lusitaniae* converted only one up to three ester molecules (Ni et al., 2014).

Particular attention was given to the capability of *M. circinelloides* MUT 44 and *M. plumbeus* MUT 2769 to reduce non-conventional substrates, usually not reduced by OYEs from bacteria and yeasts (unpublished results). Besides, the development of a bio-based technology for cyano saturated compounds may lead to potential industrial outcomes, i.e. the production of neurological active compounds as non-natural amino acids (Brenna et al. 2013). However, accordingly to previous findings (Liu et al. 2012, Reß et al. 2015), cyano compounds (substrate **1**-**5**) showed a poor rate of conversion. Although substrates **1**-**3** share a similar basic scaffold, the tested fungi were not suitable for the reduction of substrate **1** and **2**, but a moderate transformation of substrate **3** occurred (i.e. 21% by *M. circinelloides* MUT 44). The absence of substituents on the aromatic ring of substrate **3** probably helped the reduction of the C=C bond.

Fungi were more active toward cyano esters (e.g. substrate **4**-**5**). Similarly, a cyano acid was moderately (e.g. 26% at maximal) converted by 6 up to 23 ERs, whereas the two tested cyano esters were reduced by most of the enzymes, five of which have obtained yields above 50% (Reß et al. 2015). Ten up to 12 cyano esters were transformed by *S. cerevisiae* and/or by OYEs (Brenna et al. 2013).

Besides, substitutions on the aromatic ring often affect positively or negatively enzymatic kinetics, due to their steric hindrance and electronic effects. For instance, even though substrates **4** and **5** differ only for a chlorine substitution on the aromatic ring, only **5** was extensively reduced. *S. racemosum* MUT 2770 was active only toward substrate **5** (53%) whereas *M. circinelloides* MUT 44 reduced 86% and 29% of **4** and **5**, respectively. It may be hypothesized that the presence of chlorine may have facilitated the reduction by the two fungi. The role of halogens must be carefully assessed and generalizations are hardly to make among chemical classes. For instance by acting as secondary EWG, the presence of a halogen may be critical for the reduction of esters. In other cases, being in α position of the C=C double bond, it fosters the reaction (Gatti et al. 2013).

Substrate **6** is a commercial product employed as a building block material for the synthesis of fragrances, i.e. musk odorants, which are usually produced *via* chemical synthesis (Liu et al. 2014, Nacsa et al. 2015). No evidences could be found about its transformation by biological methodologies, arousing great interest in the bioconversion rates of *M. circinelloides* MUT 44, *M. plumbeus* MUT 2769 and *P. citrinum* MUT 4862. Besides having reduced 69% of substrate **6**, *M. circinelloides* MUT 44 claims for further detailed studies to define its potential as a new biocatalyst for fragrance production.

Even though the intraspecific physiological variability is well-known in the fungal kingdom (Fontaine et al. 2015, Johnson et al. 2012), no studies have investigated the diffusion of ER activity among strains belonging to the same species. In the present study the intraspecific diversity among three strains of *M. circinelloides* was considerable, and each strain displayed a different substrate profile. *M. circinelloides* MUT 2223 was barely active only on the ketone, whereas the other two strains were highly versatile reaching high conversion rates (> 75%) for three up to four tested

compounds. Slight differences could be drawn according to the EWG: MUT 44 and MUT 724 poorly reduced ketone and ester, respectively.

Besides through a molecular approach, it was possible to verify the presence of genes putatively associated to ERs in the 7 strains studied. All of them showed a common sequence of 200 bp, putatively coding for an ER. These data are not conclusive, however, since the additional information about the presence of other homologs and their role is necessary. Fungal genomes have recently demonstrated to possess several ERs genes, whose activation strictly varied in accordance to the external stimuli: the *in silico* study of 60 genomes of filamentous fungi highlighted the presence of a high numbers of OYE homologs per genome (Nizam et al. 2014). Further *in vivo* analysis followed the expression of 6 OYE genes in *Ascochyta rabiei* during oxidative stress and after chickpea infection: the fungus responded differently, activating two expression profiles, in terms of genes and timing (Nizam et al. 2014).

In conclusion by evaluating the reduction of 10 unsaturated molecules, the great potential of *M. circinelloides* MUT 44 was revealed: it coupled high conversion yields of 7 analytes, fast reactions and high versatility, e.g. being active towards all the chemical classes tested.

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