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Scope and limitations of biocatalytic carbonyl reduction with white-rot fungi

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ABSTRACT

The reductive activity of various basidiomycetous fungi towards carbonyl compounds was screened on an analytical level. Some strains displayed high reductive activities toward aromatic carbonyls and aliphatic ketones. Utilizing growing whole-cell cultures of *Dichomitus albidofuscus,* the reactions were up-scaled to a preparative level in an aqueous system. The reactions showed excellent selectivities and gave the respective alcohols in high yields. Carboxylic acids were also reduced to aldehydes and alcohols under the same conditions. In particular, benzoic, vanillic, ferulic, and *p*-coumaric acid were reduced to benzyl alcohol, vanillin, dihydroconiferyl alcohol and 1-hydroxy-3-(4-hydroxyphenyl)propan, respectively.

1. Introduction

Hydride-containing agents traditionally employed for reducing carbonyl compounds currently raise increasing environmental concerns [1]. Alternative catalytic hydrogenations require heavy metal-based catalysts and often harsh reaction conditions [2]. High selectivities, mild reaction conditions and environmental safety attract the attention to biocatalytic reductions as alluring alternative to traditional organic synthesis, especially in the food, pharmaceutical and fine chemical industries [3–6]. Nicotinamide adenine dinucleotide (NAD(P)H) dependent dehydrogenases are the catalysts of choice for the reduction of carbonyls [7]. The best-investigated sources of reductive enzymes are yeasts and bacteria, while the reductive potential of basidiomycetous fungi was disclosed only on an analytical level and for a very limited number of cultures so far [8].

In the current study, we focused on enzymes produced by white-rot fungi which represent versatile natural catalysts due to their unique ability to degrade lignin, the most abundant aromatic biopolymer on earth [9–11]. Lignin is characterized by a highly complex three-dimensional structure based on *p*-coumaryl, coniferyl, and sinapyl moieties [11]. Its degradation into low-molecular weight aromatic compounds is a complex process catalysed by extracellular enzymes and followed by intracellular metabolic bioconversions [12]. Heme-thiolate peroxygenases such as chloroperoxygenases (CPO) [13] and unspecific peroxygenases (UPO) [14], class II heme-containing peroxidases, and various copper-containing oxidoreductases including laccases [15,16] are the most active components of the extracellular fungal secretome and have been studied extensively [9,17–21]. Lignolytic enzymes responsible for intracellular degradation of low-molecular weight fragments of lignin have been the subject of intense recent studies [12]. Numerous attempts to utilize basidiomycetous enzymes both in isolated form and as whole-cell biocatalysts for various types of oxidations have been thoroughly reviewed [14,15,22]. The first reduction catalysed by a basidiomycetous fungus, *Trametes versicolor,* has been described as early as in 1959 for the transformation of aromatic carboxylic acids to the corresponding aldehydes and alcohols [23,24]. Various fungi were screened for their ability to reduce carboxylic acids of different nature, namely fluorinated benzoic, isomeric anisic, and amine containing benzoic acid derivatives on an analytical level [8,25,26]. The use of isolated enzymes currently dominates in preparative enzymatic catalysis [27–30] due to the high selectivities and yields. However, costly purification processes, cofactor regeneration steps, presence of auxiliary enzymes as well as the low stability of many isolated enzymes represent significant drawbacks [30].

All these disadvantages may be overcome when whole-cell systems are utilized. They are also simpler in application and can serve as

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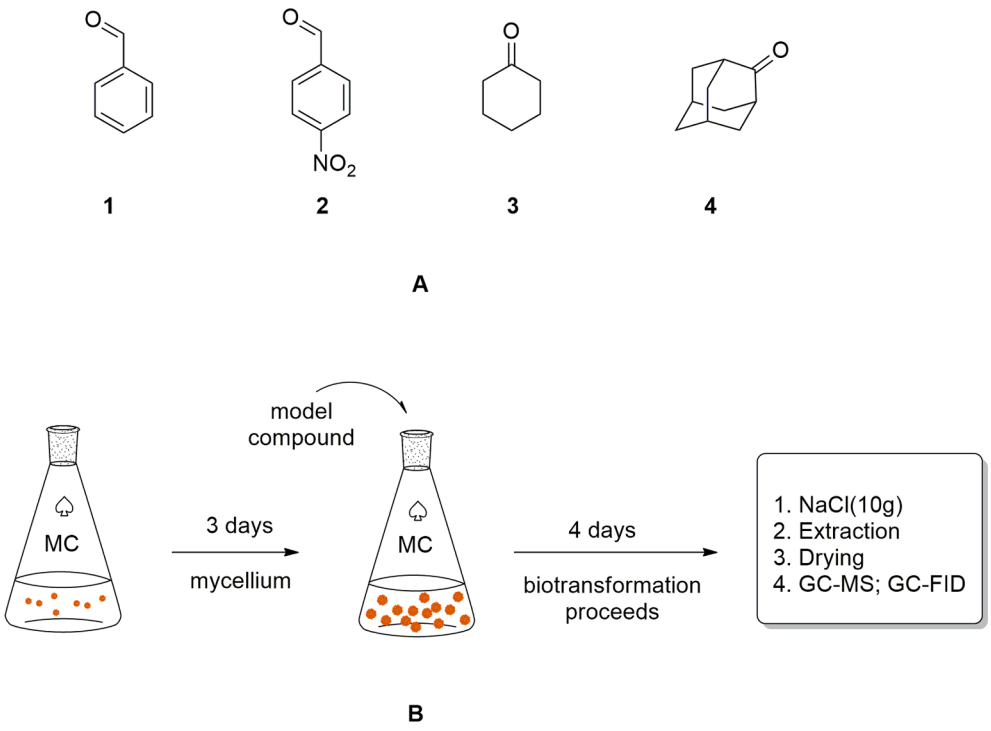


Fig. 1. Model compounds benzaldehyde (**1**), *p*-nitrobenzaldehyde (**2**), cyclohexanone (**3**), adamantanone (**4**) utilized for screening procedure (**A**) and the workflow of the screening (**B**, MC = main culture).

efficient "green" catalysts [31]. On the other hand, fungal secretomes consist of a complex mixture of enzymes of different nature that are able to catalyse a broad range of chemical transformations, and are thus not expected to be selective if whole-cell cultures are utilized. Nevertheless, examination of whole-cell transformations is typically the first step in revealing the catalytic properties of enzymes.

In this study, we reveal the reductive potential of whole-cell cultures of several white-rot fungi. In particular, *Dichomitus squalens* was chosen as this fungus has been shown to degrade chlorophenoxyacetic acids [32], and its carboxylate reductase was recently characterized [33]. Additionally, *Dichomitus albidofuscus*, which is able to oxidase aliphatic compounds [34], *Ischnoderma benzoinum* that transforms L-phenylalanine to benzaldehyde [35], and *Daedalea quercina* whose laccases are able to decolorize synthetic dyes efficiently [36] were selected for the present study. Whereas the chemistry of *Daedalea quercina* and *Marasmius cohortalis* remains largely undisclosed, fungi of the *Pleurotus* family, namely *P. sapidus*, *P. ostreatus*, and *P. cornucopiae,* display an enormously rich chemistry and various applications in biotechnology [20,37–39] and are also subject of our scrutiny.

2. Materials and methods

2.1. Chemicals

Benzaldehyde, benzoic acid, cyclohexanone, adamantanone, 3-chloro-4-methoxybenzaldehyde, *m*-methylbenzaldehyde, veratraldehyde, *m*-anisaldehyde, *m*-nitrobenzaldehyde, *p*-nitrobenzaldehyde, *p*-bromobenzaldehyde, 2-heptenal, dodecanal, undecanal, *m*-nitrobenzoic acid, *p*-nitrobenzoic acid, vanillic acid, cinnamonic acid, ferulic acid, *p*-coumaric acid (for synthesis), benzyl alcohol, cyclohexanol, 2-adamantanol, 3-chloro-4-methoxybenzyl alcohol, *m*-methylbenzyl alcohol, veratryl alcohol, *m*-anisyl alcohol, *m*-nitrobenzyl alcohol, *p*-nitrobenzyl alcohol, *p*-bromobenzyl alcohol, 1-heptanol, 1-dodecanol, 1-undecanol, vanillin, dihydroconiferyl alcohol, 1-hydroxy-3-phenylpropanol-1-ol, 1-hydroxy-3-(4-hydroxyphenyl)propan (as standards for compound identification) were purchased from Merck KGaA (Darmstadt, Germany).

2.2. Organisms

The stock culture of the fungi *Dichomitus albidofuscus* (CBS, 321.75), *Marasmius cohortalis* (DSMZ, 8257), *Pleurotus sapidus* (DSMZ, 8266), *Flammulina velutipes* (DSMZ, 1658), *Daedalea quercina* (DSMZ, 4953), *Ischnoderma benzoinum* (CBS, 231.97), *Pleurotus cornucopiae* (DSMZ, 5342), *Pleurotus ostreatus* (DSMZ, 1833), and *Dichomitus squalens* (DSMZ, 9615) were maintained on a solid medium containing 15 g·L^{-1} malt extract (Fluka, Neu-Ulm, Germany) and 15 g·L^{-1}Agar-Agar (Roth, Karlsruhe, Germany).

2.3. Submerged cultures

The culture medium was prepared by dissolving malt extract (30 g) in 1 L of deionised water. For preparation of the precultures, a 1 cm^2 agar plug from the leading mycelial edge was transferred into 100 mL medium (250 mL Erlenmeyer flask) and then homogenized with a T 25 digital Ultra-Turrax homogenizer (IKA, Staufen, Germany; 30 s, 10.000 r·min^{-1}). The precultures were grown on an incubation shaker (Orbitron, Infors HAT, Bottmingen, Switzerland; 150 r·min^{-1}, deflection 25 mm) under exclusion of light at 24 °C for 7 days. Afterwards, the precultures were homogenized, and 10% (v/v) of the homogenate was inoculated for submerged cultivation into 400 mL (1000 mL Erlenmeyer flask), 200 mL (500 mL Erlenmeyer flask) or 40 mL (100 mL Erlenmeyer flask) medium.

2.4. Screening procedure

The substrate (0.5 mmol) was added to submerged cultures of the respective fungus (40 mL) on the 3rd culture day. The reaction mixture was incubated on an incubation shaker at 150 r·min^{-1} (deflection 25 mm) under exclusion of light at 24 °C for 4 days. Afterwards, 5 g of NaCl was added to the medium, and the mixture was stirred for 10 min at 800 rpm (magnetic stirrer). For extraction 25 mL of Et_2O was added, and the

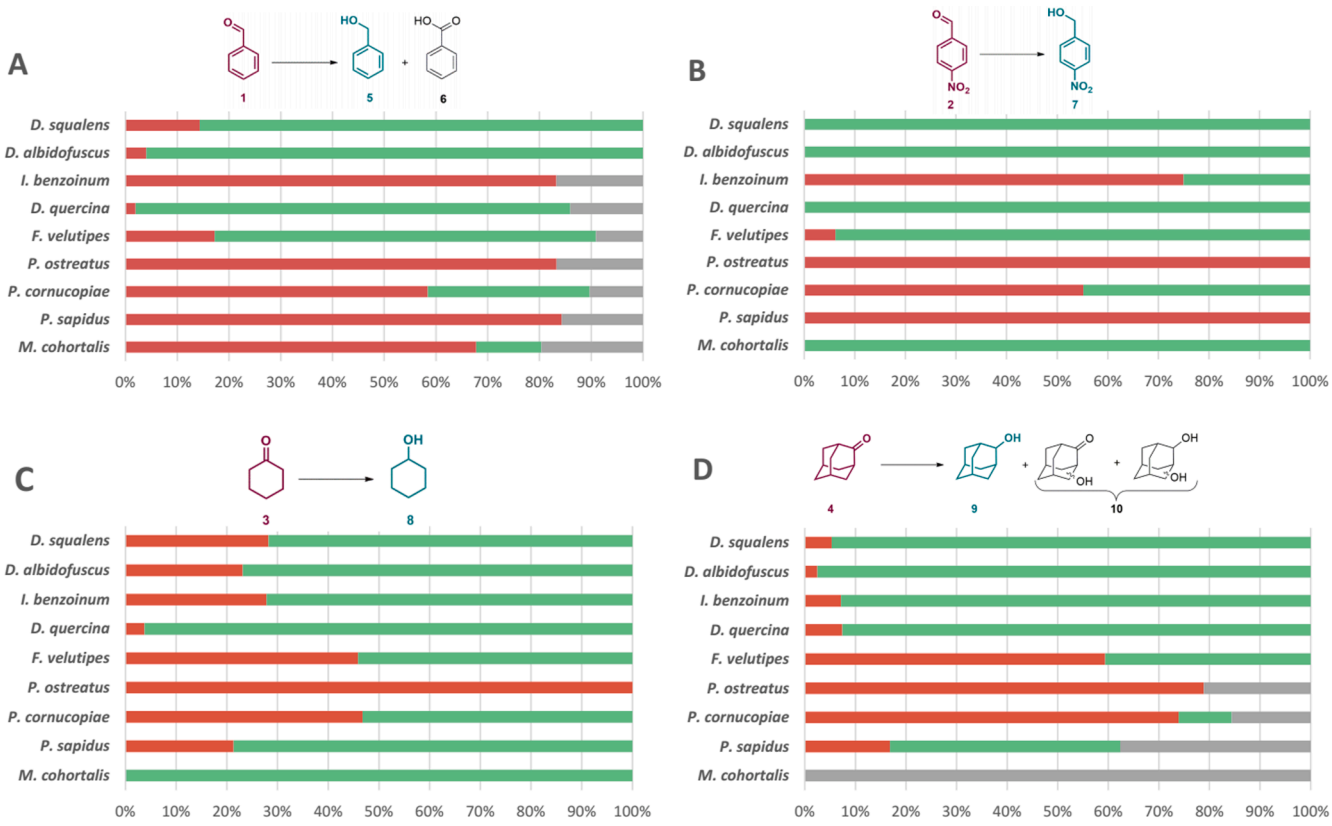


Fig. 2. Screening results for biotransformation of benzaldehyde (**1**, **A**), *p*-nitrobenzaldehyde (**2**, **B**), cyclohexanone (**3**, **C**), adamantanone (**4**, **D**) in the presence of various fungi.

resulting mixture was stirred for 20 min at 800 rpm (magnetic stirrer), and centrifuged for 5 min at 3000 × g to separate the organic layer. The extraction was repeated three times. The combined organic layers were washed with brine (1 × 30 mL) and water (1 × 30 mL), dried over Na_2SO_4 and evaporated to dryness. The resulting reaction mixtures were analysed by GC–MS (Agilent Technologies 7890A GC, column Agilent VF-WAXms (30 m × 0.25 mm, 0.25 μm) and Agilent 5975C MSD Triple-Axis mass spectrometer) and GC-FID (Agilent Technologies 7890A GC, 7683B Series Injector, column Agilent HP5 30 m × 0.2 mm, 0.25 μm). Every biotransformation was repeated three times to verify the reproducibility of the experiments. The detailed information about experimental data and yields is presented in the Electronic Supplementary Information.

2.5. General procedure for preparative biotransformation

The substrate (1 mmol) was added to submerged cultures of DAL on the 3rd culture day. The reaction mixture was incubated on an incubation shaker at 150 r·min^{-1} (deflection 25 mm) under exclusion of light at 24 °C for 4 days. 10 g of NaCl was added to media, and the mixture was stirred for 10 min at 800 rpm (magnetic stirrer). For extraction, 50 mL of Et_2O was added, and the resulting mixture was stirred for 20 min at 800 rpm (magnetic stirrer), and centrifuged for 5 min at 3000 × g to separate the organic layer. The extraction was repeated three times. The combined organic layers were washed with brine (1 × 30 mL) and water (1 × 30 mL), dried over Na_2SO_4 and evaporated to dryness. The resulting reaction mixtures were analysed by GC/MS and NMR spectroscopy. Products were purified by column chromatography on silica gel (eluent (pentane/ether) changed gradually: 10/1, 7/3, 1/1) to isolate the major components. The respective fractions were combined, concentrated in vacuum, and the ^1H and ^{13}C NMR spectra of the residuals were compared with those of reference compounds. Every biotransformation was repeated three times to verify the reproducibility of the experiments. The detailed information about experimental data and yields is provided in the Electronic Supplementary Information.

3. Results and discussion

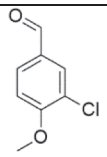
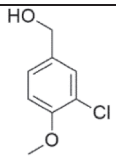
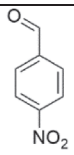
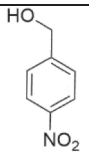
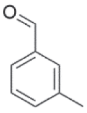
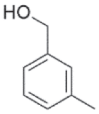
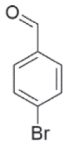
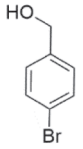
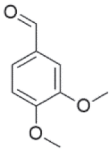
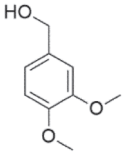
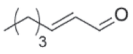
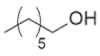
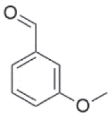
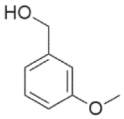
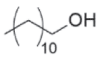
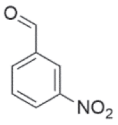
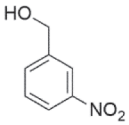
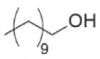
3.1. Screening of reductive activity of selected fungi

Several basidiomycetous fungi were screened for their potential to reduce benzaldehyde (**1**) and *p*-nitrobenzaldehyde (**2**) (Fig. 1, **A**) as substituted aromatics to a certain extent mimic the lignin fragments [40]. Compound **2** was chosen because of the electron-withdrawing nature of the nitro-group. As mentioned above, whole-cell cultures usually contain a complex mixture of enzymes including highly active oxidative species, especially laccases and peroxidases that are even able to oxidize non-functionalized aliphatic hydrocarbons [34]. Therefore, the aliphatic ketones cyclohexanone (**3**) and adamantanone (**4**) that may potentially be oxidized or reduced by the fungal enzymes were included in the screening. For the screening, the fungi were grown in submerged cultures for three days prior to the addition of the model compounds **1**–**4** (Fig. 1, **B**).

The results of the biotransformation experiments differed for each compound. (Fig. 2) For substrate **1**, benzylic alcohol (**5**) and benzoic acid (**6**) were found as products, while for substrate **2** only the reduced product **7** was detected. The aliphatic ketone **3** was solely reduced to the corresponding alcohol, while for compound **4** a complex mixture of CH-oxidation products **10** was detected apart from the reduction product **9**, especially with *M. cohortalis*.

The only fungus that did not show any reduction of **1** or **2** was *P. ostreatus*. All fungi, except for *P. sapidus* and *I. benzoinum* were able to reduce benzaldehyde (**1**) to benzyl alcohol (**2**) (Fig. 2, A). For **1** the reduction often competes with oxidation to benzoic acid (**6**), and only with *D. squalens* and *D. albidofuscus* solely the reduction product **5** was formed in high yields. No traces of *p*-nitrobenzoic acid as well as

Table 1
Preparative reduction of aldehydes with growing whole-cell cultures of *Dichomitus albidofuscus* (DAL).

Substrate	Product, preparative yield	Substrate	Product, preparative yield
 **11**	 **12**, 80%	 **2**	 **7**, 89%
 **13**	 **14**, 49%	 **21**	 **22**, 34%
 **15**	 **16**, 31%	 **23**	 **24**, 95%
 **17**	 **18**, 52%	 **25**	 **26**, 95%
 **19**	 **20**, 92%	 **27**	 **28**, 95%

derivatives with reduced nitro group have been detected in any reaction mixture. All fungi except *P. ostreatus* reduced cyclohexanone (**3**) efficiently to cyclohexanol (**8**) without any traces of oxidative products. Biotransformations with adamantanone (**4**) gave the most complex product spectrum. Apart from 2-hydroxyadamantane (**9**), hydroxyadamantanone and dihydroxyadamantane (**10**) were detected with fungi of the *Pleurotus* family. The reaction mixtures obtained from cultures of *M. cohortalis* contained only disubstituted derivatives of adamantane **10**.

Compared to other fungi, *D. albidofuscus* (DAL) demonstrated the highest reduction efficiency towards all of the tested model substrates (Fig. 2). Apart from that, only moderate amounts of biomass were

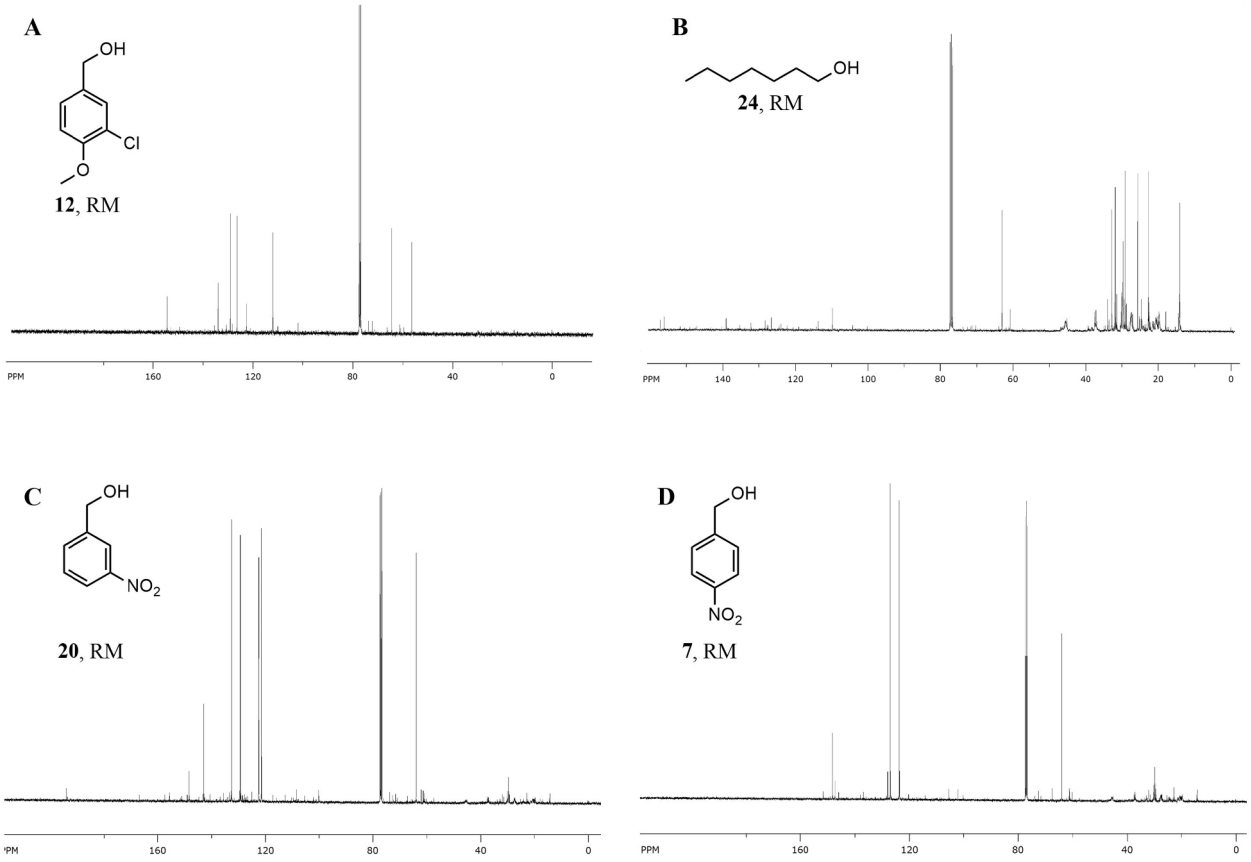


Fig. 3. ^{13}C NMR spectra of the extracts from the reaction mixtures (RM) of the reduction with the *D. albidofuscus* for 3-chloro-4-methoxybenaldehyde (**A**), 3-heptenal (**B**), *m*-nitrobenzoic acid (**C**) and *p*-nitrobenzoic acid (**D**). All resonances correspond to the signals of inset structures.

formed during the culture period which simplified the workup procedure. This fungus was thus selected for preparative experiments on the reduction of various classes of carbonyl compounds.

3.2. Reduction of aldehydes with D. albidofuscus

For studying the scope and limitations of the reduction with DAL, a number of substituted aromatic and aliphatic aldehydes (Table 1) were supplemented to its submerged cultures on the 3rd culture day. All reactions were performed with 1 mmol of substrates, and the crude extracts were analysed by GC–MS in combination with ^{1}H- and ^{13}C NMR spectroscopy in order to estimate the actual amount of non-volatile impurities. The products were than isolated from the reaction mixtures by column chromatography and compared to authentic reference compounds. The whole-cell cultures reduced 3-chloro-4-methoxybenzaldehyde (**11**) to the corresponding alcohol **12** with a preparative yield of about 80% (Table 1). Comparable observations were performed with *m*-methylbenzaldehyde (**13**), and the yield of the corresponding alcohol **14** was ca. 50%. Veratraldehyde (**15**) gave the alcohol **16** (31%), and *m*-anisaldehyde (**17**) gave 49% of the corresponding alcohol **18**. The moderate yields here could be attributed to the fact that alcohols **16** and **18** can serve as a substrates for extracellular aryl-alcohol oxidases that reduce oxygen to hydrogen peroxide [41]. The carbonyl groups of the isomeric nitrobenzaldehydes **19** and **2** were reduced to the corresponding alcohols **20** and **7** after 3 days almost quantitatively.

p-Bromobenzaldehyde (**21**) was transformed to alcohol **22** with moderate yields. We also tested the reduction of α-β-unsaturated aldehyde, namely 2-heptenal (**23**), that was transformed into the corresponding saturated alcohol **24** with high yields. The enzymatic reduction of activated double bonds has been described for the old yellow enzyme family of flavoprotein oxidoreductases [42], where the carbonyl group is maintained and only reduction of the C=C double bond is observed. The linear saturated aldehydes **25** and **27** dodecanal and undecanal were readily reduced to the corresponding alcohols **26** and **28** with high yields by DAL.

In many cases, the selectivity of the whole-cell cultures of DAL reductions was outstanding. We conclude this based on the NMR-analyses of crude extracts from the reaction mixtures where the target products clearly dominated as deduced from the spectral data of typical crude reaction mixtures (Fig. 3). The main resonances of the ^{13}C NMR spectra of the reaction mixtures of reduction of 3-chloro-4-methoxybenzaldehyde (**11**, Fig. 3**A**) and 2-heptenal (**23**, Fig. 3**B**) agreed well with the

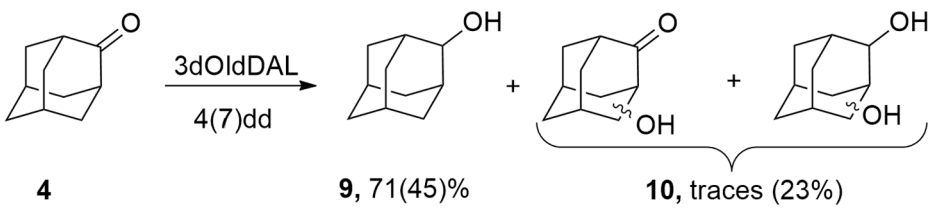


Fig. 4. Preparative biotransformation of adamantanone (**4**) by *D. albidofuscus*.

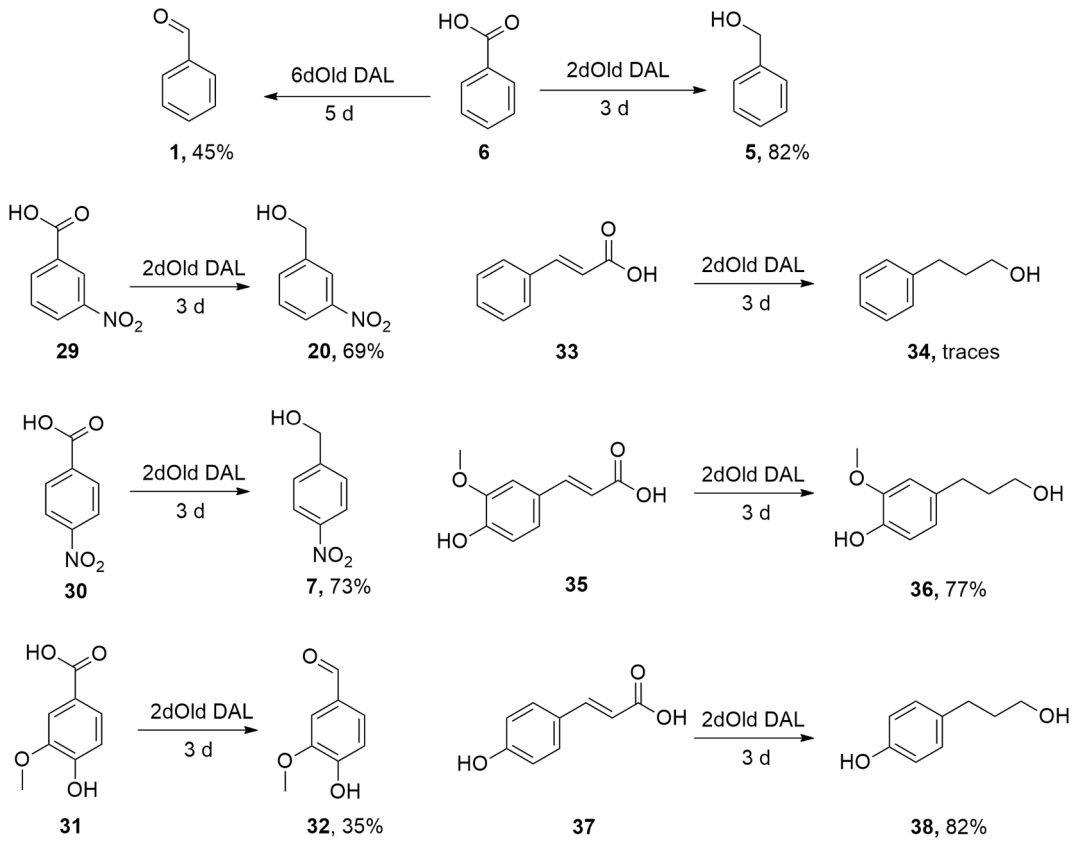


Fig. 5. Reduction of carboxylic acids by growing whole-cell cultures of *D. albidofuscus*.

spectral data of the corresponding standard samples. Despite the whole-cell culture obviously produced a large set of enzymes of different nature, the preparative reductions of aldehydes by DAL are highly selective and only trace amounts of oxidation products, namely the corresponding carboxylic acids, have been found in the reaction mixtures.

3.3. Reduction of adamantanone with D. albidofuscus

The biotransformation of admantanone (**4**) with DAL gave the alcohol **9** with an isolated yield of 71%. (Fig. 4) Traces of disubstituted derivatives of adamanatane which accumulated at prolonged reaction times were detected in the reaction mixture.

To localize the enzymes responsible for the reductions, biotransformation experiments were performed separately with supernatants and mycelia of 4 days old cultures. While no traces of alcohol **9** were found in the reaction mixtures containing culture supernatants, 22% of adamantanol (**9**) accumulated after 24 h when the reaction was performed with mycelium. The responsible enzymes are thus likely located intracellularly.

3.4. Reduction of carboxylic acids with D. albidofuscus

The reduction of carboxylic acids to the respective alcohols usually requires either prolonged heating with large amounts of hydride reagents [43–45] or involvement of heavy-metal-based catalysts [46]. With DAL, benzoic acid (**6**) was readily reduced to benzyl alcohol (**5**) during 3 days with 82% yield when **6** was added on the 2nd culture day (Fig. 5). In contrast, when **6** was added on the 6th culture day, only **1** was found in the reaction mixture and isolated in 45% preparative yield. It should be noted that this approach gives halogen-free benzaldehyde (**1**) which is essential for applications in the pharmaceutical and food industry [47].

m-(**29**) and *p*- (**30**)Nitrobenzoic acids were readily reduced to the corresponding alcohols **12** and **7** with high yields after addition of the substrate on the 2nd culture day (Fig. 3, **C** and **D**). While the carboxylic acid and (intermediate) aldehyde functions were reduced, the nitro group remained unchanged. Vanillic acid (**31**) was reduced under the same conditions to vanillin (**32**) with 35% preparative yield.

DAL cultures were also employed for the reduction of cinnamic acid (**33**) and its substituted derivatives containing coniferyl and *p*-coumaryl moieties. During three days, the double bonds and carboxyl groups of acids **35** and **37** were reduced giving dihydroconiferyl alcohol (**36**) and 1-hydroxy-3-(4-hydroxyphenyl)propan (**38**), respectively (Fig. 5). Only traces of alcohol **34** were found in the reaction mixture of transformation of cinnamic acid (**33**) under the same conditions.

Summarizing the results obtained for acids, we conclude that growing whole-cell cultures of DAL may be efficiently employed for reductive processes. Moreover, the fungus is able to reduce aromatic acid derivatives to aldehydes and alcohols with satisfactory preparative yields.

4. Conclusions

Basidiomycetous fungi are able to selectively reduce carbonyl groups not only of aromatic compounds but also of saturated ketones and aldehydes. Growing cultures of *D. albidofuscus* display remarkable selectivities in preparative “on water” reductions of aromatic carbonyl compounds with high yields at room temperature. The fact that this fungus is able to reduce benzoic acids to alcohols with high preparative yields is extraordinary as such transformations usually require either hazardous organic reagents or harsh reaction conditions in organic chemistry. Some functional groups resist the reaction conditions which may be useful for the reduction of polyfunctional aromatics. Detailed mechanistic studies as well as isolation of responsible intermediates in whole-cell culture of *D. albidofuscus* chemistry are currently underway.

Declaration of Competing Interest

The authors declare that they have no known competing financial interest or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bioorg.2021.104651.

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