Aerobic C–C Bond Cleavage Catalyzed by Whole-Cell Cultures of the White-Rot Fungus *Dichomitus albidofuscus*

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Whole-cell cultures of the basidiomycetous white-rot fungus *Dichomitus albidofuscus* exhibit varying catalytic activity towards aromatic compounds depending on the growth stage. This study reveals the catalytic behavior of mature whole-cell cultures that effectively catalyze a C–C bond cleavage oxidizing toluene, benzaldehyde and acetophenone to phenol. The reaction products were analyzed by GC-MS and NMR techniques. To exclude the *de novo* formation of phenol by the fungus, its origin has been proven by bioconversion of benzaldehyde-d_5. The key step involves an aerobic Baeyer-Villiger type rearrangement where the incorporation of oxygen into the product was confirmed based on isotope labelling experiments with $^{18}O_2$. Intermediate esters were not found in reaction mixture presumably due to the detected esterase activity in the mycelium as well as in supernatant of the whole-cell cultures. As a result, the sequence of biocatalytic reactions catalyzed by *D. albidofuscus* for the degradation of toluene via C–C bond cleavage has been disclosed.

The development of "green", environmentally friendly[1] approaches for selective oxidations in organic chemistry remains challenging.[2] In particular, the selective cleavage of single C–C bonds represents a key organic transformation both in the lab and in industry. The C–C bond cleavage typically requires either harsh reaction conditions or aggressive reagents, produces a complex mixture of low-molecular weight products and is often accompanied by deep overoxidations.[3] Remarkably, some biological systems are able to degrade organic material under very mild reaction conditions, where the C–C bond cleavages represent the key reaction steps. In particular, relatively stable extracellular enzymes produced by fungi are able to degrade robust biopolymers including lignin, cellulose,[4] chitin,[5] melanin,[6] and keratin.[7] The fungal extracellular secretome plays a main role in lignin degradation[8] and consists of a complex mixture of enzymes of different nature including heme–thiolate peroxygenases such as chloroperoxygenases (CPO) and unspecific peroxygenases (UPO), class II heme-containing peroxidases, and various copper-containing oxidoreductases including laccases.[9] Another type of enzymes involved in lignin degradation, namely the peroxidase-independent cytochrome P450 monooxygenases,[10] have enormous potential for oxidations as alternatives to traditional chemical approaches. There are numerous studies on the degradation of toxic polycyclic aromatic hydrocarbons (PAHs) by white-rot fungi.[11] PAHs are oxidized *in vitro* by laccases in the presence of a mediator generating a large quantity of quinones and polymers with low molecular masses.[12] Concerning saturated hydrocarbons, *P. strigosozonata* is able to degrade *n*-alkanes containing up to 10 carbon atoms.[13]

Despite the broad number of studies on the degradation of pollutants in various biological systems, the application of white-rot fungi in synthetic chemistry remains challenging as the knowledge on such preparative/selective transformations is still limited. Fungi may be promising active players in the field of C–C bond cleavage at the preparative level; however the lack of information on mechanisms of action, as well as of precise experiments with model substrates hampers their further utilization in organic synthesis.

Recently, we discovered the ability of the white-rot fungus *D. albidofuscus* (DAL) to oxidize adamantane to adamantanol with moderate preparative yields.[14] In the present study, we focused on the oxidation of substituted aromatics and performed labeling studies to get insights into the reaction mechanism. Toluene (**1**) represents the simplest model compound possessing aromatic and aliphatic carbon atoms. In addition to C–C bond cleavage, it allows for evaluating the reactivity of two types of C–H bonds under oxidative conditions: While the oxidation of aromatic C–H bonds gives isomeric cresols, the oxidation of the methyl group may lead to benzoic acid. Additionally, we focused on the reactivity of the simplest toluene oxidation products, namely benzyl alcohol and benzaldehyde, whose behavior helps to understand the mech-

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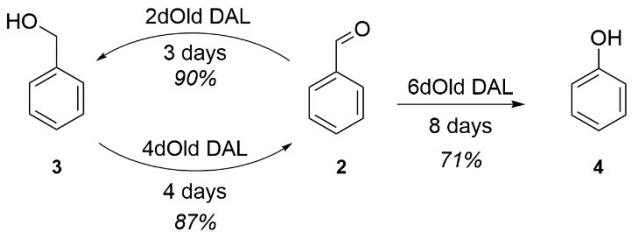
anism of the observed biotransformations. The energy of the C–H bonds in the methyl group of **1** (89 kcal/mol)[15] is lower than that of the 3°C–H bond of adamantane (98.5 kcal/mol), which reacts smoothly in presence of DAL[16] and we thus expected to observe the oxidation of the methyl group of **1**. However, **1** inhibits the growth of the fungus due to its cytotoxic effect, and no oxidation products were formed even after 8 days of cultivation. We therefore added **1** to the growing culture on the 3rd day and obtained a mixture of oxidation products including benzaldehyde (**2**), benzyl alcohol (**3**), phenol (**4**), and isomeric cresols after 3 days (Scheme 1). Thus, growing cultures of DAL display the ability to oxidize both aliphatic (**2** and **3**) and aromatic (**5**) C–H bonds of **1**. While the formation of **2** and **3**[17] and even **5**[18] is not unusual and was observed in the enzymatic oxidation of **1** in several studies, the formation of substantial amounts of the C–C bond cleavage product **4** was surprising.

After prolonged reaction times (8 days) only **4** and **5** have been found in the reaction mixtures. The above results on the oxidation of **1** by growing cultures of DAL show its complex behavior, which is not surprising for such aggressive species as white-rot fungi that produce a large set of enzymes of different nature.[19] To the best of our knowledge, the direct oxidative demethylation of toluene to phenol has not been described before in biological systems, and we performed a number of model experiments targeting to understand the mechanism of C–C bond cleavage of **1** in the presence of DAL. Taking into account the above results on deep oxidation of **1** we next attempted the oxidation of aldehyde **2** and alcohol **3** by whole-cell cultures of DAL. To control the mass balances in addition to the GC-MS analyses, the transformations were performed at the preparative level with 1 mmol of substrates. The reaction mixtures were separated by column chromatography and the structures of individual components were confirmed by ^{1}H- and ^{13}C-NMR spectroscopy. Surprisingly, a varying set of products was observed, depending on the time when benzaldehyde (**2**) was added to the growing culture (Table 1).

When **2** was added on the 2nd or 4th culture days, the main product was **3** (entry 1 and 2). The reduction of carbonyl groups has already been shown by us for a number of white-rot fungi including DAL.[20] However, the addition of **2** to the mature cultures on the 6th day resulted in **4** as main product (entries 3 and 4). During three days no biotransformation was observed if **3** was added on the 2nd culture day (entry 5). However, supplementation on the 4th as well as on 6th culture growth day resulted in the formation of **2**, followed by oxidation to **4** (entries 6 and 7). We thus conclude that depending on the age of the growing DAL, it displays either reductive or oxidative properties, *i.e.*, at the early stages of growth we observed reduction, whereas at later stages the oxidation takes place. Remarkably, instead of common enzymatic oxidations of **2** to benzoic acid[21] the deep oxidative cleavage to **4** is observed in high preparative yields, and we studied this transformation in more detail.

Table 1. Biotransformation of benzaldehyde (**2**) and benzyl alcohol (**3**) with whole-cell cultures of DAL (all yields isolated)

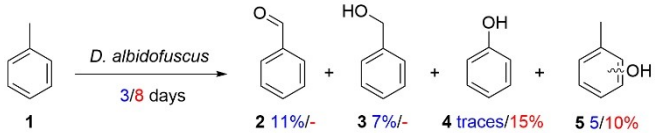


#	Starting compound	Biotransformation conditions (age of culture, d/duration of reaction, d)	Products (isolated) (yields)
1	**2**	2/3	**3** (90%)
2	**2**	4/4	**3** (75%) **4** (traces)[a]
3	**2**	6/4	**4** (45%)
4	**2**	6/8	**4** (71%)
5	**3**	2/3	**3** (100%)
6	**3**	4/4	**2** (87%), **4** (traces)[a]
7	**2**	6/8	**4** (71%)

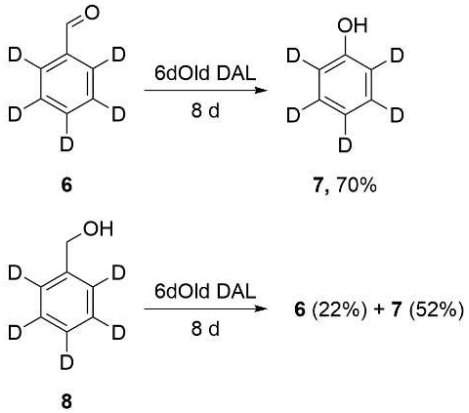
[a] Identified based on GC-MS data.

The experiments were reproduced with deuterated isotopologues of **2** and **3** (PhCHO-d_5 (**6**) and $PhCH_2OH$-d_5 (**8**)), to exclude the *de novo* formation of the products by the fungus (Scheme 2). Blank experiments, including the addition of **2** or **3** to the culture media without inoculation as well as to the whole-cell cultures after inactivation by heating, confirmed the formation of the products exclusively by the growing whole-cell cultures of DAL.

Thus, three reaction modes were identified for whole-cell cultures of DAL. The reduction of the carbonyl group of **2**



Scheme 1. Biotransformation of toluene (**1**) by whole-cell cultures of DAL.

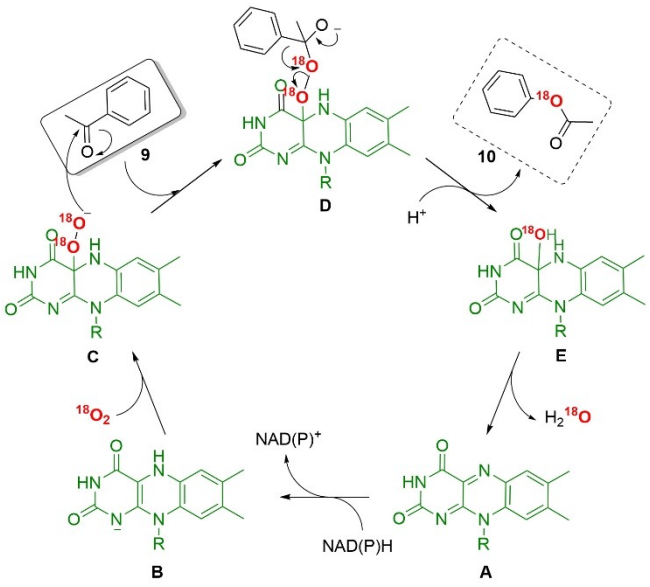


Scheme 2. Biotransformation of deuterated analogues of benzaldehyde (**6**) and benzyl alcohol (**8**) by whole-cell cultures of DAL.

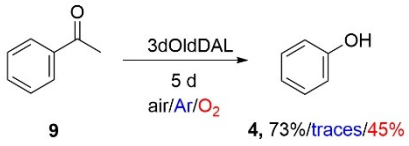
proceeds during the initial stage of growth and gives alcohol **3**, which is then oxidized back and further decarbonylated at the final stage. It should be noted that traces of **3** were present in the reaction mixture during the first four days after addition of **2** to 6 day-old cultures. Moreover, the addition of **3** to the 6 day old cultures gave **4** with the same yield. We conclude that the oxidation of **1** to phenol (**4**) takes place through the intermediate formation of aldehyde **2**, which than undergoes an oxidative cleavage at the final step. The latter transformation may be attributed to a Baeyer-Villiger oxidation that usually requires the utilization of peroxides in combination with strong acids or bases.[22] The enzymatic versions of this transformation are associated with Baeyer-Villiger monooxygenases (BVMOs).[23] Dependent on NAD(P)H BVMOs use the free, abundant, and green oxidant O_2 and only generate water as a by-product. While these flavoenzymes have been identified in a variety of bacteria[24] and ascomycetous fungi,[25] only little is known about BVMOs of basidiomycetous origin. For example, analytical screening displays that several strains of basidiomycetes are able to oxidize bicyclo[3.2.0]hept-2-en-5-one to the respective lactones.[26] Taking into account that the Baeyer-Villiger reaction is possibly responsible for the aerobic decarbonylation of **2**, we tested the fate of acetophenone (**9**) in our system, as the participation of **9** in such types of transformations is well documented.[27] In full agreement with the results reported above, phenol (**4**) was formed from acetophenone (**9**) with mature growing cultures of DAL (Scheme 3). Only traces of alcohol (product of reduction) have been detected in the reaction mixture. As the reaction proceeds more selectively and gives only phenol without any products of further oxidation, we chose ketone **9** as a model compound for kinetic and isotope labelling experiments. The reaction conditions have been optimized and the best results were obtained when the substrate was added to 3 days old growing cultures followed by 5 days of aerobic cultivation. In order to prove the true aerobic nature of the reaction we first performed the transformation of **9** under the argon atmosphere and found unreacted starting compound and only trace amounts of **4** in the reaction mixture.

To proof the participation of molecular oxygen in the observed oxidative decarbonylation, we performed the reaction under $^{18}O_2$ atmosphere. As the fungi are normally grown under air atmosphere, their behavior under the atmosphere of 100% oxygen was examined first. Therefore, we firstly disclosed the activity of DAL under pure oxygen. Despite the reaction occurs slightly faster than under air, the total yield of **4** was only 45%, but it was enough for detecting the distribution of the isotope label in the product. We found that the reaction under the $^{18}O_2$ atmosphere resulted exclusively in the formation of ^{18}O-labelled **4** that is evidenced by a characteristic m/z = 96 peak in the GC-MS spectrum of the reaction mixture. Only trace amounts of ^{16}O-phenol (m/z = 94) were identified. This strongly supports the participation of BVMO in the process, as the incorporation of $^{18}O_2$ into the phenol structure is in a full agreement with the mechanism suggested for BVMO[23c] (Scheme 4) which contains flavin adenine dinucleotide (**A**) in its active site. Formed after reduction by NAD(P)H, **B** it is able to react with molecular oxygen to give a peroxyflavin structure **C**, which forms a Criegee intermediate **D** with a carbonyl substrate **9**. The Baeyer-Villiger type rearrangement of **D** eliminates **10** and forms hydroxylated form **E**. After the rearrangement **E** spontaneously removes water to form the resting state of BVMO (**A**). However, we were not able to identify **10** in the reaction mixture, presumably due its fast hydrolysis in the reaction media.

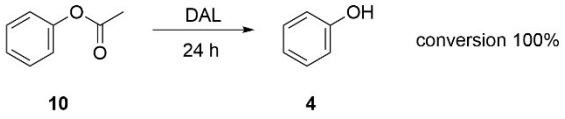
As the hydrolytic activity of fungal cultures is well known, we performed a model study on the esterase activity of growing DAL cultures. The supplementation of growing culture with phenylacetate (**10**) gives only phenol after 24 h without any traces of the initially supplemented compound (Scheme 5). The esterase activity has been measured for supernatants as well as for intracellular crude extracts (Figure 1) from the first day of culture growth. It is inhibited after supplementation of the growing culture with **9** on the 3rd day, however not completely.



Scheme 4. The postulated mechanism of BVMO action adapted to labelled oxygen-18 experiment with acetophenone (**9**).



Scheme 3. Biotransformation of acetophenone (**9**) by mature whole-cell cultures of DAL.



Scheme 5. Biotransformation of phenylacetate (**10**) by whole-cell culture of DAL.

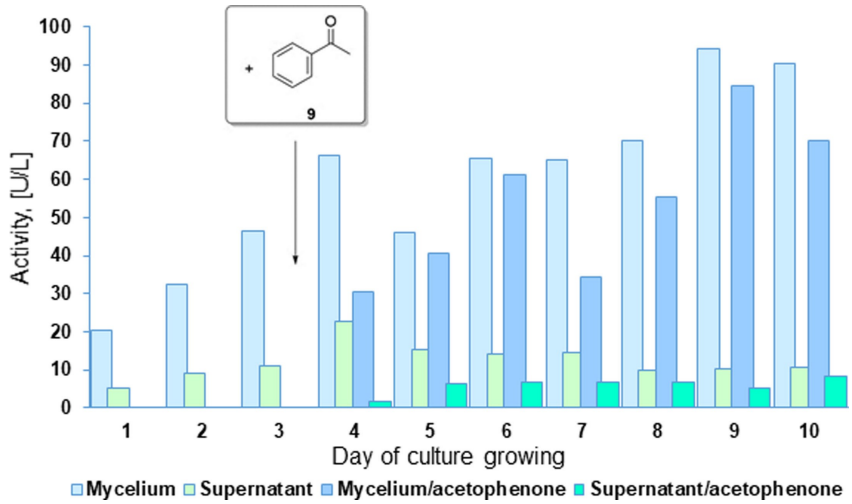
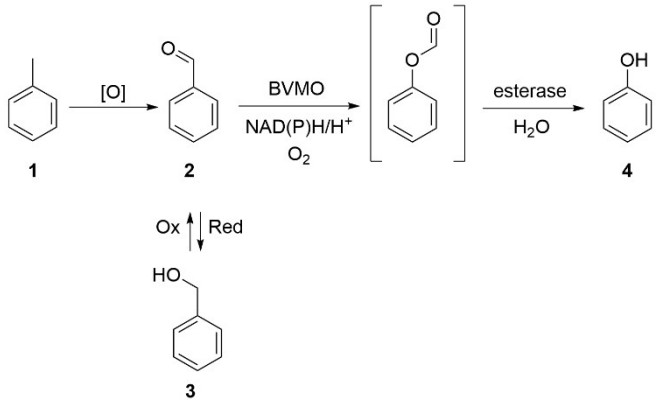


Figure 1. Esterase activity of mycelium and supernatant with and without acetophenone (**9**) supplementation.

The activity increases from the 4th to 6th day, then decreases due to phenol (**4**) formation, and then increases again.

Based on the above findings we assume that the formation of phenol includes Baeyer-Villiger type rearrangement, followed by hydrolysis as **10** formed after rearrangement is hydrolyzed quickly to **4** by esterases that are present in the reaction media. Thus, the whole sequence of the oxidation of toluene (**1**) to **4** involves the formation of benzaldehyde (**2**) that can be partially reduced to benzylic alcohol (**3**) (Scheme 6). In turn, **3** readily converts back to **2** with mature cultures.

We conclude that the whole-cell growing cultures of the white-rot fungus *Dichomitus albidofuscus* display reductive activity towards aromatic compounds at the early stages of growth, while mature cultures effectively oxidize organic substrates of different nature. The oxidation of benzaldehyde (**2**) as well as of acetophenone (**9**) to phenol (**4**) represents an effective aerobic C–C bond cleavage reaction that occurs under mild conditions and likely involves a Baeyer-Villiger type rearrangement at the penultimate step. High esterase activity of the reaction media is responsible for the formation of phenol from intermediate esters. Besides the preparative importance of such transformation for bioorganic synthesis, the results may be useful for better understanding of the mechanisms of biodegradation of organic compounds in presence of aggressive fungal species, where the sequences of reductive, oxidative, and hydrolytic reactions may take place as we have shown herein.



Scheme 6. Stepwise biotransformation of toluene (**1**) to phenol (**4**) by whole-cell cultures of DAL.

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Conflict of Interest

The authors declare no conflict of interest.

Keywords: benzaldehyde · biocatalysis · oxidation · oxidative decarbonylation · white-rot fungi

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