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Biocatalytic Cleavage of Alkenes with O_2 and *Trametes hirsuta* G FCC 047

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Alkenes possessing a C=C double bond adjacent to an aromatic ring were cleaved to yield the corresponding carbonyl compounds by use of molecular oxygen as the sole oxidant and a cell-free extract of the wood-degrading fungus *Trametes hirsuta* FCC 047 as catalyst. The oxygen pressure required was optimized. Special adapted equipment allowed 96 reactions to be performed in parallel under controlled oxygen pressure. A broad spectrum of aryl-alkenes was successfully converted into the corresponding ketones/aldehydes with excellent chemoselectivity under a controlled oxygen atmosphere (2 bar).

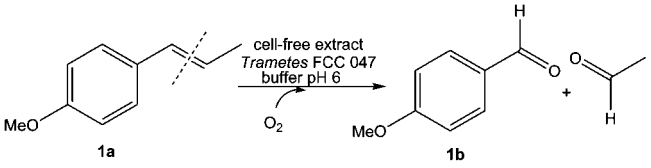
Introduction

Ketones or aldehydes are accessible by many synthetic pathways, either as intermediates or even as final products. Alkenes are possible precursors for aldehydes and ketones, and alkene cleavage to produce the corresponding carbonyl groups is consequently a widely investigated reaction in organic chemistry.[1–3] In this context ozonization is one of the most widely employed processes. However, safety hazards (in particular on large scales) and the need both for special equipment and for reducing reagents in molar amounts (Zn/AcOH or Me_2S) complicate this reaction.

Nowadays the development of metal-independent "green" oxidation methods represents a major challenge in organic synthesis, but only a limited number of papers in the field of biocatalytic alkene cleavage have been published. Laccases[4] and peroxidases, for instance, cleaved alkenes as a side reaction in the presence of hydrogen peroxide (chloroperoxidase,[5] horseradish peroxidase,[6] myeloperoxidase or *Coprinus cinereus* peroxidase[7]). Oxygenases transformed a very narrow substrate spectrum (tryptophan 2,3-dioxygenase,[8] indole 2,3-dioxygenase,[9] protocatechuate 3,4-dioxygenase,[10] ligno stilbene-α,β-dioxygenase,[11–13] β,β-carotene 15,15′-monooxygenase,[14] carotenoid cleavage dioxygenase AtCCD1[15] and β-diketone dioxygenase[16]). We recently reported biocatalytic alkene cleavage with hydrogen peroxide as source for molecular oxygen and an enzyme preparation obtained from the wood-degrading fungus *Trametes* species FCC 047. The reaction was performed under physiological reaction conditions – room temperature and aqueous buffer.[17,18] Here we describe further developments of this biotransformation to perform the reaction under constant controlled O_2 pressure, together with further optimizations of the reaction conditions. Finally we present an extended substrate spectrum and improved conversions.

Results and Discussion

trans-Anethole (**1a**) was shown to be the substrate of choice for the biocatalytic alkene cleavage, affording *p*-anisaldehyde (**1b**) as sole product (Scheme 1).[17,18] We therefore chose this compound for optimization of the reaction conditions and to test special equipment for controlling the O_2 pressure. The starting material concentration was 6 g L^{-1}, to ensure preparative significance for a biocatalytic transformation.



Scheme 1. Biotransformation of *trans*-anethole (**1a**) into *p*-anisaldehyde (**1b**).

Pressure Equipment

Two aluminium plates (15 × 15 × 1 cm) were connected with four screws (see Supporting Information for an image), holding a Plexiglas® cylinder (internal diameter: 10 cm, external diameter: 11 cm, length: 27 cm) in between. The apparatus was equipped with one manometer to control the pressure and two O_2-tolerant valves. To perform the reaction, the equipment was connected to an oxygen bottle and

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placed on an orbital shaker. The reaction vessels ("riplate LV" 5-mL deep-well plate) allowed screening of a total of 96 samples at the same time. The riplate LV could not be used for volatile substrates such as 2-methyl-1-phenylprop-1-ene (**4a**), styrene (**5a**), 4-methylstyrene (**6a**), or 5-*o*-tolylpent-2-ene. For these substrates 4-mL glass vials with septa were used, holes being punched through the septa to permit oxygen exchange. The 4-mL vials were placed on a wooden plate and introduced into the Plexiglas® cylinder. To our delight, quantitative conversion of *trans*-anethole (**1a**) was achieved both with a "riplate LV" 5-mL deep-well plate and with 4-mL glass vials under oxygen pressure.

O_2 Pressure

With the equipment described above we could adjust the O_2 pressure to a desired value, and so the biotransformation with *trans*-anethole (**1a**) as substrate was performed at 1, 2, 3, 4, and 6 bar. The best pressure value turned out to be 2 bar (Figure 1). Without biocatalyst no conversion was observed in all instances.

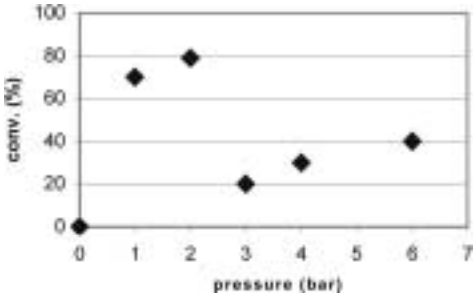


Figure 1. Alkene cleavage at varied O_2 pressure.

Screening of Bacterial Strains

Being interested whether bacteria could also perform alkene cleavage under similar conditions, we tested 128 different strains for the transformation of *trans*-anethole (**1a**) at room temperature (25 °C) and 2 bar O_2 pressure, varying the pH between 4 and 7. Unfortunately, no hit was found (for a list of tested bacteria see the Supporting Information).

Organic Solvents

One of the usual disadvantages of biocatalysis in aqueous solution is bad solubility of the organic substrates to be converted. The use of co-solvents can improve the solubility and therefore lead to more effective transformations. Acetonitrile is a frequently employed organic solvent in biotransformations.[19,20] We studied the influence of acetonitrile, propan-1-ol, and butan-1-ol on the conversion of *trans*-anethole (**1a**) at a 15% (v/v) organic solvent concentration.[18] Comparison of the results with the degrees of conversion achieved without organic solvent (72%) showed that butan-1-ol caused a significant decrease in conversion (43%). On the other hand, the enzyme kept still good activity in the presence of acetonitrile (61% conversion). Propan-1-ol turned out to be the only co-solvent showing a positive effect (82% conversion).

Time Study

Monitoring of the reaction course with a substrate concentration of 6 $g L^{-1}$ and ca. 1 mg of crude protein showed that complete conversion was achieved after 25 hours (Figure 2).

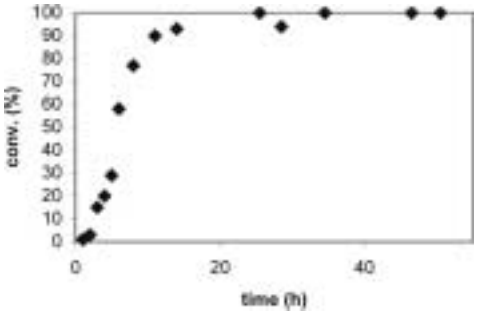


Figure 2. Time course of alkene cleavage.

Influence of Light

The mechanism of this alkene cleavage was previously unknown. Experiments to isolate the involved enzyme were unsuccessful, so no characterization of the active center was possible, nor any proposal for a suitable mechanism based on an enzyme structure. This notwithstanding, O_2 activation obviously plays a fundamental role in this mechanism. Of course, the enzyme is responsible for this activation to a certain extent, but light could contribute in part to oxygen activation by increasing the proportion of singlet oxygen. Therefore, if singlet oxygen were needed at the starting point of the mechanism, it might be expected that the reaction should be faster in the presence of light. To our surprise, however, the results showed just the opposite effect, with the reaction rate of the transformation of *trans*-anethole (**1a**) decreasing on exposure of the reaction mixture to light. When the reaction was stopped after 5 hours, 29% conversion was obtained for the illuminated sample but 44% conversion in darkness, indicating that light even seemed to inhibit the reaction.

Substrate Spectrum

Testing of various alkenes allowed the substrate spectrum to be elucidated, and hence which alkenes were accepted for alkene cleavage and what were the structural requirements for substrates to be converted. As mentioned above, the best substrate for the biocatalytic alkene cleavage with *Trametes hirsuta* G FCC 047 was *trans*-anethole (**1a**), affording quantitative conversions (>99%) with perfect chemoselectivity

(> 99%) within 24 hours. The alkenes to be tested were chosen with consideration of the following aspects: i) substituents on the phenyl ring (amino, nitro, methyl, *tert*-butyl, chloro, or bromo in *ortho*-, *meta*-, and *para*-positions), ii) substitution pattern at the double bond (mono-, 1,1-di-, 1,2-di-, trisubstituted alkenes), iii) length of alkyl chain at the double bond [1-phenylpent-1-ene (**14a**), styrene (**5a**)], iv) substrates with isolated double bonds, v) cyclic alkenes, and vi) heteroaryl alkenes with five- and six-membered rings.

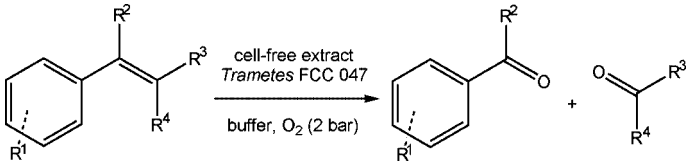
From the substrate pattern it became clear that *Trametes hirsuta* G FCC 047 could not cleave double bonds that are not conjugated to an aromatic ring, nor bulky 1,2-disubstituted alkenes such as *trans*- and *cis*-stilbene (Table 1). 1-Methyl-(2-prop-1-enyl)-1*H*-pyrrole underwent spontaneous alkene cleavage; furans were not accepted.

Table 1. Unsuitable substrates for alkene cleavage with *Trametes hirsuta* G FCC 047.

2-Bromostyrene	4-Allylanisole
5-*o*-Tolylpent-2-ene	Safrole
4-Acetoxystyrene	4-Phenylbut-1-ene
3-Vinylaniline	Ethyl 3-benzoylacrylate
trans-β-Methylstyrene	Maleic anhydride
(1,2-Dimethylpropenyl)benzene	Diethyl fumarate
trans-Stilbene	Diethyl maleate
cis-Stilbene	β-Ionone
β-Ionol	1-Methyl-2-(prop-1-enyl)-1*H*-pyrrole
2-(Prop-1-enyl)furan	2-(Prop-2-enyl)furan

Relative to styrene (**5a**), substituents in the *p*-position of styrene led to an increase in conversion except in the case of 4-aminostyrene (**2a**, Table 2). 4-Methoxystyrene (**3a**) was the most reactive of the *p*-substituted styrene derivatives. A

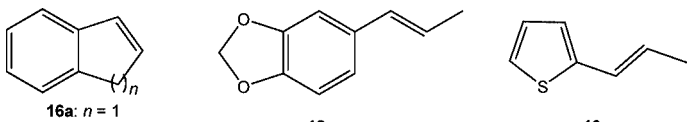
Table 2. Phenylalkenes cleaved by *Trametes hirsuta* G FCC 047 in buffer (pH 6) at 2 bar oxygen pressure.



R^1	R^2	R^3	R^4	Substrate	Product	Conversion (%)	Chemoselectivity[a] (%)
p-OMe	H	Me	H	*trans*-anethole (**1a**)	*p*-anisaldehyde (**1b**)	>99	>99
p-NH_2	H	H	H	4-aminostyrene (**2a**)	4-aminobenzaldehyde (**2b**)	3	>99
p-OMe	H	H	H	4-methoxystyrene (**3a**)	*p*-anisaldehyde (**1b**)	55	90
H	H	Me	Me	2-methyl-1-phenylprop-1-ene (**4a**)	benzaldehyde (**4b**)	13	83
H	H	H	H	styrene (**5a**)	benzaldehyde (**4b**)	22	>99
p-CH_3	H	H	H	4-methylstyrene (**6a**)	4-tolualdehyde (6b)	33	94
p-*t*Bu	H	H	H	4-*tert*-butylstyrene (**7a**)	4-*tert*-butylbenzaldehyde (7b)	23	92
m-NO_2	H	H	H	3-nitrostyrene (**8a**)	3-nitrobenzaldehyde (**8b**)	5	67
m-CH_3	H	H	H	3-methylstyrene(**9a**)	3-tolualdehyde (**9b**)	20	92
m-Cl	H	H	H	3-chlorostyrene (**10a**)	3-chlorobenzaldehyde (**10b**)	49	80[b]
o-Cl	H	H	H	2-chlorostyrene (**11a**)	2-chlorobenzaldehyde (**11b**)	77	85[c]
o-CH_3	H	H	H	2-methylstyrene (**12a**)	2-tolualdehyde (**12b**)	20	91
H	CH_3	H	H	α-methylstyrene (**13a**)	acetophenone (**13b**)	27	>99
H	H	C_3H_7	H	1-phenylpent-1-ene (**14a**)	benzaldehyde (**4b**)	27	64
H	H	CN	H	cinnamonitrile (**15a**)	benzaldehyde (**4b**)	1	>99

[a] The chemoselectivity is given as the ratio of formed aldehyde/ketone to all novel formed compounds. [b] GC-MS analysis indicated that the side products are the corresponding epoxide, diol, and 1-(3-chlorophenyl)-2-hydroxyethanone, together with two unidentified products. [c] GC-MS analysis indicated that the side products are the diol and the epoxide, together with two unidentified products.

Table 3. Alkenes **16a**–**19a** converted by *Trametes hirsuta* G FCC 047 in buffer (pH 6) at 2 bar oxygen pressure.



16a: *n* = 1
17a: *n* = 2 **18a** **19a**

Substrate	Product	Conversion (%)	Chemoselectivity[a] (%)
Indene (**16a**)	2-(2-oxoethyl)benzaldehyde (**16b**)	47	82
1,2-Dihydronaphthalene (**17a**)	2-(3-oxopropyl)benzaldehyde (**17b**)	30	69
Isosafrole (**18a**)	piperonal (**18b**)	4	100
2-(Prop-1-enyl)thiophene (**19a**)[b]	thiophenecarbaldehyde (**19b**)	93	92

[a] The chemoselectivity is given as the ratio of formed aldehyde to all novel formed compounds. [b] Substrate **19a** already showed spontaneous cleavage in the absence of catalyst (71% conversion and 48% chemoselectivity).

strongly electron-withdrawing group in the *m*-position, as in the case of 3-nitrostyrene (**8a**), disfavored conversion into the corresponding aldehyde. It is noteworthy that the reactivities of 3- and 2-chlorostyrene (**10a** and **11a**) were more than three and two times greater, respectively, than that of styrene (**5a**). Even a trisubstituted C=C bond, as in the case of 2-methyl-1-phenylprop-1-ene (**4a**), as well as a 1,1-disubstituted C=C bond, as in the case of α-methylstyrene (**13a**), were accepted. The enzyme also accepted long aliphatic chains attached at the double bond, as in the case of 1-phenylpent-1-ene (**14a**). Cyclic alkenes were also converted: indene (**16a**), for instance, was transformed more rapidly than 1,2-dihydronaphthalene (**17a**, Table 3). The substrate possessing the five-membered heteroaryl ring – thiophene (**19a**) – already reacted spontaneously, but improved conversion and chemoselectivity values were observed in the presence of catalyst (Table 3). Improved conversions in relation to our previous reports[17,18] (e.g., for **1a**, **3a**) can be attributed to a constant controlled O_2 pressure over the total reaction time.

Conclusions

The development of "green" chemical oxidation processes belongs to the current "hot topics" in organic chemistry. We have reported an essentially clean oxidation reaction for alkene cleavage that can be performed in aqueous solution under very mild conditions by use of the most innocuous oxidant – molecular oxygen. With special laboratory equipment, 96 experiments could be performed in parallel at constant O_2 pressure. Performing the reaction under light led to decreased conversion. Investigation of the substrate spectrum emphasized the requirement that the C=C double bond has to be conjugated to an aromatic ring. The biotransformations proceeded in general with high chemoselectivity. The scope and limitations of the substrate spectrum have been demonstrated: various substituted styrene derivatives were successfully cleaved, but cyclic alkenes such as indene (**16a**) or 1,2-dihydronaphthalene (**17a**) were also well accepted.

Experimental Section

General: NMR spectra were recorded in $CDCl_3$ with a Bruker AMX 360 at 360 (^{1}H) and 90 (^{13}C) MHz. Chemical shifts are reported relative to TMS (δ = 0.00 ppm) with $CHCl_3$ as internal standard [δ = 7.23 (^{1}H) and 76.90 (^{13}C)], coupling constants (J) are given in Hz. Ultrasonication experiments were performed in a Branson Digital Sonifier model 250. TLC was run on Merck 60 silica gel plates (F254) and compounds were visualized by standard techniques. Flash chromatography was performed on Merck 60 silica gel (230–400 mesh). Petroleum ether, acetone, and ethyl acetate were used as eluents. Solvents were dried and freshly distilled by conventional procedures. Petroleum ether (PE) had a boiling range of 60–90 °C unless otherwise noted.

Cells of *Trametes hirsuta* G FCC 047 were prepared as described previously.[18] The biocatalytic alkene cleavage could be performed either with freshly harvested cells or with lyophilized cells.

General Procedure for Biocatalytic Alkene Cleavage: Experiments were in general performed in triplicate. Lyophilized cells (25–30 mg) of *Trametes hirsuta* G FCC 047 were rehydrated with Bis-Tris buffer (900 μL per sample, 50 mM, pH 6). The cells were disrupted in centrifugal tubes by ultrasonication (Amplitude 50%, 1 s pulse, 4 s pause, program 1 min 40 s). The pellet was removed by centrifugation (8000 rpm, 20 min, 4 °C), and the supernatant was transferred into the reaction vessel (riplate LV or 4 mL glass vials with septa). Substrate (6 μL) was added to each vessel, and oxygen was flushed through the reactor. The pressure was adjusted to 2 bar. After 24 h at 170 rpm and 25 °C, the reaction was stopped by extraction with ethyl acetate (600 μL + 500 μL). The combined organic layers were dried with Na_2SO_4 and analyzed by GC and GC/MS.

Screening of Bacterial Strains: Experiments were performed by the general procedure described above but with use of whole cells. Lyophilized cells of each microorganism (bacterial strains from DSM, ATCC, and NCIMB collections) were rehydrated with the corresponding buffer (25 °C, 150 rpm, 30 min), and *trans*-anethole (**1a**) as substrate (6 μL, 5.9 mg, 40 μmol) was directly added to the mixture. Every strain was tested at various pH values: pH 4 (AcONa/AcOH 50 mM), pH 5 (AcONa/AcOH 50 mM), pH 6 (Bis-Tris buffer 50 mM), pH 7 (Bis-Tris buffer 50 mM).

O_2 Pressure: The reactions were performed with *trans*-anethole (**1a**, 6 μL, 5.9 mg, 40 μmol) as substrate at varied pressures (1, 2, 3, 4 and 6 bar) by the general procedure described above.

Organic Solvents: The experiments were performed analogously to the general procedure but with addition of different organic solvents (17 μL) before the reaction was started.

Influence of Light: Experiments were performed as in the general procedure. A lamp (PAR 38 EC Spot, OSRAM CONCENTRA, 120 W, made in EC, 230 V, 448) was placed over the equipment at a distance of 50 cm. Because of the heat produced by the lamp, the Plexiglas® (methacrylate) cylinder was cooled with a ventilator to keep the reaction temperature at room temperature. For purposes of comparison, the reaction was also performed in darkness at the same time, half of the samples being covered with perforated (to permit the exchange of oxygen) aluminum foil.

Supporting Information (see also the footnote on the first page of this article): A picture of the equipment and substrates synthesis and analytics are shown.

Acknowledgments

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