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Benzylic biooxidation of various toluenes to aldehydes by peroxidase

Rainer Russ,[a,*] Thomas Zelinski[b] and Timm Anke[a]

[a]*LB Biotechnologie, Universität Kaiserslautern, Paul-Ehrlich-Str. 23, D-67663 Kaiserslautern, Germany*
[b]*BASF-AG, Forschung Feinchemie, D-67056 Ludwigshafen, Germany*

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Abstract—A catalytic method is described for the oxidation of toluene and substituted derivatives to the corresponding benzaldehydes by hydrogen peroxide, using peroxidase. In most cases the respective benzoic acid was produced as a byproduct. The reaction proceeds under mild conditions in an aqueous medium.

Substituted benzaldehydes are often used as feedstock in industrial chemistry. The selective oxidation of aromatic methyl groups to the respective aldehyde is, however, difficult.[1] The chemical oxidation of the methyl group commonly proceeds directly to the carboxylic acid. We therefore chose to investigate enzymatic methods, because enzymes can be chemoselective. We started with the laccase/2,2-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) system of Potthast et al.[2] However, with toluene and laccase from different fungi, such as *Bjerkandera adusta*, *Coriolus* sp., *Phellinus* sp., and *Pleurotus ostreatus*, we found no transformation at all, which is in accordance with the findings of Fritz-Langhals and Kunath.[3] Subsequently, we tried several peroxidases with hydrogen peroxide as the oxidant. Using lignin peroxidase from *Phanerochaete chrysosporium*[4] or *Coprinus cinereus*,[5] we found no transformation of toluene. Chloroperoxidase from *Caldariomyces fumago* gave a slight transformation to benzyl alcohol and benzaldehyde, as reported earlier.[6,7] Finally, peroxidases isolated from a *Coprinus* species[†] of our strain collection were able to catalyze the transformation of toluene to benzaldehyde and, only to a minor extent, benzoic acid (Table 1).

The transformation of 21 different substituted methyl aromatics by hydrogen peroxide and *Coprinus* peroxidase was tested. Only three of the compounds tested, *p*-cymene (4-isopropyl-toluene), *m*-cresol, and *p*-cresol, were not at all transformed into the corresponding benzaldehydes. All other 18 compounds were transformed into the respective benzaldehydes whereby the efficiency of the reaction varied (Table 1). Suitable substituents comprised methyl, halogen, methoxy, and nitro groups. It seems that the position of the substituent was more important than its composition. *Ortho* or *para* positions of the substituent to the methyl group were preferred against *meta*, except for the nitrotoluenes. *o*-Nitrobenzaldehyde was obtained in a low yield, whereas *m*-nitrobenzaldehyde was formed with yields comparable to the *p*-isomer. In the case of *o*-nitrotoluene, an interaction of the intermediate methyl cation radical with the nitro group perhaps prevented the formation of the aldehyde. *o*-Nitrotoluene was the only substrate that produced the alcohol derivative. The cresols were found not suitable for catalytic conversion by *Coprinus* peroxidase and hydrogen peroxide, probably due to polymerization reactions as described for lignin peroxidase.[4] The preparation with *o*-cresol immediately turned yellow after addition of the enzyme. A weaker discoloration to yellow was observed for *m*- and *p*-cresol, as well as toluene, 3-chloro-, 4-methoxy-, and 4-fluorotoluene. *p*-Cymene was transformed into two compounds that were not identified. A molecular mass of 132 and 136 inferred that none of the compounds was either an aldehyde- or carboxyl-derivative of *p*-cymene.

Keywords: oxidation; chemoselectivity; biotransformation; biocatalysis; toluene; benzaldehyde.

* Corresponding authors. Tel.: +49-6221-4038-430; fax: +49-6221-4038-440. Present address: LION bioscience AG, Waldhofer Str. 98, D-69123 Heidelberg, Germany.; e-mail: russ@lionbioscience.com

† *Coprinus* sp., a member of the 'ink-cap' mushroom family, was fermented in 20 l soy meal medium. Mycelia and soy meal were removed by centrifugation. The resulting supernatants were cleared by ultrafiltration (pore size 0.16 μm). The filtrates were then concentrated by ultrafiltration using a membrane with a 10 kDa cutoff. The partial purification of the peroxidase was performed by FPLC. Two subsequent runs on Q-Sepharose at different pH-values (5 and 7.3, respectively) resulted in a 12 fold activity enrichment (specific activity with ABTS 7.6 U/mg of protein) and the total removal of laccase activity. Manuscript in preparation.

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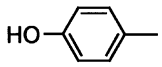
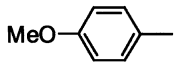
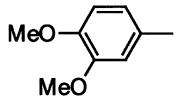
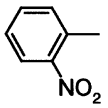
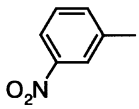
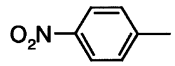
Table 1. Benzylic oxidation of various toluenes by hydrogen peroxide catalyzed by means of peroxidase[a]

parent compound	residual substrate concentration	product	product concentration
Toluene	0.06 mM	Benzaldehyde Benzoic acid	0.26 mM 0.1 mM
o-Xylene	< 0.05 mM	*o*-Tolualdehyde *o*-Toluic acid	0.57 mM 0.34 mM
m-Xylene	< 0.05 mM	*m*-Tolualdehyde	0.05 mM
p-Xylene	< 0.05 mM	*p*-Tolualdehyde *p*-Toluic acid	0.28 mM 0.15 mM
Mesitylene	0.11 mM	3,5-Dimethylbenzaldehyde	0.1 mM
1,2,3-Trimethyl-benzene	0.26 mM	2,3-Dimethylbenzaldehyde 2,3-Dimethylbenzoic acid	0.21 mM 0.15 mM
1,2,4-Trimethyl-benzene	0.29 mM	2,4-Dimethylbenzaldehyde 2,5-Dimethylbenzaldehyde 3,4-Dimethylbenzaldehyde Dimethylbenzoic acid	0.33 mM 0.13 mM 0.02 mM approx. 0.28 mM
4-Isopropyl-toluene (*p*-Cymene)	0.19 mM	4-Isopropylbenzaldehyde two unidentified products	< 0.05 mM
4-tert.-Butyltoluene	0.34 mM	4-tert.-Butylbenzaldehyde 4-tert.-Butylbenzoic acid	0.06 mM 0.33 mM
4-Fluorotoluene	< 0.05 mM	4-Fluorobenzaldehyde	0.36 mM
4-Bromotoluene	0.2 mM	4-Bromobenzaldehyde 4-Bromobenzoic acid	0.6 mM 0.5 mM
2-Chlorotoluene	< 0.05 mM	2-Chlorobenzaldehyde 2-Chlorobenzoic acid	0.36 mM 0.31 mM
3-Chlorotoluol	< 0.05 mM	3-Chlorobenzaldehyde 3-Chlorobenzoic acid	0.13 mM 0.26 mM
4-Chlorotoluene	< 0.05 mM	4-Chlorobenzaldehyde 4-Chlorobenzoic acid	0.24 mM 0.35 mM
o-Cresol	< 0.05 mM	Salicylaldehyde Salicylic acid	0.08 mM < 0,05 mM
m-Cresol	0.08 mM	3-Hydroxybenzaldehyde 3-Hydroxybenzoic acid	< 0.05 mM < 0.05 mM

Table 1. (*Continued*)

parent compound		residual substrate concentration	product	product concentration
p-Cresol		< 0.05 mM	4-Hydroxybenzaldehyde 4-Hydroxybenzoic acid	< 0.05 mM < 0.05 mM
4-Methylanisole		0.19 mM	*p*-Anisaldehyde *p*-Anisic acid	0.5 mM 0.2 mM
3,4-Dimethoxy-toluene		0.9 mM	3,4-Dimethoxybenzaldehyde 3,4-Dimethoxybenzoic acid	0.15 mM < 0.05 mM
2-Nitrotoluene		1.22 mM	2-Nitrobenzyl alcohol 2-Nitrobenzaldehyde 2-Nitrobenzoic acid	0.13 mM 0.05 mM <0.05 mM
3-Nitrotoluene		1.21 mM	3-Nitrobenzaldehyde 3-Nitrobenzoic acid	0.3 mM 0.12 mM
4-Nitrotoluene		1.1 mM	4-Nitrobenzaldehyde 4-Nitrobenzoic acid	0.3 mM 0.09 mM

[a] Procedure: 10 μl of the respective parental compound dissolved in acetone (100 mM) was diluted in 350 μl of tartaric acid buffer (200 mM; pH 5). 4.5 μl of hydrogen peroxide (50 mM), 45 μl water, and 25 μl of a partially purified peroxidase from *Coprinus* sp. (no laccase activity, peroxidase activity approx. 30 U/ml i.e. 1 ml of enzyme solution oxidizes 30 μmol ABTS [2,2-Azino-bis(3-ethylbenzthiazoline-6-sulfonic acid] per minute) were added. The vials were incubated at room temperature. 4,5 μl Hydrogen peroxide (50 mM) was added with a syringe through the membrane of the vial in use at 10 min intervals (9 times overall). At the halftime of the transformation experiment, an extra 25μl of peroxidase was added. Control reactions without addition of enzyme or with denatured enzyme resulted not in the formation of any detectable product. The concentration of the substances was determined by comparison with authentic standards by HPLC measurements. The identity of the products was confirmed by GC–MS.

In summary, the method presented here used *Coprinus* peroxidase and hydrogen peroxide to oxidize a variety of toluene derivatives to their corresponding aldehydes. Further studies are underway to characterize the enzyme responsible, as well as improving the reaction conditions to provide better yields.

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