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Journal of Molecular Catalysis B: Enzymatic 10 (2000) 435–444

JOURNAL OF MOLECULAR CATALYSIS B: ENZYMATIC

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Enzymatic oxidation of alkenes

M.-L. Niku-Paavola*, L. Viikari

VTT, Biotechnology and Food Research, Tietotie 2, P.O. Box 1501, FIN-02044 VTT, Espoo, Finland

Received 28 July 1999; received in revised form 26 October 1999; accepted 5 November 1999

Abstract

Laccase (EC 1.10.3.2) from the white-rot fungus *Trametes hirsuta* was used to oxidize alkenes. The oxidation was the effect of a two-step process, in which the enzyme first catalyzed the oxidation of primary substrate, the mediator, and then the oxidized mediator oxidized the secondary substrate, the alkene. Three different mediators were studied in the oxidation of aliphatic and cyclic alkenes.

All the alkenes tested were oxidized, but the degree of conversion depended on the alkene and mediator used. The mediators differed from each other in optimal reaction conditions and in specificity towards a given alkene. The best results were obtained by using hydroxybenzotriazole as mediator. Aliphatic polyunsaturated and aromatic allyl alcohols were completely oxidized within 2 h at 20°C. Aliphatic allyl alcohols were oxidized up to 70% at 45°C for 20 h, whereas a conversion of 60% was achieved in 5 h under oxygen atmosphere. By contrast, the oxidation degree of other alkenes, such as allyl ether, *cis*-2-heptene and cyclohexene, remained low with all the mediators and did not exceed 25%. The major oxidation products in all cases were the corresponding ketones or aldehydes.

Keywords: Enzymatic catalysis; Mediated oxidation; Oxidation of alkenes; Laccase–mediator system (LMS)

1. Introduction

Biocatalytic methods are emerging because of their regio- and stereo-selectivity and mild physiological conditions. Enzymatic methods could compete with chemical methods, especially in syntheses that cannot be successfully carried out by chemical catalysts. Examples of beneficial biocatalytic reactions are oxidations of alkanes, alkenes, aromatics, and heteroatoms [1]. These transformations are generally carried out by microbial cultures. Only in a few cases have oxidations been performed by isolated enzymes. Chloroperoxidase (EC 1.11.1.10) has been used for enantioselective epoxidation of alkenes and olefins [2]. Laccase (EC 1.10.3.2) has been used together with a mediator to oxidize selectively aromatic methyl groups and benzyl alcohols to aldehydes [3–5]. This type of ''mediated oxidation'', i.e., the combination of an enzyme catalyzing oxidation, laccase, with an electron transferring primary substrate can be used for the further oxidation of compounds not otherwise oxidized by this enzyme [6]. Medi-

* Corresponding author. Tel.: +358-9-456-5120; fax: +358-9-455-2103.

E-mail address: marja-leena.niku-paavola@vtt.fi (M.-L. Niku-Paavola).

1381-1177/00/$ - see front matter
PII: S1381-1177(99)00117-4

ated oxidation has been successfully used, e.g., for degradative oxidation of lignin [7,8]. In this work, the potential of the laccase–mediator system (LMS) in the oxidation of alkenes was studied.

2. Experimental

The enzyme used was the purified laccase of *Trametes hirsuta* VTT D443 [9]. Enzymatic treatments were carried out at pH 4.5 in 25 mM succinate buffer. Laccase was dosed according to its activity towards 2,2′Azino-bis[3-ethylbenzothiazoline-6-sulfonic acid diammonium salt] (ABTS) [9]. The dosages were in the range 0.1–10 mg (laccase protein)/mmol (alkene).

Mediators 1-hydroxy-1*H*-benzotriazole (HBT) (Sigma), ABTS (Boehringer), and violuric acid (Fluka) were used in the concentration range of 0.01–0.35 mmol/mmol alkene.

Alkenes used were: *cis*-2-heptene (Aldrich); cyclohexene (Fluka); cyclohexenone (Merck); allyl ether (Aldrich); methylacrylate (Merck); (+)-α-pinene (Sigma); *cis*-3-hexen-1-ol (Sigma); *cis*-2-hexen-1-ol (Aldrich); cinnamyl alcohol (Aldrich); and nerol, geraniol, and linalool obtained from Prof. A. Koskinen, University of Oulu, Finland. The alkene concentration in the tests was 20 mM.

Other substrates tested were 20 mM solutions of neopentyl glycol (2,2-dimethyl-1,3-propandiol, Riedel), *i*-propanol, propanol, hexanol, and butanol, all from Merck.

2.1. Mediated oxidation

Alkene, mediator, and enzyme were mixed with buffer to give a final volume of 2 ml in a sealed reaction bottle containing 20 ml air under normal pressure. Some samples were purged with additional O_2. The incubation was carried out at 20°C or 45°C for 10 min to 48 h with vigorous stirring. The reaction was stopped after boiling for 5 min. The products were extracted with diethylether, analysed for conversion by gas chromatography (GC) and identified by gas chromatography–mass spectrometry (GC–MS).

GC was performed using an HP-Innowax column (60 m × 0.25 mm i.d., film thickness 0.50 μm). The initial oven temperature was 50°C and the temperature was increased by 10°C/min to 240°C. Detection was made by FID.

GC–MS was performed using a Varian 3400 gas chromatograph interfaced to an Incos-50 mass-selective detector (Finnigan). The column was HP-5 (50 m × 0.2 mm i.d., film thickness 0.33 μm). The oven temperature was held at 80°C for 1 min, then increased to 320°C by 5°C/min and held at 320°C for a further 5 min. In the identification, a mass spectral library (Wiley Registry of Mass Spectral Data with Structures, 6th edn., Palisade, USA) was used. The identification was based on 90% similarity between the spectra of the unknown and reference compounds.

3. Results

3.1. Alkene oxidation by HBT-mediated laccase-catalyzed reaction

Laccase catalyzed oxidation of the alkenes only in the presence of the mediator. Neither laccase nor HBT alone was able to cause oxidation. The degree of conversion depended on the alkene used. Treatment at 20°C for 20 h resulted in a low conversion, maximally around 10%, of *cis*-2-heptene, cyclohexene, cyclohexenone, allyl ether, and methylacrylate. Under the same reaction conditions, α-pinene, *cis*-2- and *cis*-3-hexenols were oxidized up to 45–50%, whereas the oxidation degree was 90–100% of the theoretical for linalool, geraniol, nerol and cinnamyl alcohol (Table 1).

Table 1
Oxidation of alkenes by laccase–HBT mediator reaction
For each mmol alkene, 10 mg laccase and 0.35 mmol HBT, reaction at 20°C for 20 h.

Alkene	Conversion (%)[a]	Reaction products, relative amount[b]
Cyclohexene	10	2-Cyclohexen-1-one, 4%; unidentified, 6%
(+)-α-Pinene	45	Verbenone, 26%; pinocarvone, 3%; *trans*-sobrerol, 2%; *p*-cymen-8-ol, 2%; unidentified, 12%
Cis-2-hexen-1-ol	51	*Trans*-2-hexenal, 34%; *cis*-2-hexenal, 3%; *trans*-hexenol, 8%; unidentified, 5%
Linalool	99	*Cis*- and *trans*-furanoid linalool oxide, 61%; unidentified, 38%
Geraniol	97	*Cis*-pyranoid and *trans*-pyran linalool oxide, 46%; unidentified, 51%
Nerol	93	*Cis*-pyranoid and *trans*-pyran linalool oxide, 51%; unidentified, 42%
Cinnamyl alcohol	100	Cinnamaldehyde, 40%; benzaldehyde, 43%; unidentified, 17%

[a] Based on unconverted substrate.
[b] Percentages of the original substrate amount.

The main reaction products of alkenes were the corresponding ketones or aldehydes but other products were also detected (Tables 1 and 2). The allyl alcohol *cis*-2-hexen-1-ol was oxidized to *trans*-aldehyde with traces of *cis*-aldehyde and *trans*-2-hexenol. The main oxidation product of pinene was verbenone, but low amounts of pinocarvone, *trans*-sobrerol, and *p*-cymen-8-ol were also detected. Similarly, cinnamyl alcohol was oxidized to cinnamaldehyde and benzaldehyde. The polyunsaturated alcohols, linalool, geraniol and nerol, were converted to linalool oxides with a pyran or pyranoid configuration. Some of the reaction products remained unidentified. The amounts of unidentified end products were rather high especially in the case of polyunsaturated alcohols (Table 1).

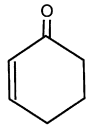
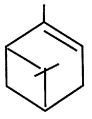
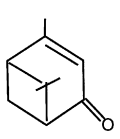
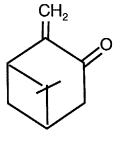
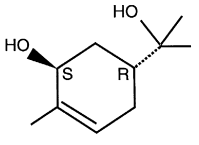
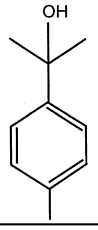
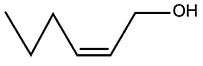
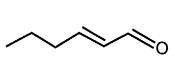
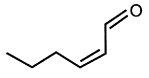
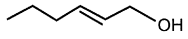
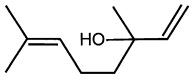
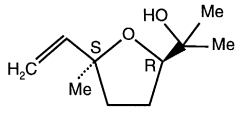
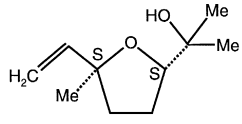
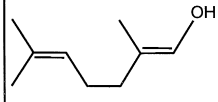
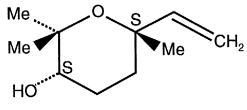
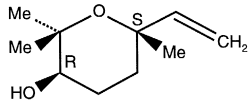
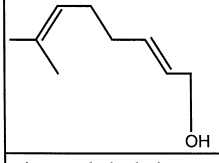
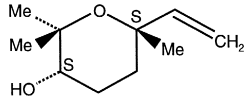
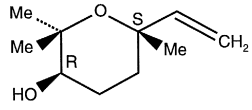
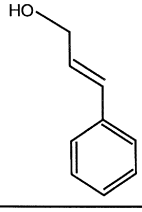
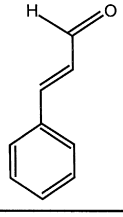
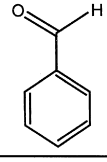
3.2. Reaction products as a function of time

The highest conversions in laccase–mediator reactions were obtained for allyl alcohols and their oxidation was therefore studied in more detail. By analysing the end products at different reaction stages, it was confirmed that aldehyde is the first main product to appear (Fig. 1). During the oxidation of *cis*-2-hexenol, *trans*-hexenal was detected after 2 h reaction. The amounts of aldehyde and by-products increased when the reaction proceeded. However, after 20 h at 20°C, the residual amount of the starting compound was still 50% (Fig. 1A). In the case of geraniol, the starting compound was hardly detectable after a reaction time of 2 h. The aldehyde products of geraniol, geranial and neral, appeared during the first 10 min of reaction. The amounts of the aldehydes as well as those of unidentified products increased steadily until the starting compound was completely oxidized. Thereafter, the amounts of aldehydes started to decrease. Linalool oxides appeared among the reaction products within a reaction time of 2 h. After 20 h, aldehydes were no longer detectable (Fig. 1B). The aldehyde products of cinnamyl alcohol, cinnamaldehyde and benzaldehyde, were also detected in only 10 min at 20°C. The amounts of aldehydes increased until cinnamyl alcohol was completely oxidized after 2 h. If the reaction time was extended, the amounts of aldehydes slightly decreased and that of unidentified compounds increased (Fig. 1C).

3.3. The effect of reaction conditions on the conversion

Different reaction conditions were studied in order to improve the efficiency of conversions. The degrees of conversion of *cis*-2-heptene, cyclohexene, cyclohexenone, allyl ether, and methylacrylate remained low with all the HBT and laccase concentrations investigated (e.g., Fig. 2A). By contrast, the conversions of pinene and *cis*-3-hexen-1-ol were improved by increas-

Table 2
Oxidation products of alkenes by laccase–HBT mediator reaction

Parent compound	Products
cyclohexene 	2-cyclohexen-1-one 
(+)-α-pinene 	verbenone  pinocarvone  *trans*-sobrerol  *p*-cymen-8-ol 
cis-2-hexen-1-ol 	*trans*-2-hexenal  *cis*-2-hexenal  *trans*-hexenol 
linalool 	*cis*- and *trans*-furanoid linalool oxide  
geraniol 	*cis*-pyranoid and *trans*-pyran linalool oxide  
nerol 	*cis*-pyranoid and *trans*-pyran linalool oxide  
cinnamyl alcohol 	cinnamaldehyde  benzaldehyde 

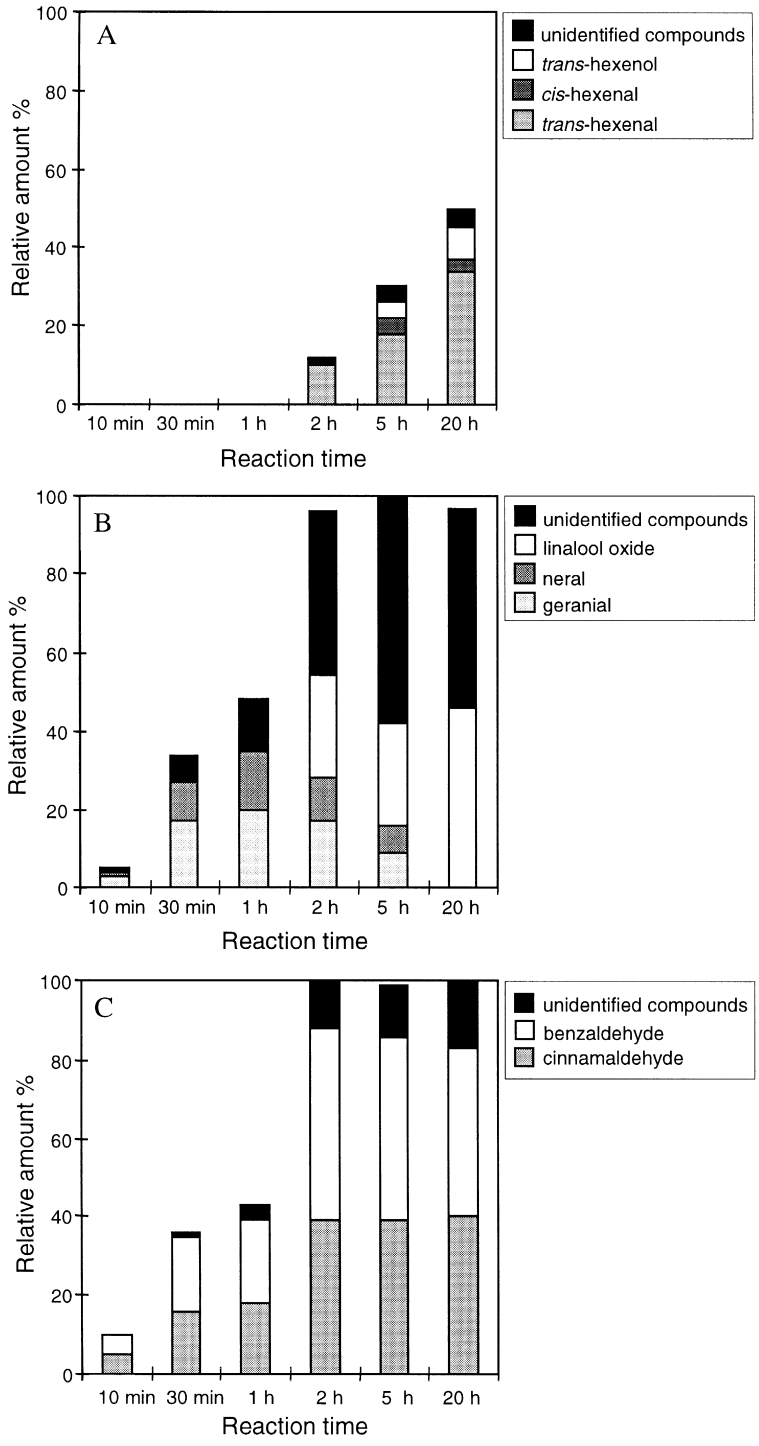


Fig. 1. Oxidation products during HBT-mediated laccase reaction. (A) Oxidation products of *cis*-2-hexen-1-ol. (B) Oxidation products of geraniol. (C) Oxidation products of cinnamyl alcohol. For each mmol alkene, 10 mg laccase and 0.35 mmol HBT; reaction at 20°C for 20 h. The relative amounts of products are percentages of the original substrate amount.

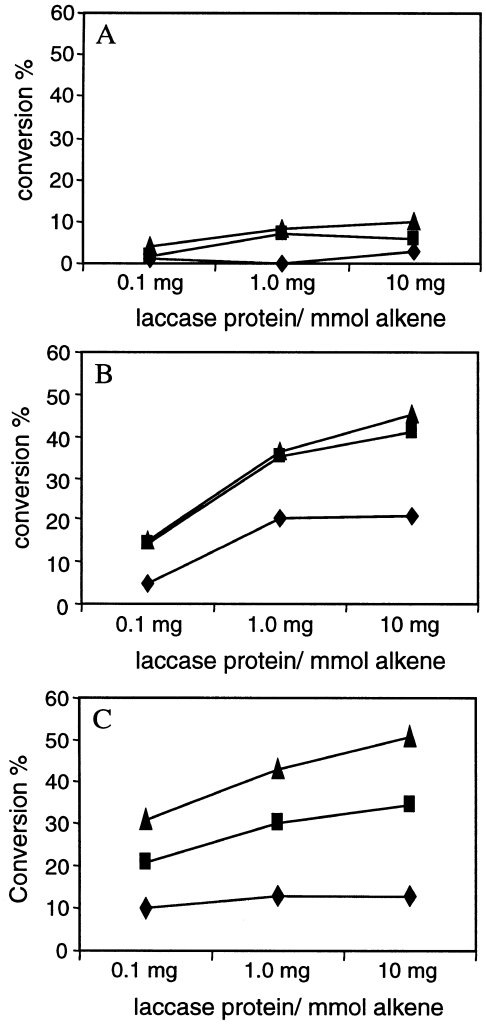


Fig. 2. Conversion of alkenes in the laccase–HBT reaction. (A) Conversion of cyclohexene. (B) Conversion of pinene. (C) Conversion of *cis*-3-hexen-1-ol. Laccase 0.1–10 mg/mmol alkene; HBT mmol/mmol alkene 0.01 (♦-♦); 0.1 (■-■); 0.35 (▲-▲); reaction at 20°C for 20 h.

ing the mediator and enzyme concentrations (Fig. 2B,C). The optimal concentrations appeared to depend on the alkene used. For example, the conversion of pinene was enhanced only up to an HBT concentration of 0.1 mmol/mmol alkene, whereas the conversion of *cis*-3-hexen-1-ol steadily increased with the higher HBT and enzyme concentrations. A further enhancement was obtained by increasing the reaction temperature and time. The conversion of *cis*-3-hexen-1-ol was clearly higher at

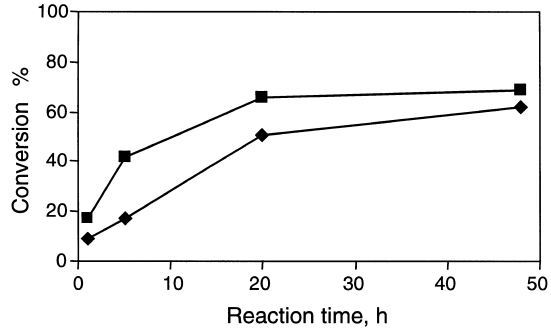


Fig. 3. Conversion of *cis*-3-hexen-1-ol in the laccase–HBT reaction. For each mmol alkene, 10 mg laccase and 0.35 mmol HBT; reaction at 20°C (♦-♦), at 45°C (■-■).

45°C than at 20°C and reached 70% during 48 h treatment (Fig. 3). When the reaction mixture was saturated with pressurized oxygen, the degree of conversion for *cis*-3-hexen-1-ol increased appreciably; approximately 40% and 60% at 20°C and 45°C with a reaction time of 5 h, respectively (results not shown).

Enzymes are often inactivated in organic solvents. Fig. 4 shows that the laccase activity in the reaction mixture decreased by 37% and 77% after a reaction time of 5 h at 20°C and 45°C, respectively. After 24 h, the corresponding inactivations were 70% and 100%, respectively. In the absence of mediator and alkene, laccase was rather stable during 5 h both at 20°C and 45°C. However, after 24 h, the inactivations were 26% and 49%, respectively. Thus, the inactivation of enzyme is one of the possible reasons for the limited degree of conversion and should be

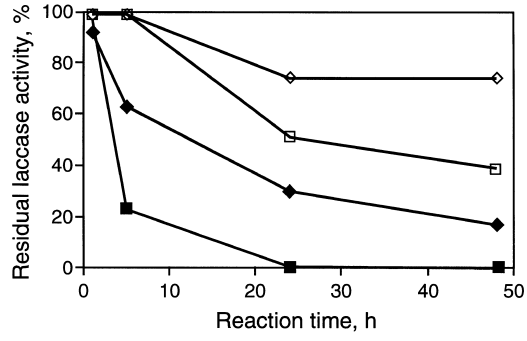


Fig. 4. Stability of laccase in the *cis*-3-hexen-1-ol–HBT reaction. For each mmol alkene, 10 mg laccase and 0.35 mmol HBT; reaction at 20°C (♦-♦), at 45°C (■-■); laccase alone at 20°C (◇-◇), at 45°C (□-□).

considered in relation to enzyme dosages, reaction temperatures, and time.

3.4. Comparison of different mediators in the oxidation of alkenes

Three different mediators, HBT, ABTS, and violuric acid, were compared for their oxidation efficiency in the LMS. The mediators appeared to differ from each other in optimal reaction conditions and in specificity towards a given alkene. The maximal degrees of conversion with HBT, as well as with violuric acid, were obtained by using 10 mg enzyme and 0.35 mmol mediator/mmol alkene while the corresponding values for ABTS were 0.1 mg and 0.01 mmol (Fig. 5). All the mediators had low efficiency towards allyl ether and cyclohexene; the conversions did not exceed 25% (Fig. 5A,B). Pinene underwent 45% conversion with HBT and ABTS but almost none with violuric acid (Fig. 5C). The conversion of *cis*-3-hexen-1-ol was 40% and 50% with violuric acid and HBT, respectively, but only 8% with ABTS (Fig. 5D). Furthermore, geraniol and cinnamyl alcohol were oxidized 97% and 100%, respectively, in HBT-

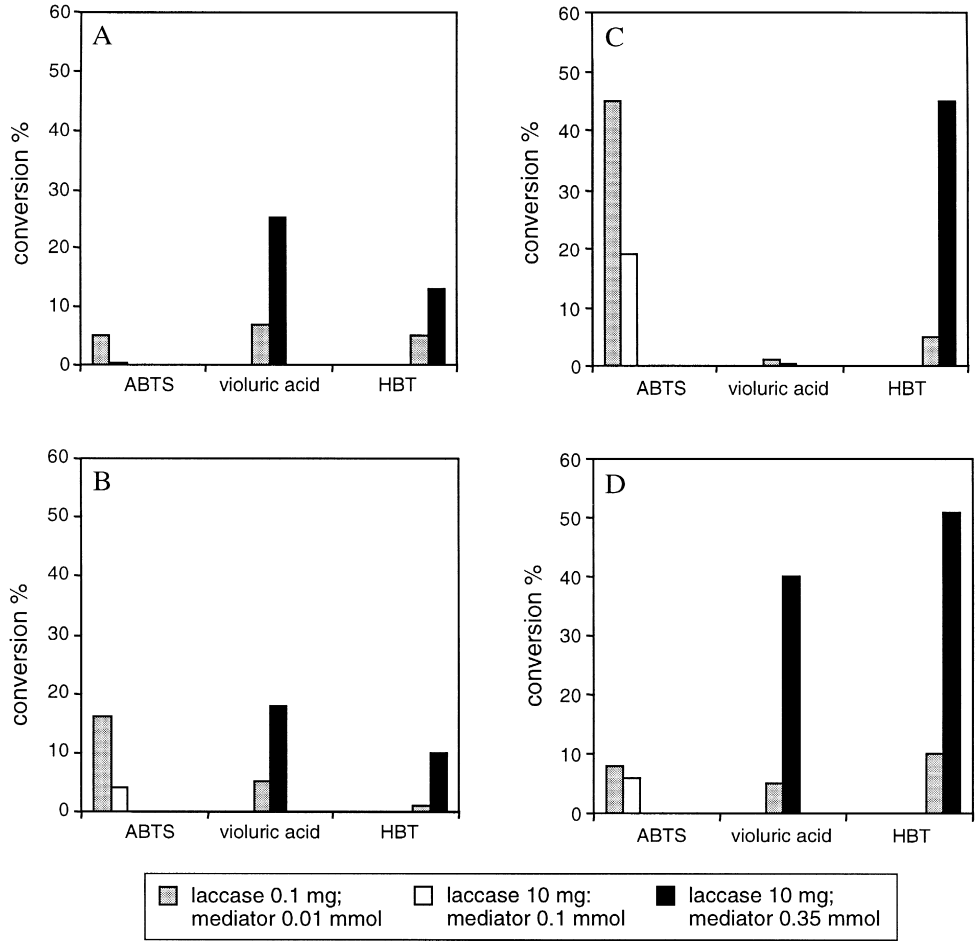


Fig. 5. Conversion of alkenes with different mediators. (A) Conversion of allyl ether. (B) Conversion of cyclohexene. (C) Conversion of pinene. (D) Conversion of *cis*-3-hexen-1-ol. Laccase 0.1–10 mg/mmol alkene; ABTS 0.01–0.1 mmol/mmol alkene; violuric acid and HBT 0.1–0.35 mmol/mmol alkene; reaction at 20°C for 20 h.

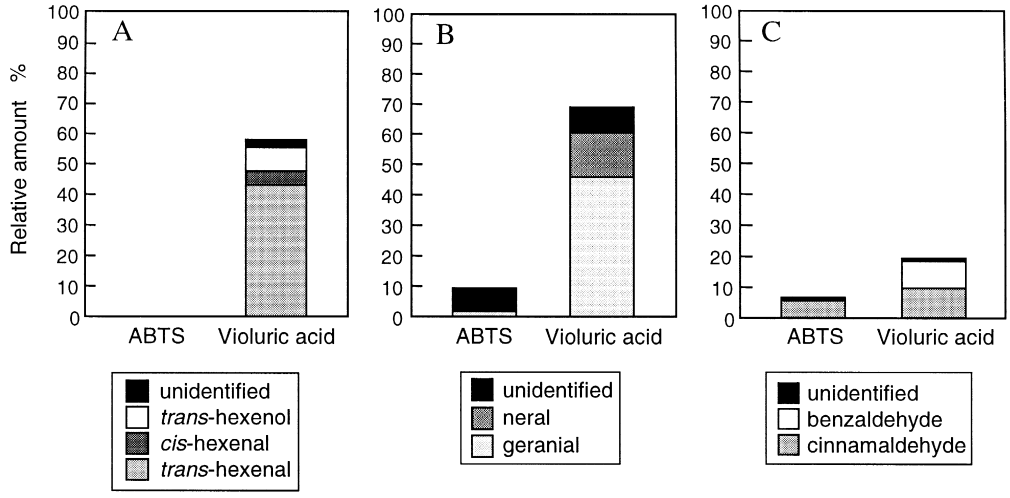


Fig. 6. Oxidation products of alkenes in ABTS- and violuric acid-mediated laccase reaction. (A) Oxidation products of *cis*-2-hexen-1-ol. (B) Oxidation products of geraniol. (C) Oxidation products of cinnamyl alcohol. In ABTS-mediated reactions for each mmol alkene, 0.1 mg laccase and 0.01 mmol mediator. In violuric acid-mediated reactions for each mmol alkene, 10 mg laccase and 0.35 mmol mediator. Reactions at 20°C for 20 h.

mediated reactions at 20°C for 2 h (Fig. 1B,C), whereas the corresponding figures were 70% and 20% with violuric acid and 10% and 7% with ABTS at 20°C for 20 h (Fig. 6A,B). With all three mediators, the main reaction products of alkenes were the same, i.e., the corresponding aldehydes or ketones.

3.5. Specificity of the mediated oxidation

The highest degrees of conversion, 70–100%, in the laccase-HBT reaction were obtained by using allyl alcohols as secondary substrates. By contrast, the degrees of oxidation for saturated alcohols, such as *i*-propanol, propanol, hexanol, butanol and neopentyl glycol, were low, at most 5% (results not shown). The reaction products were aldehydes as in the case of allyl alcohol. To be effective, the laccase–mediator oxidation appeared to require an unsaturated substrate.

4. Discussion

In this work, the mediated oxidation by a laccase purified from *T. hirsuta* was studied in the oxidation of alkenes, which are not natural substrates for this enzyme. The oxidation was the effect of a two-step process, in which the enzyme first catalyzed the oxidation of primary substrate, the mediator, and then the oxidized mediator oxidized the secondary substrate, the alkene. Three different mediators, HBT, ABTS, and violuric acid were tested. Several aliphatic and cyclic alkenes were used as secondary substrates.

All the compounds tested as secondary substrates were oxidized, but the degree of conversion depended on the substrate and mediator used. The mediators differed from each other in optimal reaction conditions and in specificity towards a given alkene. The effective proportion of enzyme to mediator was far lower with ABTS than with HBT and violuric acid. However, the degree of maximum conversion was also generally low with ABTS. The specificity of mediators towards a given alkene resulted in approximately 25% conversion of allyl ether with violuric acid whereas the degree of conversion with the other mediators was approximately 10%. Furthermore, violuric acid was unable to act as a mediator in the oxidation of

pinene whereas the degree of conversion was 45% with the other mediators.

The highest degrees of conversion in the mediated oxidation were obtained when allyl alcohols were oxidized using HBT as mediator. Aliphatic polyunsaturated and aromatic allyl alcohols were completely oxidized within 2 h at 20°C. Aliphatic allyl alcohols were oxidized up to 70% during 20 h at 45°C. However, a conversion of 60% was achieved in a reaction time of 5 h if the reaction was carried out under oxygen atmosphere instead of normal air. This demonstrates the importance of adequate transfer of oxygen in the laccase reactions.

The oxidation degree of alkenes, such as allyl ether, *cis*-2-heptene, and cyclohexene, remained low with all mediators and did not exceed 25%. The degree of conversion for saturated alcohols, however, was still lower at only 5%.

The LMS has been evaluated earlier in the oxidation of lignin. It has been shown that the efficiency of mediated oxidation depends on the origin of the laccase and on the structure of the mediator [10], as well as on the structure of lignin [9]. There are stability differences between laccases during the reaction, and thus, between their performances [10]. Although laccases are rather unspecific enzymes, they obviously have individual requirements concerning the structure of the mediator. For a given laccase, there is considerable variation in the extent of oxidation of lignin depending on the mediator [10]. Correct spatial and electronic structure of the secondary substrate is also needed for the reaction to proceed [4]. According to current theories, laccase oxidizes mediators to radicals. The oxidation potential and stability of the radicals will affect their ability to oxidize the secondary substrate [11]. The oxidation potentials of HBT and ABTS are the highest of the known mediators, but only slightly higher than that of violuric acid. HBT and violuric acid contain an N–OH group which forms radicals capable of transferring electrons but also of becoming deoxygenated as stoichiometric oxidants. ABTS is an azo-compound and forms stable and electrochemically reversible cation radicals and dications [12]. The former reacts with phenolic and the latter with non-phenolic structures of lignin. It has been noticed that at low mediator/secondary substrate molar ratio, ABTS is an activator for laccase, whereas at increased mediator ratio ABTS becomes a competing co-substrate [13]. The different oxidative characteristics would make the mediators differently active towards a secondary substrate with a specific structure. The variable reaction product yields obtained here, by the use of either HBT, violuric acid, or ABTS as mediators, were obviously due to the different reactivity of the oxidized mediator in each case.

According to recommendations, mediated oxidation of lignin should be performed at 45°C [7]. However, it was observed that during mediated oxidation of alkenes at 45°C, only about 20% of the laccase activity remained after 5 h. In the absence of mediator and alkene, laccase was stable. In order to maintain enzyme activity during oxidation of alkenes, it is not practical to use temperatures higher than 45°C or reaction times longer than 5 h. Thus, the oxidation conditions should be optimized with respect to oxygen supply; reaction time and temperature; as well as concentrations of enzyme, mediator and alkene.

The main reaction products of alkenes were the corresponding ketones or aldehydes with all three mediators tested. The mediated oxidation by laccase obviously proceeded by radical mechanisms leading to allylic oxidation. However, in the case of geraniol, aldehydes disappeared during an extended reaction whereas linalool oxides appeared. The conversion of geraniol into linalool oxides is possible if geraniol undergoes an epoxidation at the nonallylic double bond followed by a β-elimination of HO^-. The potential epoxide intermediate was not identified in this work. It has been proposed recently that epoxidation does occur during laccase-catalyzed oxidation in the presence of mediator [13]. This was concluded when isoeugenol methyl ether was converted to veratraldehyde

during *Pycnoporus* laccase-catalyzed oxidation in the presence of ABTS.

Correspondingly, chemical oxidation of cinnamyl alcohol, geraniol, and nerol resulted in aldehyde products. In the case of copper-catalyzed air oxidation, the alcohols were 70–90% converted into the corresponding aldehydes during 1 h at 70–90°C [14].

Whole microbial cell cultures have been the conventional catalysts in bio-organic oxidations. Purified oxidative enzymes have been used very seldom. Oxidation by the LMS presented here for alkenes has earlier been applied in the delignification of kraft pulp using mediators such as ABTS and HBT [7,8]. Recently, *Coriolus versicolor* laccase was used in ABTS-mediated oxidation for the selective oxidation of methyl-substituted benzyl alcohols [3,4]. Overall yields of the corresponding aldehydes were 90–98% during a 24–48 h reaction at room temperature. Aromatic aldehydes were also obtained when methyl benzenes were oxidized by *Trametes versicolor* laccase and various N–OH-compounds as mediators [5]. Complete oxidation of 1-methyl-3, 4-methoxybenzene was obtained with laccase and HBT in a 22 h reaction at 45°C. The authors claimed that allyl alcohols, e.g., 3-methyl-2-buten-1-ol and cinnamyl alcohol, can also be analogously oxidized but no data for these conversions were presented. In these oxidations [3–5], laccase doses were similar but mediator equivalents four times higher than in the *T. hirsuta* laccase oxidations presented here. It appears that the efficiency of the mediated oxidation depends on the compatibility of the laccase, mediator, and secondary substrate.

Acknowledgements

This work was financially supported by Neste Oy, Finland, and the Technology Development Centre, Finland. Thanks to MSc K. Latva-Kala for performing and interpreting the GC and GC–MS analyses and to R. Arnkill and A-L. Ruskeepää for their technical assistance.

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