

Oxidoreductases from *Trametes* spp. in Biotechnology: A Wealth of Catalytic Activity

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Received: September 13, 2006

Revised version: April 27, 2007

Accepted: May 4, 2007

Summary

Those oxidoreductases that are part of the ligninolytic complex of basidiomycete and ascomycete fungi have played an increasingly important role in biotechnological applications during the last decade. The stability of these extracellular enzymes, their good solubility, and a multitude of catalyzed reactions contribute to this trend. This review focuses on a single genus of white-rot basidiomycetes, *Trametes*, to highlight the numerous possibilities for the application of this microorganism as well as three of its enzymes: laccase, cellobiose dehydrogenase, and pyranose 2-oxidase. Whereas laccase is without doubt a major player in biotechnology, the two other enzymes are less well known, but represent emerging biocatalysts with potential. Both cellobiose dehydrogenase and pyranose 2-oxidase are presumed to participate in lignin breakdown and will be used to exemplify the potential of less prominent oxidoreductases from this genus.

Key words: *Trametes*, laccase, cellobiose dehydrogenase, pyranose 2-oxidase, biodegradation, biosensors, biotechnology, food technology

Introduction

Extensive efforts have been dedicated to evaluate the possibilities offered by ligninolytic enzymes from *Trametes* (syn. *Coriolus*, *Polyporus*) species for analytical, industrial or environmental applications. The genus *Trametes* is probably the most actively investigated in the phylum of Basidiomycota for lignolytic enzyme formation and application. Like other white-rot fungi, several reasons account for the attractiveness of *Trametes*, chief among them is the constitutive, extracellular secretion and the

non-specific nature of the lignolytic enzymes, which obviates the need for adaptation to the target molecule. Further, they can grow on cheap media such as corn cobs, straw, peanut shells and even sawdust (1,2), while their hyphal growth form allows them to extend far from their original starting point, an attractive property in bioremediation of soils. The lignolytic enzymes that make *Trametes* very attractive are laccase (Lac, EC 1.10.3.2) and manganese peroxidase (MnP, EC 1.11.1.13), while lignin peroxidase (LiP, EC 1.11.1.14) has only rarely been reported in *Trametes*. Manganese peroxidase, which will

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not be explicitly reviewed here, catalyzes the oxidation of Mn^{2+} to Mn^{3+} , which is normally the oxidizing agent that acts on target molecules. Various aspects of fungal laccases in general have been reviewed, including functional aspects (3–6), molecular and catalytic properties (7–11) and their technological application (12,13). In screening for either laccases or manganese peroxidase, *Trametes* spp. have been shown to produce multiple isoforms of these enzymes expressed under different culture conditions (14–16). Two other oxidoreductases have also been found in *Trametes*, the not so commonly known cellobiose dehydrogenase (CDH, EC 1.1.99.18) and pyranose 2-oxidase (P2O, EC 1.1.3.10). Since they are supposed to be involved in lignin degradation, they have been included in this review. General reviews of these enzymes are available (17–19).

Trametes versicolor, a representative fungus of this genus, is among the first fungi for which the occurrence of laccase was reported (20) and its laccase has already been marketed by several companies. Most of the previous reviews on advances in biotechnological and industrial applications of lignolytic fungi have focused on white-rot fungi as a group in general, despite the fact that this is a taxonomically and phylogenetically heterogeneous group. Quite often this bias has led to overestimating the importance of the best studied white-rot fungus, namely *Phanerochaete chrysosporium*. Since *P. chrysosporium* typically produces lignin peroxidases, this has therefore been considered the most important lignin-degrading enzyme. Nevertheless, studies have shown that most of the white-rot fungi, including some from the genus *Trametes*, produce very low quantities or do not produce LiP at all, although they are efficient lignin degraders (6,21–23). Studies on the application of *Trametes* spp. or their lignolytic enzymes have mainly been directed towards the development of socioeconomically important products or waste treatment. A large body of evidence shows that *Trametes* is among the most versatile of white-rotters with ongoing intensive research into applications in bioremediation, effluent treatment, the pulp and paper industry, the food industry, synthetic chemistry, biofuels, cosmetics, biosensors and the textile industry, amongst others. This review focuses on the genus *Trametes*, highlighting advances in research and possible industrial applications of the whole microorganism or of the three above-mentioned enzymes.

Laccase

History and catalysis

Laccase (benzenediol:oxygen oxidoreductase, EC 1.10.3.2), together with ferroxidases (EC 1.16.3.1) and ascorbate oxidase (EC 1.10.3.3), form the family of multicopper oxidases (MCOs), which in turn belong to the highly diverse group of blue copper proteins. A recent study, involving phylogenetic analysis of about 350 available MCO sequences, classified this enzyme family into laccases *sensu stricto* (basidiomycete and ascomycete laccases), fungal pigment MCOs, fungal ferroxidases, ascorbate oxidases, insect laccases, plant laccase-like MCOs, and bacterial laccase-like enzymes including bilirubin oxidases (24). The first fungal laccase was reported by G.

Bertrand for *Russula cyanoxantha* and *R. delica* in 1896 (25). Since then, most of the laccases that have been isolated, characterized, and applied originate from basidiomycetes and, to a lesser extent, from ascomycetes. The laccases from these organisms have thus formed our current picture of laccases.

Fungal laccases typically have high redox potential and thereby can efficiently oxidise a broad range of aromatic compounds like monophenols, diphenols, polyphenols, methoxy-substituted phenols, aromatic amines and diamines, benzenethiols and even some inorganic compounds such as iodine by using oxygen as an electron acceptor (26). They also catalyze decarboxylation and demethylation reactions (27). Laccase is one of the few enzymes able to catalyze the four-electron reduction of molecular oxygen to water, a reaction involving a cluster of four copper atoms that forms the catalytic core of the enzyme. The X-ray structures of four basidiomycete laccases have been elucidated, namely the enzymes from *Coprinus cinereus* (28), *Trametes versicolor* (29), *Rigidoporus lignosus* (30) and *Cerrena maxima* (31). The available structures confirm the results of previous spectroscopic studies undertaken on the laccase from the plant *Rhus vernicifera* (32). Three main steps in laccase catalysis can be distinguished: first, type-1 (T1) copper, which is near the surface of the enzyme and acts as primary oxidation site, is reduced by a suitable substrate, then the electron is transferred internally to a trinuclear cluster made up of one type-2 (T2) and two type-3 (T3) copper atoms. During the sequential oxidation of four substrate molecules, an oxygen molecule is concomitantly reduced to water at the trinuclear cluster, which is located about 12 Å away from T1 (29). Hydrogen peroxide is not detected during steady state laccase catalysis, indicating a complete four-electron reduction to water (33). The redox potential was determined in bioelectrochemical studies for several *Trametes* laccases, namely those from *T. versicolor* (34), *T. hirsuta* (35) and *T. villosa* (36). The potential of the T1 site is about 780 mV *vs.* the normal hydrogen electrode (NHE) and can therefore be classified as high-potential laccases. The redox potential of the T2 site in *Trametes hirsuta* laccase was elucidated by cyclic voltammetry studies to be about 400 mV *vs.* NHE (37).

Typically, *Trametes* secretes a series of laccase isoforms under appropriate induction conditions. The isoforms may have isoelectric points ranging from 3.0 to 7.5, they are glycosylated (reported values range from 10 to 15 % of molecular mass), and consist of 520 to 550 amino acids, having an overall molecular mass between 60 000 and 65 000. These enzymes have acidic pH optima as is typical for most basidiomycete laccases. A selection of kinetic data from *Trametes* laccases is given in Table 1 (38–48), data for other fungal laccases are presented in the work of Baldrian (3).

Applications of isolated laccase or the whole organism

Due to their catalytic properties and high stability under various operating conditions, laccases have been used in a wide range of analytical, industrial, and environmental applications. Purified laccase from *Trametes* is quite often restricted to low-volume/high-value applications, whereas laccase containing crude extract or the whole organism is preferentially used in high-volume processes.

Table 1. Kinetic parameters of laccases and laccase isoforms from *Trametes* species

Species (Ref. no.)	Isoforms	M _r /kDa	pH optimum / pH used in assay							
			ABTS	FeCy	2,6-DMP	Guaiacol	Catechol	HQ	SGZ	
<i>T. gallica</i> (38)	Lac I	60	2.2/3.0		3.0/3.0	4.0/4.0				
<i>T. gallica</i> (38)	Lac II	60	2.2/3.0		3.0/3.0	4.0/4.0				
<i>T. hirsuta</i> (39)		70			within 3.7–4.9 for all measured substrates					
<i>T. hirsuta</i> (40)		73	2.5/4.0		4.0/4.0	/4.0	/4.0		/4.0	
<i>T. hirsuta</i> (41)		55		3.5/4.9		4.5/4.9	4.5/4.9	4.5/4.9		
<i>T. multicolor</i> (42)	Lac II	63	<2.5/3.5	2.5/2.5	4.0/4.0	4.5/4.5	4.0/4.0	4.5/4.5		
<i>T. ochracea</i> (39)		64			within 3.5–4.5 for all measured substrates					
<i>T. pubescens</i> (43)	LAP 2	65	< 3/3.0	3.0/3.0	3.0/3.0	3.0/3.0	3.5/3.5	3.5/3.5	4.5/4.5	
<i>T. pubescens</i> (44)	Lac 1	67	/4.5	2.5/4.5			/4.5	/4.5		
<i>T. pubescens</i> (44)	Lac 2	67		2.5/4.5			/4.5	/4.5		
<i>T. sanguinea</i> (45)		62			5.0/5.0					
<i>T. trogii</i> (46)		70	/3.4		/3.4		/3.4	/3.4		
<i>T. versicolor</i> (47)		68	2.5/4.5		3.5/4.5					
<i>T. villosa</i> (48)	Lac 1	63	2.7/2.7						5.5/5.5	
<i>T. villosa</i> (48)	Lac 3	63	6.0/4.0						5.5/5.5	
			K _M /μM							
			pI	ABTS	FeCy	2,6-DMP	Guaiacol	Catechol	HQ	SGZ
<i>T. gallica</i>	Lac I	3.1	12			420	405			
<i>T. gallica</i>	Lac II	3.0	9			410	400			
<i>T. hirsuta</i>		4.2		180			63	142	61	
<i>T. hirsuta</i>		7.4	57			61	11	40		143
<i>T. hirsuta</i>		4.0		167			63	146	61	
<i>T. multicolor</i>	Lac II	3.0	14	40	186		436	845	362	
<i>T. ochracea</i>		4.7		96			90	110	74	
<i>T. pubescens</i>	LAP 2	2.6	14	43	72		360	470	390	6
<i>T. pubescens</i>	Lac 1	5.3	50	700				200	500	
<i>T. pubescens</i>	Lac 2	5.1	60	200				600	200	
<i>T. sanguinea</i>		3.5								
<i>T. trogii</i>		3.3, 3.6	30			410				
<i>T. versicolor</i>			37			15				
<i>T. villosa</i>	Lac 1	3.5								
<i>T. villosa</i>	Lac 3	6.0–6.5								
			k _{cat} ^{b,c} /s ⁻¹							
			T ^a /°C	ABTS	FeCy	2,6-DMP	Guaiacol	Catechol	HQ	SGZ
<i>T. gallica</i>	Lac I	25	375 ^b							
<i>T. gallica</i>	Lac II	25	370 ^b							
<i>T. hirsuta</i>		?		400			430	390	450	
<i>T. hirsuta</i>		25	260			45	2.2	7.5		107
<i>T. hirsuta</i>		25		404			424	403	455	
<i>T. multicolor</i>	Lac II	25	510	856	203		130	223	126	
<i>T. ochracea</i>				150			90	80	110	
<i>T. pubescens</i>	LAP 2	25	690	850	400		180	460	320	280
<i>T. pubescens</i>	Lac 1	20	150	280				60	240	
<i>T. pubescens</i>	Lac 2	20	400	370				160	170	
<i>T. sanguinea</i>		30				712 ^b	456 ^c	541 ^c	421 ^c	
<i>T. trogii</i>		25	198			109		30 ^b	33 ^b	
<i>T. versicolor</i>		35	351 ^b			206 ^b				
<i>T. villosa</i>	Lac 1	30	317 ^b							116 ^b
<i>T. villosa</i>	Lac 3	30	266 ^b							40 ^b

^aassay temperature; ^bturnover number calculated from specific activity; ^cturnover number calculated from relative activity; ABTS 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonate); FeCy ferrocyanide; 2,6-DMP 2,6-dimethoxyphenol; HQ hydroquinone; SGZ syringaldazine

Biosensors and biofuel cells

The importance of using biosensors for environmental pollutants is becoming recognized in the literature, with emphasis being given to the detection and control of relevant substances like phenols (49), which are released into the environment by a large number of industries, such as manufacturers of plastics, dyes and drugs, and during pulping and paper production. A phenol-detecting microbiosensor was developed by Freire *et al.* (50), who used a carbodiimide/glutaraldehyde coupling reaction for the immobilization of *T. versicolor* laccase on carbon fibre electrodes. Similarly, laccase from *T. hirsutus* was used by Shleev *et al.* (51) for monitoring lignin and its degradation products. The biosensor consisted of a laccase-modified graphite electrode used in the flow injection mode and was suitable for measuring lignin and its degradation compounds from pulp and paper industry.

Roy *et al.* (49) developed laccase-based cross-linked enzyme crystals (CLECs) used for biosensors to detect phenols at very low concentrations (50–1000 μmol). An additional advantage was obtained by using CLECs: the biosensor had its pH optimum in the range of 5.5 to 6.0, permitting measurements at nearly neutral pH conditions.

Biofuel cells were regarded as 'green' sources of electrical power during the 1980s and the early 1990s (52). Ambitious efforts were undertaken, but the power densities of biofuel cells were still at least a thousand-fold smaller than those of conventional power generators. Although the cells did not succeed in their intended purpose, a useful application might be the construction of membraneless, miniature, disposable glucose/O₂ cells to power autonomous, implanted, medical sensor-transmitters (53). Fungal laccases appear to be well suited for this task, being less substrate-specific and having a higher anodic onset potential for oxygen reduction than plant laccases. On the other hand, *Trametes* laccases have acidic pH optima, which limit their use in neutral solutions. Nevertheless, laccase from *T. versicolor* was immobilized on the cathode of several biofuel cells to provide electrical energy (54–56).

Pharmaceutical applications

There is growing interest in applying laccases from *Trametes* for the synthesis of medicines or compounds of pharmaceutical importance. Laccases from *T. pubescens* were used for the selective oxidation of stilbenic phytoalexin *trans*-resveratrol (3,5,4'-trihydroxystilbene) and gave 18 % of the dehydrodimer, a yield that compares favourably to that for the chemically catalyzed dimerization (57). *trans*-Resveratrol is produced by plants (58) and is thought to act as an anticarcinogen, while its oligomers viniferins are reported to exhibit antimicrobial, anti-HIV and anti-inflammatory activities (59). *Trametes pubescens* laccase was also used in a laccase-mediated oxidation of the steroid hormone 17 β -estradiol to identify reaction products of biological relevance (60), in the laccase catalysed C-C coupling of 5,6,7,8-tetrahydronaphthalen-2-ol to produce dimers that might be used as ligands in asymmetric catalysis (61), and in the selective modification of the saponin asiaticoside, an anti-inflammatory

compound isolated from *Centella asiatica*, by laccase-TEMPO (2,2,6,6-tetramethylpiperidine-1-oxyl) mediated oxidation of the trisaccharide unit (62).

In contrast to the above-mentioned reactions where purified laccase was used as biocatalyst, a study employing *T. rigida*, *T. villosa* or *T. versicolor* mycelium investigated the possibility of producing enantiomerically pure sulphoxides, which are building blocks for the synthesis of pharmaceuticals and biologically active compounds (63). The mycelium, but not the cell-free culture medium oxidised (phenylpropyl)sulphide, producing (S)-(phenylpropyl)sulphoxide with high enantiomeric excess (e.e. ≥ 99 %). This fact almost rules out the suggested participation of laccase in this reaction, but favours cell wall associated enzymes, most likely peroxidases.

Personal care

A new usage for laccase has been published in a patent describing a multistage procedure for whitening teeth with formulations containing laccase from *T. hirsuta* (64). Laccase-based hair dyes cause less irritation as compared to current hair dyeing methods, which use H₂O₂ as an oxidizing agent in dye formulations (65). Consequently, several patents have been published for application of laccases for oxidative hair dyeing (66–69). The formulations for dyeing keratin fibres such as those in human hair contain an oxidising dye precursor, a coupling agent and laccase as the oxidising agent. Laccase has also been successfully used to oxidize, polymerize, and detoxify urushiol, thereby reducing the effect of poison ivy dermatitis (70).

Pulp and paper industry

The use of white-rot fungi, especially *T. versicolor*, to delignify and brighten kraft pulps started a boom of research activities in the 1990s for the application of lignolytic enzymes in the pulp and paper industry. The important events leading to industrial scale trials of *T. versicolor* laccases are given in the review by Call and Mücke (71), who developed the so-called Lignozym[®]-process. This bleaching process was developed by combining laccase from *T. versicolor* with a special group of mediators containing N-OH-, N-oxide-, oxime- or hydroxamic acid-compounds, and was applied to soft and hard wood. The laccase-mediator system continues to be intensively studied in flax pulp bleaching and residual lignin modification (72,73). An interesting current development involves the addition of laccases obtained from *T. hirsuta* to unbleached thermomechanical pulp in a process designed to add value to pulp fibres by modifying their surfaces (74). Another interesting development is the use of the polyoxometalate-laccase (POM) system as an oxidative catalyst in the oxygen bleaching of kraft pulp (75). In the catalytic cycle, POM oxidises the residual lignin in the pulp and the reduced POM is reoxidised by laccase at the same stage. In fact, as early as the mid-1990s, POMs were proposed as highly selective renewable reagents or catalysts for kraft pulp bleaching (76). Various manganese-substituted polyoxotungstanates, [SiW₁₁Mn(H₂O)O₃₉]⁵⁻, [PW₁₁Mn(H₂O)O₃₉]⁴⁻, and [SiW₁₁VO₄₀]⁵⁻, were applied as catalysts for oxygen delignifica-

tion of unbleached eucalyptus kraft pulp with laccase of *T. versicolor*. Unlike the modest results that are obtained in the laccase-mediator system (LMS) at 45–60 °C, a removal of about 50 % of residual lignin was achieved with $[\text{SiW}_{11}\text{Mn}(\text{H}_2\text{O})\text{O}_{39}]^{5-}$ and $[\text{SiW}_{11}\text{VO}_{40}]^{5-}$ when the kraft pulp treatment was carried out with polyoxometalate at 110 °C (lignin oxidation stage) and with laccase at 45 °C (catalyst reoxidation stage) in separate stages.

Recently, there has been a growing interest in using nonwood lignocellulose in the pulp and paper industry. Application of a laccase-mediator system (*T. versicolor* laccase and 1-hydroxybenzotriazole) to a nonwood lignocellulose resulted in up to 90 % delignification, 82 % ISO brightness (compared with 37 % in the initial pulp, and 60 % in the peroxide-bleached control) and a very low kappa number (near 1) (77). Results were also good when the laccase-mediator treatment was performed in a bioreactor under pressurized oxygen. The pulp properties obtained were better than those obtained with conventional thermomechanical bleaching of flax pulp and demonstrate that enzymatic bleaching can feasibly substitute chlorine-containing reagents in the production of paper pulps. For photocopying paper, the *T. versicolor* laccase was more effective than those of *Fomes lividus* and a *Thelephora* sp. Ink paper treatment with *T. versicolor* resulted in a marked reduction of the kappa number and also increased brightness (78). In earlier studies, delignification of *Eucalyptus globules* wood with *T. versicolor* was more effective than that with *P. chrysosporium* (79). Extractives associated with pitch, mainly triglycerides and diglycerides, were effectively removed by *T. versicolor*, demonstrating that it can play a role in controlling pitch in the pulp and paper manufacturing industries (80). *Trametes cingulata* can depolymerise and significantly reduce the kappa number of industrial soft wood kraft pulp brownstock in only one treatment, without a mediator (81). *T. cingulata* does not produce detectable levels of any known lignolytic enzyme, prompting speculation as to the existence of a yet unknown depolymerization-polymerization enzyme system (82).

Call and Mücke (71) extended the application of Lignozym® to the deinking of waste paper and to the treatment of pulp and paper wastewater from production processes and noted that *T. versicolor* laccases were among the best in decolourising kraft effluent. A bagasse-based paper mill effluent containing 2840 colour units was decolourised up to 60 % by *T. versicolor* cultures (83). In another screening experiment, 11 strains of white-rot fungi were tested for their ability to decolourise kraft bleach plant effluent, with *T. versicolor* emerging as the best (84). Over 80 % of the colour of the effluent was removed within 3 days in the shake culture in the presence of glucose, while in the batch reactor, which received an effluent of 7000 colour units, a maximum colour reduction of 93 % was obtained in 48 h, while the chemical oxygen demand was reduced by 35 %. *T. versicolor* successfully decolourized different effluent streams of a small pulp mill utilizing agro-residues, reducing an initial value of 18 500 colour units by 97 % and the initial chemical oxygen demand by 69 % (85). Likewise, *Trametes elegans* was successfully used for direct treatment of spent black liquor from pulping processes (86).

Textile industry applications

In the textile industry, oxidoreductases have been successfully applied in cotton fibre whitening, dye finishing and waste effluent treatment. The use of laccases from *Trametes* is growing very fast, with applications ranging from effluent treatment to improving the quality of textile fibres. The traditional technology for producing denim fabric or jeans reduces the strength of the yarn considerably since it involves washing of the fabrics in the presence of pumice to generate the desired erosion followed by partial bleaching of the fabric with sodium hypochlorite, a neutralization step, and a rinsing step. In addition, this process causes substantial environmental pollution. Recently, enzymatic bleaching has been successfully achieved. Novozyme launched an industrial application, known as Denilite, in 1996. In this process indigo dyed jeans are bleached by laccase bleaches with the help of a mediator molecule (87). The degradation of indigo, both in effluents and on fabrics, with laccase preparations from *Polyporus* sp. and *Sclerotium rolfsii* has also been reported (88). Campos *et al.* (89) further reported degradation of indigo and bleaching on fabrics using purified laccases from *T. hirsuta* and *S. rolfsii* in combination with redox-mediators. Recently, the use of laccase from *T. hirsuta* for pretreatment of cotton has resulted in an increase in the whiteness (90). Laccase is also applied to finished dyed cotton fabric to give a worn-out look (91) or different fabric appearances (92,93).

The possibility to use laccases for *in situ* dye synthesis is currently being explored. For this reason, the fabric is soaked in a precursor dye before adding laccase, which then oxidizes the dye, leading to stronger binding. For example, a laccase from *T. hirsuta* covalently attached to polyethylene glycol successfully prevented re-depositioning of dyes in the solution on fabrics and garments (94).

Textile effluent treatment

There are more than 100 000 commercially available dyes with over 7×10^5 tonnes of dyestuff produced annually (95). Much research has focused on the removal of colour load from textile effluents. The structural similarity of most of the commercially available dyes to lignin constituents, which are the substrates of lignolytic enzymes, has led to extensive exploration of the genus *Trametes* for dye decolourization. Although the molecular design of the dyes is aimed at resisting fading upon exposure to sweat, light, chemicals and microbial attack, these dyes are susceptible to decolourization and degradation by a number of *Trametes* species. The decolourization of dyes by white-rot fungi was first reported by Glenn and Gold (96), who developed a method to measure ligninolytic activity of *P. chrysosporium* based on the decolourization of a number of sulphonated polymeric dyes. Subsequently, other workers adapted the dye decolourization test for evaluating the ability of white-rot fungi to degrade dyes and other xenobiotics (97,98). Although *P. chrysosporium* was the first among the white-rot fungi shown to be able to decolourize dyes, recently a number of species of *Trametes*, including *T. versicolor*, *T. hirsuta*, *T. villosa*, *T. modesta*, *T. pubescens*, *T. trogii*, and *T. multicolor*, have been shown to degrade all classes of dyes. Again, although *T. versicolor* is a species of *Trametes*,

which has been most widely studied for such applications in recent years, other members of the genus, such as *T. modesta* and *T. hirsuta* have begun to challenge it with respect to both expression of lignolytic enzymes and performance (99,100).

Culture conditions have been repeatedly shown to influence dye degradation or decolourization, with the carbon and nitrogen source being of special importance (101–104). Swamy and Ramsay (105) observed that, regardless of the MnP and laccase concentrations at the time of dye addition, nitrogen limitation was required for effective dye decolourization. On the other hand, a direct correlation between lignolytic enzyme production and industrial effluent decolourization was given by Wesenberg *et al.* (106), suggesting a differential inducing effect of the effluent on the production of lignolytic enzymes. In recent works comparing different culture conditions, Rodríguez Couto *et al.* (107–110) observed that it was possible to increase the decolourization of dyes by *T. hirsuta* using agricultural wastes (grape seeds, waste from groundnut processing, banana peels and waste from the brewing industry) under solid-state fermentation, confirming earlier reports by Lorenzo *et al.* (111) who observed similar pattern with *T. versicolor*. Addition of natural lignin molecules like syringic acid, guaiacol, catechol and metal ions (Mn^{2+} and Cu^{2+}) also enhances the decolourization effect of *T. versicolor* (112). Studies on the influence of culture conditions on dye decolourization suggest the need to optimize the physiological conditions in order to maximize performance. In other words, although laccases and manganese peroxidases are the major enzymes involved, other enzymes also influence the decolourization and degradation process. Additionally, the fact that laccases and Mn peroxidase exist in multiple isoforms, which are expressed under different culture conditions, can also add to the observed different performance. It is therefore important to investigate the conditions under which isoforms are expressed.

Studies with crude enzyme extracts and pure enzyme preparations tend to demonstrate a different performance. For example, a crude preparation of MnP from *T. versicolor* decolourized azo dyes (Amaranth, Reactive Black 5 and Cibacron Brilliant Yellow) but it did not decolourize an anthraquinone dye (Remazol Brilliant Blue R, RBBR), while a purified laccase from the same strain decolourized RBBR five to ten times faster than the azo dyes (113). It therefore follows that the structures of dyes and the available enzymes play an important role in determining the outcome of the reactions. In the same study, the authors noted that Amaranth and Reactive Black 5 were decolourized more rapidly by MnP, possibly due to the fact that they have a hydroxyl group in the *ortho* position and a sulphonate group in the *meta* position relative to the azo bond. Using laccase from *T. hirsuta*, Almansa *et al.* (114) observed the influence of structure on dye degradation. Hydroxy-substituted dyes were the most susceptible to enzyme/mediator action during their studies. Similarly, Nyanhongo *et al.* (115) observed that decolourisation was determined by the nature of the enzyme involved, its redox potential in relation to the respective dye and steric effects. In earlier studies with *T. versicolor*, Kadhim *et al.* (116) also noted that during

degradation of phenolic compounds molecules with chlorine substitutions in *ortho* and *para* positions were easily attacked, unlike compounds with substitutions in the *meta* position.

Slow reaction rates with some laccase substrates have been overcome by the addition of redox mediators. Laccases react with mediators and generate highly reactive free radicals that indirectly oxidise heterogeneous phenolic and non-phenolic compounds. Camarero *et al.* (117) showed that lignin-derived compounds were efficient laccase mediators for decolourization of different types of recalcitrant dyes. The presence of mediators increased the rate of decolourization and also enabled the decolourization of Reactive Black 5 (diazo dye) and Azure B (heterocyclic dye), which could not be decolourized in the absence of mediators. Phenolic aldehydes, ketones, acids, and esters related to the lignin units were among the best mediators, including *p*-coumaric acid, vanillin, acetovanillone, vanillate, and above all, syringaldehyde and acetosyringone. Although many studies have shown the ability of different mediators to enhance the decolourization of dyes by different *Trametes* species, there is need to identify those that can be used for all classes of dyes. In other interesting developments, some substrate dyes have been shown to act as mediators during dye decolourization (118). Further investigation is needed to define the structures of these laccase mediator dyes, so that the efficiency of mixed dye effluents can be predicted. Some ions also act as mediators. For MnPs it has been demonstrated that Mn^{3+} ions are stabilized by chelators such as oxalic acid (119), and that these chelated Mn^{3+} are highly reactive low molecular mass, diffusible redox-mediators (120), yet not much work has been done to evaluate its practical significance in dye decolourization. The main factor hampering the development of a Mn-based degradation system is the lack of an efficient and cheap source of hydrogen peroxide.

Although many metals usually used in textile dyes can inhibit fungi, enzymes from *T. trogii* decolourized Remazol Brilliant Blue R in the presence of these metal ions. Mechichi *et al.* (121) and Rong *et al.* (122) also noted that the decolourization of dyes with *T. trogii* occurred in the presence of metal ions that are found in textile industry effluents. Laccase from *T. hirsuta* remained stable for 7 days in the presence of 1 mM of Zn^{2+} , CrO_4^{3-} , Cd^{2+} , $Cr_2O_7^{2-}$, Fe^{2+} , Cu^{2+} and Hg^{2+} (123). However, copper and iron chelators and some anionic detergents found in textile industrial effluents inhibited *Polyporus* sp. and *T. villosa* by up to 20 %, showing the challenges that can be encountered when dealing with textile dye effluent waste.

Although it has been demonstrated beyond any doubt that all classes of dyes can be degraded or decolourized, very few studies identified the decolourization or degradation products or at least evaluated the degradation process. In one of the few studies in which *T. villosa* was shown to decolourize dye effluent, it was demonstrated that the toxic and mutagenic compounds were reduced, as evaluated by the Microtox assay and Ames mutagenicity test (124). However, in a similar study, although 95 % colour removal was achieved in a non-sterile, undiluted carpet dye effluent by *T. versicolor*, the toxicity was unchanged as determined by the Microtox

assay (125). This observation therefore puts a question on most of the previous degradation studies, including those undertaken with other microorganisms, where the adverse effects after degradation were not evaluated nor were the products identified.

Recently a number of publications have appeared on various reactor designs for dye effluent treatment using the species of *Trametes*, namely the rotating biological contacting reactor (126–128), the submerged membrane fungal reactor (129), and the expanded-bed reactor (130). Rodríguez Couto *et al.* (131,132) have shown that agricultural wastes and stainless steel sponge can be used as carriers to immobilize fungal mycelia in the bio-reactor system. A novel submerged membrane reactor with *T. versicolor* proved efficient in removing dyes in a textile wastewater and fouling problems could be avoided through cleaning (133). Successful development of reactors is determined by full understanding of the optimal physiological conditions required for the expression of required enzymes. Studies targeting the regulation of laccase gene transcription in *Trametes* (134) provide the necessary understanding.

Other promising technological developments with potential for enzymatic treatment of effluents include the immobilization of laccase (135). A wide range of complex textile dyes were decolourized efficiently with no limitation to a certain structural group of dyes in an enzyme-reactor with immobilized *T. modesta* laccase (136). However, the attack of dyes with hydroxyl groups at the *ortho* or *para* position relative to the azo bond were preferentially oxidized compared to those with hydroxyl groups at the *meta* position.

Biodegradation of pentachlorophenols

Pentachlorophenol (PCP) has been used extensively as a wood preservative, fungicide, bactericide, herbicide, algaecide, and insecticide. *T. versicolor* has been widely studied for its ability to degrade PCP. In earlier studies the best degradation results of PCP by fixed films of white-rot fungi in rotating tube bioreactors were obtained with *T. versicolor* (62 %), followed by *P. chrysosporium* (38 %) (137). In another field-scale bioremediation of pentachlorophenol by *T. versicolor*, PCP residues decreased from between 800–1000 to 4 mg/kg over a period of 2.5 years (138). *T. hirsuta* was also shown to completely mineralize PCP (139). In a screening experiment for bio-augmentation of contaminated soils to remove PCP with *Irpex lacteus*, *Bjerkandera adusta* and *T. versicolor*, the last emerged the best. *Trametes versicolor* effectively degraded the diphenyl ether herbicides chlornitrofen (2,4,6-trichloro-4'-nitrodiphenyl ether) and nitrofen (2,4-dichloro-4'-nitrodiphenyl ether), which constitute the largest class of diphenyl ether herbicides (140). However, cytochrome P450 and not the extracellular ligninolytic enzymes (lignin peroxidase, manganese peroxidase and laccase) catalyzed the oxidation of both these compounds.

Biodegradation of polycyclic aromatic hydrocarbons

Polycyclic aromatic hydrocarbons (PAHs) are common industrial pollutants, resulting from processes such as coal gasification, coking, and wood preservation (141). Laccase from *T. versicolor* immobilized on kaolinite was efficient in oxidising anthracene and benzo[a]pyrene

with or without mediators (142). Laccase from *T. versicolor* degraded effectively 90–100 % of anthracene and benzo[a]pyrene within 24 h, while laccase from *T. trogii* degraded nitrobenzene and anthracene (143). Whereas in earlier studies *P. chrysosporium* had converted anthracene to anthraquinone, a dead-end metabolite, four *Trametes* strains removed anthracene without significant accumulation of the quinone (144). Laccase of *T. versicolor* in combination with 1-hydroxybenzotriazole (HBT) was able to oxidise acenaphthene and acenaphthylene completely after a 70-hour incubation (145). In earlier studies, the presence of 1-HBT significantly increased the oxidation, by the laccase from *T. versicolor*, of the PAHs acenaphthylene, acenaphthene, fluorene, anthracene, benzo[a]pyrene, and perylene (146). Recently Tekere *et al.* (147) also demonstrated that degradation of PAH with *Trametes pocas*, *T. versicolor* and *T. cingulata* was comparable to *P. chrysosporium*.

Biodegradation of polychlorinated biphenyls

Polychlorinated biphenyls (PCBs) are synthetic compounds that have one to ten chlorine atoms attached to an aromatic biphenol frame, creating 209 theoretical congeners. Twenty to sixty of these are found in commercial products (148). PCBs were widely used for a variety of industrial purposes from 1929 to 1978 (149). Due to their low chemical reactivity, heat stability, non-flammability, and high electrical resistance, they were widely used as dielectric fluids in capacitors and transformers, hydraulic fluid, solvent extenders, and flame retardants (150). A method for dechlorination of materials containing toxic organic chlorinated compounds using *T. versicolor* laccase was developed by Taspinar and Kolankaya (151). Schultz *et al.* (152) found that although *T. versicolor* degraded mono-, di- and trichloro hydroxy PCBs completely, the degradation of tetra- to hexachloro hydroxy PCBs required the presence of mediators. In a screening study with high concentrations of PCBs (up to 3000 mg/L), *T. versicolor* performed better than *P. chrysosporium* and *Lentinula edodes*, degrading both low and high concentrations (153). Manganese peroxidase and laccase from *T. versicolor* effectively degraded 50 % of Delor 106 PCB (154). In other studies, *T. versicolor* laccases degraded hydroxy PCBs (toxic metabolites of PCBs) more rapidly than laccases from *Pleurotus ostreatus* (155). Recently *T. multicolor* was also shown to degrade 2,5-dichlorobiphenyl effectively (156).

Biodegradation of explosives

Biodegradation studies in the field of explosives have been largely directed towards 2,4,6-trinitrotoluene (TNT) since it has been the major explosive massively produced and widely used for both civil and military purposes since the First World War. It is by far the major explosive contaminating former sites used for the production or application of ammunition (157–159). A comprehensive list of fungi able to degrade TNT, found in Hawari *et al.* (157), is a testimony of a widespread ability among these organisms. Success has been hampered by the fact that the fungi are inhibited by low concentrations of TNT. Studies on the possible application of *Trametes* are recent and have been largely directed towards immobilization of TNT degradation products. The adoption of this approach has been due to the observation that biodegra-

duction of TNT predominantly results in the accumulation of aminodinitrotoluenes (AMDNT), azoxy compounds and diaminonitrotoluenes (DAMNT), which are both toxic and carcinogenic. Another motivation for developing fungus-based technologies is the fact that laccases are involved in the formation of humic material (160) and that laccase-oxidized molecules either undergo nonenzymatic reaction among themselves or with molecules in the surrounding milieu, which are not necessarily laccase substrates, leading to the formation of polymers (161). Dawel *et al.* (162) provided the first evidence for the coupling of 2,4-diamino-6-nitrotoluene onto guaiacol using *T. versicolor*. Later Thiele *et al.* (163) demonstrated the ability of laccases from *T. villosa* to facilitate irreversible coupling of TNT and its products to humic and catechol compounds. More recently, Nyanhongo *et al.* (164,165) evaluated the ability to immobilize TNT biotransformation metabolites onto different humic monomers by laccase-producing fungal isolates of *T. modesta*, *T. versicolor* and *T. hirsuta*. Incorporation of humic monomers, especially ferulic acid, guaiacol and catechol, drastically reduced the accumulation of aminodinitrotoluenes during the biotransformation of TNT. Immobilization experiments with the major individual TNT metabolites, using pure *T. modesta* and *T. hirsuta* laccase in the presence of either guaiacol, syringic acid, ferulic acid or catechol, showed that immobilization decreased in the order 2-hydroxylaminodinitrotoluenes (HADNTs) > AMDNTs > DAMNTs, although azoxy compounds were effectively immobilized. HADNTs, which are the first TNT biodegradation metabolites, were completely immobilized in the presence of humic monomers. The study concluded that for effective immobilization to be achieved, incorporation of humic monomers during the initial stages of degradation by laccase mediators is essential. Acute toxicity tests conducted during the biodegradation and immobilization process in *T. modesta* cultures also showed a decrease in toxicity. This strategy is attractive for application in highly contaminated soils and in process wastewater, as it will drastically reduce the spread of TNT and its degradation products. The practical application of such a strategy is quite feasible since humic substances are readily available in nature and they can be easily added to TNT wastewaters or sediments.

Biodegradation of various pollutants

The use of fungal strains in biofilters targeted at removing organic pollutants from air is quite attractive since they secrete extracellular enzymes. *T. versicolor* has been shown to be efficient in removing certain organic pollutants from waste gas (166).

Fungi have also been used for bioremediation of soils. *T. versicolor* proved to be more efficient than *P. chrysosporium* and *Pleurotus ostreatus* in the degradation of oil from contaminated soils degrading 25 g/kg soil in 12 months (167).

Fermentation of wood hydrolysates for ethanol production is made difficult by the presence of inhibitory compounds. Use of a laccase from *T. versicolor* to treat lignocellulosic hydrolysates from willow that had been pretreated with steam and SO₂ led to detoxification by removing monoaromatic phenolic compounds, which

would otherwise inhibit *Saccharomyces cerevisiae* in the subsequent fermentation (168). The removal of these compounds resulted in increased ethanol production.

Canola meal (the oil-free residue from rape seed oil extraction) is a good and cheap source of protein for animals and is a particularly rich source of the sulphur amino acids, methionine and cysteine, but contains very high levels of glucosinolates, fiber, phytate, and phenolics, which reduce its value for animal feed. Lacki and Duvnjak (169–171) used an enzyme preparation from *T. versicolor* containing laccase to remove soluble phenolic acid esters and other phenolic compounds from commercial canola meal. A decrease by more than 98 % (judged by the reduction of sinapic acid ester) together with an only slight reduction of the meal protein content was reported to be more efficient than other tested methods.

Cellobiose Dehydrogenase

History and *in vivo* function

Cellobiose dehydrogenase [cellobiose:(acceptor) 1-oxidoreductase, EC 1.1.99.18] is an extracellular flavocytochrome, discovered in 1974 by Westermark and Eriksson in *Trametes versicolor* (172) and shortly thereafter in *Phanerochaete chrysosporium* (173) during screening on kraft lignin-cellulose agar containing cellobiose. The typical darkening of the agar below and around the mycelium after 5 days of growth indicated the oxidation of phenolic substances by laccase activity (Bavendamm reaction; 174), but there was also a bleached zone (indicating quinone reduction) dividing the darkened area into an inner and an outer circle. An enzyme mixture was extracted from the bleach zone. It oxidised the laccase substrate guaiacol due to laccase activity, but after the addition of cellobiose, the brown colour of the oxidised guaiacol disappeared. The enzyme responsible for this reduction was isolated and is now called cellobiose dehydrogenase.

Cellobiose dehydrogenase (CDH) is secreted by several wood-degrading, phytopathogenic and saprophytic fungi from the phyla of Basidiomycota and Ascomycota (17,18) in the presence of cellulose or cellobiose during the exponential phase of growth together with cellulolytic, hemicellulolytic, or ligninolytic enzymes (175). In the *Trametes* family CDH occurrence has been reported for *T. versicolor* (176), *T. cotonea*, *T. gibbosa*, *T. hirsuta*, *T. incerta*, *T. maxima*, *T. meyenii*, *T. multicolor*, *T. pubescens*, *T. suaveolens*, and *T. villosa* (177). Although its *in vivo* function is not totally clear, CDH is certainly involved in the degradation of the two most prominent biopolymers, cellulose and lignin. This was elegantly demonstrated by creating a CDH-deficient *T. versicolor* mutant that had a greatly decreased ability to colonize fresh or seasoned native birch wood (178). A mechanism proposed by Kremer and Wood (179) suggests the degradation or depolymerization of cellulose, hemicellulose, and lignin by the generation of hydroxyl radicals in a Fenton type reaction *via* the enzyme-catalysed reduction of Fe³⁺ to Fe²⁺. This mechanism could also provide fungi without special lignin degrading enzymes a limited lignolytic activity (*i.e.* brown-rot (180), and plant pathogenic fungi).

The production of reactive oxygen species by CDH has been confirmed by Mason *et al.* (181,182). The authors suggest that CDH can produce both reagents needed for Fenton chemistry, namely H_2O_2 and ferrous iron complexes, which may migrate into and disrupt the lignocellulose matrix.

Molecular and catalytic properties

Typically, CDH is a monomeric protein with a bipartite domain organisation, consisting of an N-terminal heme domain containing a cytochrome *b*-type heme and a C-terminal flavin domain with a noncovalently bound flavin-adenine-dinucleotide (FAD). The flavin domain is loosely related to several other FAD containing oxidases and dehydrogenases in the glucose-methanol-choline (GMC) oxidoreductase family. Based on the reported sequences, a phylogenetic tree of all currently known CDH sequences was constructed (183).

Comprehensive characterization of *Trametes* CDH has been performed for four species, namely *T. versicolor* (176), *T. villosa*, *T. pubescens* (177), and *T. hirsuta* (184). The molecular mass, determined by SDS-PAGE, is nearly identical, being 97 000 for the CDH of *T. versicolor*, 98 000 for that of *T. villosa*, and 100 000 for that of *T. pubescens*. Although not reported, it is likely that the enzymes are glycosylated like other members of the CDH family (185,186). The isoelectric points are 4.2 (*T. versicolor* CDH), 4.4 (*T. villosa* CDH), and 4.3 (*T. pubescens* CDH), these values being consistent with the reported values of other basidiomycete CDHs.

The catalytic cycle of CDH is divided into a reductive half-reaction and an oxidative half-reaction. During the reductive half-reaction the natural substrate β -D-cellobiose and higher cellodextrines are oxidised at the anomeric C1 carbon atom to yield cellobionolactone, which is further hydrolysed in bulk water to the corresponding aldonic acid (187). *In vitro* the β -1,4-linked disaccharides lactose and mannobiose are acceptable substrates, whereas the α -1,4-linked maltose or the monosaccharide glucose are poor substrates, as indicated by the relatively low values of their specificity constants (*i.e.*, k_{cat}/K_m).

The reduced enzyme can be reoxidized by two-electron acceptors such as oxygen, 2,6-dichloroindophenol (DCIP), methylene blue, and several quinones, or one-electron acceptors like the 2,3'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid) (ABTS) cation radical, complexed metal ions such as Fe^{3+} and Mn^{3+} , and even cytochrome *c* (176). Kinetic parameters for four *Trametes* CDHs are shown in Table 2.

Applications

The catalytic properties of *Trametes* and other basidiomycete CDHs are used in several interesting applications in two main areas: analytical usage in biosensors and enzymatic assays for the detection and quantification of substrates and analogous molecules, as well as biotechnological applications in pulp and paper processing, bioremediation/biodegradation, and biocatalysis. The high cost of CDH has discouraged the use of CDH in industrial processes, although heterologous expression of several CDHs in *Pichia pastoris* might change the situation.

Table 2. Kinetic parameters of cellobiose dehydrogenases from *Trametes* species

	Species (Ref. no.)														
	<i>T. hirsuta</i> (184)			<i>T. pubescens</i> (177)			<i>T. versicolor</i> (176)			<i>T. villosa</i> (177)					
Electron donors^a	pH in assay, K_m/mM, k_{cat}/s^{-1}														
Cellobiose	5.0	0.042	10.7	4.5	0.21	21.9	4.5	0.12	6.1	4.5	0.21	23.6			
Cellobiose	5.0	0.22	3.5												
Cellotetraose	5.0	1.40	9.5	4.5	0.42	15.0				4.5	0.47	14.2			
Cellopentaose				4.5	0.46	13.9				4.5	0.51	14.7			
Lactose	5.0	2.13	6.9	4.5	2.4	25.8				4.5	3.7	26.9			
Maltose				4.5	390	1.9				4.5	350	2.1			
Glucose				4.5	890	1.3				4.5	1300	1.9			
Electron acceptors^b	pH optimum, pH in assay, $K_m/\mu M$, k_{cat}/s^{-1}														
Dichloroindophenol	5.0	5.0	12.2	20.9	4.5	4.5	4.9	25.6	4.5	11	37	4.5	4.5	9.9	38.1
1,4-Benzoquinone					4.5	4.5	19	27.5				4.5	4.5	11	20.8
TBBQ	5.0	38.9	11.7		4.5	4.5	18	24.2	4.5	26	25	4.5	4.5	41	16.9
Cytochrome <i>c</i>	5.0	1.3	1.1		3.5	3.5	0.7	29.4	4.5	7.8	10.2	3.5	3.5	1.8	31.5
ABTS cation radical					4.0	4.0	0.3	18.1				4.0	4.0	0.2	16.1
Ferricyanide	5.0	214	7.0		4.0	4.0	8.6	27.8	4.5	10	5.3	4.0	4.0	5.5	36.9
Ferricenium ion					4.5	4.5	1.0	34.7				4.5	4.5	1.2	35.6

^ameasurements were performed at 25 °C (*T. hirsuta* CDH, *T. versicolor* CDH) and 30 °C (*T. villosa* CDH, *T. pubescens* CDH) with 2,6-dichloroindophenol as electron acceptor

^bmeasurements were performed with cellobiose at 25 °C (*T. hirsuta* CDH, *T. versicolor* CDH) and with lactose as electron donor at 30 °C (*T. villosa* CDH, *T. pubescens* CDH)

ABTS 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonate); TBBQ 3,5-di-*tert*-butyl-1,2-benzoquinone

Biosensors

Although soluble CDH has been applied in enzymatic assays for the determination of cellobiose produced in the cellulase saccharification process (188,189), to monitor chromatographic cellobiohydrolase purification (190), and to quantify lactose in milk (191), the usage in biosensors is much more promising.

The first approaches towards the detection of cellobiose and lactose had been based on mediated electron transfer (MET). Therefore, the so-called second-generation biosensors were created by immobilizing CDH from *P. chrysosporium* on a polyvinyl pyridine polymer functionalised with osmium complexes and polyamine groups (Os-pVP-PA) on a rotating disc electrode (192,193), a polyvinyl pyridine polymer functionalised with osmium complexes and ethylamine groups (Os-pVP-EA) (194), and an osmium-based hydrogel on a graphite electrode. Each of them was used together with a second electrode containing an oligosaccharide dehydrogenase in a dual-enzyme electrode cell (195). Due to the catalytic properties of basidiomycete CDHs, the sensitivity for glucose is several orders of magnitude lower than for cellobiose. The discrimination of reducing monosaccharides is an interesting feature allowing selective measurements of β -1,4-linked disaccharides in the presence of usually interfering monosaccharides. In a more recent approach, *T. versicolor* CDH was used to detect lactose with a third-generation biosensor using the excellent direct electron transfer (DET) characteristics of the enzyme (196). With the enzyme simply immobilized on a graphite electrode surface by physical adsorption, the electrode was used in a wall-jet amperometric cell. A detection limit of $1 \mu\text{M}$ lactose with a linear range of detection from 1 to $100 \mu\text{M}$ makes it the currently most sensitive biosensor for lactose.

First-generation biosensors were constructed for the detection of electron acceptors. The underlying principle is the reduction of the analyte by CDH followed by re-oxidation at the electrode, thus giving an amperometric signal. The detection of *ortho*- and *para*-diphenolic compounds was accomplished with CDH directly absorbed onto graphite electrodes. The sensor efficiently discriminated between diphenols and monophenols, and was sensitive enough to detect these at concentrations as low as 5 nM (197,198). The same electrode was used in an enzyme flow immunoassay as the label detector. The substrate 4-aminophenol was oxidised at the electrode surface forming 4-iminoquinone, which was reduced back by CDH in the presence of cellobiose. This cycling of the analyte was used to amplify the signal (199). Stoica *et al.* (200) used a modified graphite electrode for the detection of catecholamines in the flow injection mode. The sensitivity, linear range, and amplification factor were modulated by the cellobiose concentration in the buffer, the cellobiose being used to recycle the catecholamines between the graphite electrode and the reduced CDH. The result of this is an amplified response, enabling detection limits below 1 nM .

Biotechnology

The application of CDH in processes for the industrial, enzymatic conversion of carbohydrates requires purified enzyme. The high enzyme costs have to be off-

set by efficient usage or highly priced products. To use the enzyme efficiently, a regeneration system employing a redox mediator and laccase was suggested for the production of lactobionic acid (201). The process provides a high total turnover number for both enzymes, mainly depending on the applied redox mediator. The highest specific productivity of a typical conversion was reported to be $32 \text{ g}/(\text{L}\cdot\text{h})$, which is high compared to other enzymatic processes (202). A schematic presentation of the regeneration cycle, proposed for several flavoprotein dehydrogenases/oxidases, can be seen in Fig. 1.

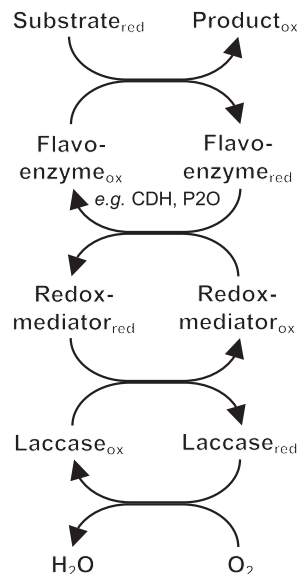


Fig. 1. Schematic presentation of the proposed regeneration scheme for cellobiose dehydrogenase and pyranose 2-oxidase employing laccase for efficient redox mediator recycling

The use of this enzyme system for the production of a lactose-free galactooligosaccharide mixture was also proposed. After transgalactosylation, the residual lactose is converted to lactobionic acid by the enzymatic reaction described above, the lactobionic acid being easily removed by ion-exchange chromatography. The product obtained was of considerably higher purity than other available products (203).

Waste removal and bioremediation

The application of CDH in the removal of compounds harmful to the environment has been proposed and two degradation routes have been suggested: a non-specific, free radical mechanism for depolymerisation by hydroxyl radicals, produced in a Fenton type reaction (204), and the formation of carboxylate radicals in the presence of oxalate and ferric iron (205). However, the use of CDH in this area is limited to the whole-cell approach and the effects described are a result of the action of several enzymes including CDH.

Enzymatic treatment of paper pulp

Several authors suggested that CDH could be used as part of an enzyme complex to modify or break down cellulose and lignin in the pulp and paper industry (206). Their suggestion was to use it similarly to other

ligninolytic enzymes in the presence of complexed iron. A synergistic interaction of CDH with another ligninolytic enzyme, manganese peroxidase, has been reported (207). Reports on the successful use of CDH in kraft pulp delignification/bleaching involve the *Schizophyllum commune* enzyme (208) and the *Humicola insolens* enzyme (209). The degree of bleaching has been moderate in all these experiments. Additionally, the unspecific attack of hydroxyl radicals also affects cellulose fibres. The supplementation of Douglas fir kraft pulp with CDH, cellobiose, and iron resulted in a substantial reduction in the degree of polymerisation of the pulp cellulose (210). The use of basidiomycete CDH for bleaching is not feasible because of the high pH applied and the high enzyme costs, but pretreatment with whole organisms is a promising strategy.

Pyranose 2-Oxidase

History and catalysis

The enzyme pyranose 2-oxidase (P2O) (pyranose: oxygen 2-oxidoreductase; EC 1.1.3.10), which catalyzes the oxidation of several aldopyranoses at position C2 to yield the corresponding 2-ketoaldoses (aldos-2-uloses, osones), was first discovered by Ruelius *et al.* in 1968 (211). It is widely distributed among wood-degrading basidiomycetes (212–214). It has been purified and characterized from several microorganisms, including *Phanerochaete chrysosporium* (215), *Phlebiopsis gigantea* (216), *Pleurotus ostreatus* (217), and unidentified basidiomycete no. 52 (218). In *Trametes* sp., P2O was first described by Machida and Nakanishi (219) in *Trametes (Coriolus) versicolor*, and later in *T. multicolor* (220) and *T. pubescens* (221). *Trametes* P2O exhibits significant oxidative activity on a number of carbohydrates present during wood degradation and transfers the two electrons obtained to molecular oxygen, thus forming hydrogen peroxide (19). From ultrastructural and immunocytochemical studies, it is known that P2O is primarily localized in the periplas-

mic space (222). Only during autolysis, which occurs at later stages of development, it is located extracellularly, and under these circumstances it is primarily associated with the fungal cell walls or with extracellular slime. This preferential distribution in the peripheral regions of the fungal cell wall cytoplasm is consistent with previous reports of H₂O₂ production in white-rot fungi under ligninolytic conditions (223,224). The hydrogen peroxide produced by P2O may be used as cosubstrate by two lignin-degrading enzymes, namely lignin peroxidase and manganese peroxidase. Thus the main metabolic role of P2O appears to be a constituent of the fungal ligninolytic system (222).

T. multicolor P2O is a rather large, homotetrameric protein that contains covalently bound flavin adenine dinucleotide (FAD) (225). The *in vivo* substrates of P2O are presumably D-glucose, D-galactose, and D-xylose, which are abundant in lignocellulose and which are oxidized to 2-keto-D-glucose (D-*arabino*-hexos-2-ulose, 2-dehydro-D-glucose), 2-keto-D-galactose (D-*lyxo*-hexos-2-ulose, 2-dehydro-D-galactose), and 2-keto-D-xylose (D-*threo*-pentos-2-ulose, 2-dehydro-D-xylose), respectively. In addition, P2O also exhibits significant activity with a number of other carbohydrates, including L-sorbose, D-glucono-1,5-lactone, and D-allose (226). The substrate selectivity, however, varies to some extent among P2Os isolated from different fungi. *T. multicolor* P2O almost exclusively oxidizes sugars at position C2, whereas *T. versicolor* P2O can also oxidize the C3 atom (227). The enzyme-substrate complex for *TmP2O* was simulated in docking experiments by Hallberg *et al.* (225); these experiments suggested a more favourable positioning with the C2 of glucose in proximity of the electron withdrawing N5 of the FAD. After reduction of the FAD, the electrons are transferred to molecular oxygen, resulting in the formation of hydrogen peroxide (19), or to other electron acceptors shown in Table 3. The reported glycoside transfer reaction of *Phanerochaete gigantea* P2O (19) was not found in *Trametes multicolor* P2O (C. Leitner, unpublished results).

Table 3. Kinetic parameters of pyranose 2-oxidases from *Trametes* species

	Species (Ref. no.)			
	<i>T. multicolor</i> (220)	<i>T. multicolor</i> (220)		<i>T. versicolor</i> (219)
Electron donors ^a	<i>K_m</i> /mM	<i>k_{cat}</i> /s ⁻¹	Rel. act./%	Rel. act./%
D-Glucose	0.74	54	100	100
L-Sorbose	38	53	99	3.4
D-Xylose	30	30	56	2.6
D-Galactose	9.2	3.1	5.7	0.9
Electron acceptors ^b				
Oxygen	0.09	71	100	100
1,4-Benzoquinone	0.30	270	380	
2,6-Dichloroindophenol	0.094	150	210	2.5
ABTS cation radical	0.07	21	30	

^ameasurements were performed at 30 °C (*T. multicolor* P2O) and 37 °C (*T. versicolor* P2O) measuring the generation of hydrogen peroxide from dissolved molecular oxygen in a peroxidase coupled assay at pH=6.5 (*T. multicolor* P2O) and 7.0 (*T. versicolor* P2O)

^bmeasurements were performed with glucose as electron donor at the same temperatures and pH values as in (^a) except 1,4-benzoquinone and 2,6-dichloroindophenol (*T. multicolor* P2O, pH=4.5)

Applications

Biocatalysis and food technology

For the last two decades, P2O has received increased attention as the key biocatalyst in several biotechnological applications. P2O was used as the key biocatalyst in the Cetus process, which was patented in 1981 for the conversion of D-glucose to D-fructose *via* the intermediate 2-keto-D-glucose (228–230). Despite the fact that this process never went beyond pilot plant scale, there is still some interest in it (231–233), because in a similar way D-galactose can be converted to D-tagatose (234,235). D-Tagatose has the potential for use as a low-calorie sweetener with sweetness comparable to that of sucrose, it is non-cariogenic and also exhibits a prebiotic effect (236).

Several 2-ketosugars can be efficiently produced by coupling the pyranose 2-oxidase *via* a redox mediator (e.g. benzoquinone) to laccase as a regenerating enzyme (201). The scheme of the regeneration system is shown in Fig. 1.

When pyranose 2-oxidase is added to dough intended for use in the preparation of baked products, it oxidizes dough constituents and thereby serves to improve the strength of gluten structures in the dough or baked products. This results in an overall improved strength of the dough, in addition to better rheological and handling properties. Furthermore, the gluten strengthening effect mentioned above may result in an increased volume and an improved crumb structure and softness of the baked product, as well as an increased strength, stability and reduced stickiness of the dough, thus resulting in improved machinability. The effect on the dough may be particularly advantageous when poor quality flour is used. The improved machinability is of particular importance in connection with dough that is to be processed industrially (237).

Personal care

In oxidative hair dyes, usually an oxidation dye (*p*-phenylenediamine and *p*-aminophenol in alkaline solution) and an oxidizing agent (hydrogen peroxide) are mixed upon use and applied to hair. Both, the alkaline dye solution and the hydrogen peroxide lead to serious skin irritation and hair damage. By using P2O instead of hydrogen peroxide in neutral solution, these problems can be avoided (238).

Analytcs and biosensors

Pyranose 2-oxidase was suggested for the quantitative determination of two important substrates. In a glucose assay it has two main advantages over the commonly used glucose oxidase: a higher affinity for the substrate and no selectivity for either the α - or β -anomer of glucose, which improves the response time of the reaction. The detection of 1,5-dehydroglucitol, a natural glucose analogue and important clinical marker for hyperglycemia or renal dysfunction, was proposed by Yabuuchi *et al.* (239). Several assays were developed and are used in clinical trials (240–246).

To construct a P2O biosensor, the enzyme was immobilised on electron mediating osmium redox polymers (247). This 'wiring' of the enzyme to the electrode

enabled a measurement of glucose that was independent of oxygen concentration. This was possible because the high electron collecting efficiencies of the osmium redox polymers compete very well with molecular oxygen, the natural reoxidising agent for P2O.

Conclusions and Evaluation of Future Potential

One aim of this review was to show the multitude of applications of *Trametes*, either the intact organism or isolated enzymes thereof, which have been proposed, tested at pilot-scale, or realized in a variety of biotechnological applications. Based on the defined catalytic properties of the purified oxidoreductases laccase, cellobiose dehydrogenase, and pyranose 2-oxidase, low-volume high-cost analytical applications like biosensors were developed. The application of homogenous enzymes or of mixtures with defined composition also ensures specific reactions of substrates without side-products being formed, but only in a small fraction of the examples shown was the profit high enough to justify the use of the expensive biocatalysts. Sometimes, specialised mediators are used to enhance or enable desired reactions. Although *Trametes* possesses efficient machinery for the production of the three mentioned enzymes, heterologous expression in suitable fungal or yeast hosts might reduce enzyme costs further. Together with advances in reaction engineering, this aspect will enable broader usage. A third field of application is bioremediation or waste treatment. Certainly, the pressure for low-cost treatments is most pronounced in these high-volume applications, favouring the use of the whole microorganism or unpurified culture broth. As demonstrated by several reviewed articles, the concerted attack of *Trametes* ligninolytic enzymes can handle substances that otherwise would be difficult to degrade. It is quite reasonable to propose that, based on the numerous catalytic properties of the described enzymes, many new applications will be found in the near future, thereby highlighting a versatile genus of white-rot fungi: *Trametes*.

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