ELSEVIER

Journal of Molecular Catalysis B: Enzymatic 6 (1999) 29–39

JOURNAL OF MOLECULAR CATALYSIS B: ENZYMATIC

Immobilization of laccase from *Cerrena unicolor* on controlled porosity glass

J. Rogalski [a,*], A. Dawidowicz [b], E. Jóźwik [a], A. Leonowicz [a]

[a] *Department of Biochemistry, Maria Curie-Skłodowska University, M.C.-Skłodowska Place 3, 20-031 Lublin, Poland*
[b] *Department of Chemical Physics, Maria Curie-Skłodowska University, M.C.-Skłodowska Place 3, 20-031 Lublin, Poland*

Received 26 August 1997; accepted 29 April 1998

Abstract

White-rot basidiomycete *Cerrena unicolor* grown in non-induced and induced conditions was tested for production of laccase, lignin peroxidase (LiP) and manganese-dependent peroxidase (MnP). A typical correlation between the concentration of phenolic compounds in the culture fluid and the extracellular laccase activity was observed. The heterogeneous crude laccase preparation obtained after the non-induced fermentor cultivation was immobilized both on controlled porosity glass (CPG) activated by γ-aminopropyltriethoxysilane (APTES) and on CPG with its surface covered by dextran layers. The laccase activities were tested in the aqueous solution for the native and immobilized preparations using different pH and temperature conditions. Laccase activities were additionally examined for native and immobilized forms of laccase preparations in the aqueous solution containing organic solvents. The greatest activity toward the substrate used in the presence of organic solvents was shown by the laccase preparation coupled with the CPG covered by a dextran layer. Potential inhibitors such as thioglycolic acid, thiourea and EDTA used in 1-mM concentration did not show inhibiting properties towards the laccase preparations.

Keywords: *Cerrena unicolor*; Controlled porosity glass; Immobilization; Laccase; Organic solvents

1. Introduction

Bioconversion of lignin is one of the most substantial processes of the carbon cycle which occurs in the biosphere. In spite of the importance of lignin degradation, the pathways of its decomposition have not yet been completely described [1,2]. According to the literature [1–10], the white-rot fungi *Trametes* (*Coriolus*, *Polyporus*) *versicolor*, *Phanerochaete chrysosporium* and *Phlebia radiata* belong to the most efficient lignin-degrading microorganisms. Their enzymatic systems and especially such enzymes as lignin peroxidase (LiP), manganese-dependent peroxidase (MnP) or laccase are the main biocatalysts taking part in the process of biodegradation. Fungal laccase (benzendiol: oxygen oxidoreductase, EC 1.10.3.2) catalyses the demethylation reaction [11] (which is an important and initial step of the biodegradation process of the polymer chain), and subsequently decomposes the lignin macromolecule [12], by splitting aromatic rings [13] and C_α–C_β bonds in the phenolic substructures [14]. In the process

* Corresponding author

1381-1177/99/$ - see front matter
PII: S1381-1177(98)00117-9

of oxidizing of many compounds (mainly of phenolic type), laccase shows broad specificity in the detoxification process of a number of aquatic and terrestrial xenobiotics [15], industrial waste waters [16] and biotechnological industry products [17]. In this respect, increasing laccase production and activity is a fundamental task in view of its industrial application.

The production of laccase by *T. versicolor* and *P. radiata*, the fungi known as the best laccase producers, can be stimulated by low molecular-weight compounds [18,19], lignin preparations [20,21] and toxic compounds of aromatic origin [20,22]. The removal of oxygenated products of high toxicity, originating from the used laccase inducers, is in these cases the most expensive step during the laccase concentration and purification procedure. Consequently, the strenuous purification process decreases the biotechnological potential of *Trametes* and *Phlebia*.

Recently, Leonowicz et al. [23] found a new fungal source of extracellular laccase, *Cerrena unicolor*, which produces this enzyme under non-induced conditions at the same rate as that of *Trametes* or *Phlebia* after induction.

Two points are discussed in this paper. The first is the optimization of extracellular laccase production with *C. unicolor* C-139 which was grown in a shake flask and fermentor culture under both induced and non-induced conditions. The other is the activity and kinetic properties of native and immobilized forms of crude enzyme preparations obtained from the *C. unicolor* culture medium following partial concentration.

Due to its complex chemical composition (polymer of phenylpropionic units), lignin is not degraded readily by enzymatic systems of living organisms. On the basis of the observation that lignin dissolves in certain organic solvents, previous research indicates a possible application of lignin-degrading enzymes in non-aqueous solutions [24,25]. In this context, the possible application of laccase from *C. unicolor* in non-aqueous media is also examined.

2. Materials and methods

2.1. Strain and culture conditions

C. unicolor C-139 was obtained from the culture collection of the Regensberg University. The fungus was maintained in 2% (w/v) malt agar slants. As an inoculum, pieces of agar were grown in the Lindenberg and Holm medium [26] in non-agitated conical flasks for 14 days at 28°C. The mycelial mats were subsequently collected and homogenized in a Waring blender.

After inoculation with 4% (v/v) crumbled fungal mats, the cultures were run at 28°C in 300-ml wide-mouthed Erlenmeyer flasks (each of them containing 100 ml of culture medium) placed on a rotary shaker (180 rpm).

In order to investigate the effect of certain inducers on the growth and activity of *C. unicolor*, the aromatic compounds (veratric acid, ferulic acid and 2-amino-1,4-dimethylbenzole) were added on the ninth day after inoculation; their final concentration was 1.0 mM or 0.02 mM [20]. The solutions of the inducers were filter-sterilized using a 0.2-μm filter.

The fermentor scale cultivation was performed at 28°C in a 5-l Bioflo III (New Brunswick) fermentor containing 3 l of the sterilized (0.075 MPa, 30 min) Lindenberg and Holm medium with 1% glucose. The fermentor was inoculated with crumbled fungal mats as described above (10% of total volume), aerated at 2-l air per minute and stirred at 300 rpm. Antifoam 289 emulsion (Sigma, St. Louis, USA) was occasionally added to the fermentor cultures.

2.2. Determination of enzyme activities

Syringaldazine (Aldrich, USA) as the substrate for laccase [27] and veratryl alcohol (Aldrich) as the substrate for LiP [28] were used in the culture liquids to determine laccase and LiP, respectively. Both activities were measured in nkat per liter of the culture filtrate. MnP activity was assayed by means of the phenol red method

[29] which was modified in order to avoid the application of NaOH in the reaction mixtures. During these measurements the change of absorbance A_{520} for 5 min at 30°C was studied as described before [30]. MnP activity was measured in arbitrary units (U) per milliliter of the culture filtrate.

The activity of immobilized laccase was measured using polarographic analysis with a Clark-type oxygen electrode (Rank, Cambridge, Great Britain) connected to a linear TZ 4100 recorder (Laboratorni Pristroje, Praha, CSRS) [22]. The Michaelis constant (K_m) values were calculated from the double reciprocal plots [31] using ENZFITTER (1987, Biosoft, USA) software and an IBM-AT computer (Siemens, PCD-2M).

2.3. Determination of phenolic compounds

The total amount of phenolic compounds in the culture broth was determined calorimetrically with diazosulphanilamide (DASA) [32].

2.4. Preparation of supports for enzyme immobilization

The obtained enzyme was immobilized on two types of support materials prepared from controlled porosity glass (CPG): CPG activated by γ-aminopropyltriethoxysilane (APTES) (support I) and CPG with the surface covered by two polysaccharide layers—one formed of cross-linked DEAE–dextran and the other prepared from cross-linked dextran (support II). The CPG was obtained according to the method described elsewhere [33].

2.4.1. CPG activation by APTES—support I preparation

The support ($S_{BET} = 87\ m^2\ g^{-1}$; $D = 71$ nm) was activated by APTES from Sigma according to the method which allows for coupling of a significant amount of aminopropyl radicals on the glass surface [34]. The activated support (APTES-CPG) was subsequently used for laccase immobilization.

2.4.2. Coverage of the CPG surface by polysaccharide layers—support II preparation

2.4.2.1. Formation of DEAE–dextran layer. A total of 1.5 g DEAE–dextran (Pharmacia, Uppsala, Sweden) (MW 500 kD) dissolved in 25-ml doubly distilled water (pH adjusted to 11.5) was added to 10 g of CPG at room temperature. After evaporation of the water under vacuum the support was dried at 80°C for 15 h. The formed DEAE–dextran layer was cross-linked by diethyleneglycol–diglycidyl ether (EDG-2) (IChP, Warsaw, Poland) in an aqueous medium according to the procedure described in Ref. [35].

2.4.2.2. Formation of the second layer composed of native dextran. The second layer of polysaccharide was introduced following the procedure described above. Instead of DEAE–dextran, pure dextran (MW 110 kD) (POLFA Pharmaceutical Factory, Kutno, Poland) was employed as in Ref. [36].

2.5. Immobilization of laccase

The crude preparation of laccase was covalently coupled with support I using glutaraldehyde (Merck, Germany) (material I-GA) or 1,1′-carbonyldiimidazole (CDI; Fluka-AG, Switzerland) (material I-CDI) as described by Lappi et al. [37] and Kaufman and Pierce [38], respectively.

The attachment of laccase preparation to support II (CPG covered by polysaccharide layers) was carried out using CDI (material II-CDI) as described in Refs. [38,39].

2.6. Chemicals

2,6-Dimethoxyphenol (2,6-DMP), caffeic acid, ferulic acid, syringic acid and guaiacol

were obtained from Aldrich (Steinham, Germany), vanillic acid, veratric acid from Sigma and methoxyhydroquinone from EGA-Chemie (Steinheim/Albud, Germany). The organic solvents: dimethylsulphoxide (DMSO), dioxane (LiChrosolv), ethanol (LiChrosolv, 99.5%), acetone (LiChrosolv) were obtained from Merck (Darmstadt, Germany). Formamide, ethylene glycol, thioglycolic acid, NaN_3, and EDTA were obtained from Merck, whereas thiourea was obtained from Sigma.

3. Results and discussion

3.1. Fermentation profile and induction of enzymes

C. unicolor was found to be a pathogen on two northern hardwood tree species causing a canker-rot [40]. In a non-induced culture this fungus produces an amount of extracellular laccase comparable with the laccase level formed by *Phlebia* [19], *Trametes* [22] or *Pleurotus* [41] under induced conditions.

The increase of laccase as well as LiP and MnP production by the above mentioned fungi is stimulated by low molecular aromatic compounds and lignin preparations. The question which arises is whether the same substances affect in a similar way the production of extracellular ligninolytic enzymes by *C. unicolor*.

Table 1 presents the dynamics of culture parameter of *C. unicolor* (performed under shake flask conditions) induced with veratric or ferulic acid or xylidine (at 1 and 0.02 mM) on the ninth day of the cultivation period (when more than 60% of glucose was already consumed).

Table 1
Dynamics of culture parameters during *C. unicolor* growth in a shake flask

Time [days]	Type of inducer	Inducer concentration [mM]	Glucose concentration [mg/ml]	Laccase activity [nkat/l]	MnP activity [U/ml]
0	–	0.0	10.0	0.0	0.0
3	–	0.0	7.9	10.0	0.0
5	–	0.0	6.1	55.6	0.0
7	–	0.0	5.2	130.4	0.0
8	–	0.0	3.9	368.4	0.0
9	–	0.0	2.6	498.7	0.0
10	–	0.0	1.9	686.2	0.0
	veratric acid	1.0	1.9	489.3	0.0
		0.02	1.6	877.0	0.0
	ferulic acid	1.0	1.2	1930.0	2.35
		0.02	1.3	673.2	0.0
	xylidine	1.0	2.4	0.0	0.0
		0.02	1.6	551.2	0.0
12	–	0.0	0.9	3650.2	0.0
	veratric acid	1.0	1.0	1980.0	1.98
		0.02	0.9	2210.1	1.14
	ferulic acid	1.0	0.8	3810.1	5.9
		0.02	0.9	2919.5	0.22
	xylidine	1.0	2.2	0.0	0.0
		0.02	0.7	3720.1	0.0
14	–	0.0	0.3	8870.1	0.4
	veratric acid	1.0	0.8	7980.4	1.86
		0.02	0.6	8960.1	3.4
	ferulic acid	1.0	0.4	9420.3	7.0
		0.02	0.5	8970.6	1.3
	xylidine	1.0	2.1	0.0	0.1
		0.02	0.6	9670.3	0.0

Table 1 also includes the data for the non-induced cultivation. As shown in Table 1, under non-induced cultivation the laccase activity appears in the culture broth medium on the third day and subsequently shows a gradual increase. The MnP was found in a comparable growth medium only after 14 days. A different situation was observed in the case of induced cultivation. All inducers (except for xylidine) positively affect the production of MnP. In the case of ferulic acid (1 mM), MnP activity is observed in the medium on the 10th day of cultivation (the second day after the induction), whereas after induction with 0.02 mM ferulic acid or veratric acid only on the 12th day of the process. It should be mentioned that MnP activity is not observed in the control cultivation. In induced and in non-induced cultures alike, the fungus does not produce LiP activity. The data listed in Table 1 show the positive influence of both acids and xylidine (0.02 mM) on the activity of the extracellular laccase. In comparison with the control laccase levels, the induced and non-induced cultures differ by less than 10%.

Some of the inducers used in the *C. unicolor* cultivation have a significant influence on the MnP production but they do not seem to be very significant in the case of laccase production. Considering the above and the toxic properties of oxidized inducers, optimalization of the fermentor cultivation conditions of *C. unicolor* was carried out in the absence of inducing substances. The obtained results are presented in Fig. 1.

As observed in shake cultures, in fermentor the laccase activity reaches a level of about 7000 nkat on the 10th day of cultivation; this corresponds with the beginning of the stationary phase (compare white rings and black triangles). Such a great increment of activity (from 500 to 7000 nkat between the sixth and 10th days) is usually observed in the case of the induced *T. versicolor* or *P. radiata* cultivation.

On the basis of literature [42,43], it is known that *Phanerochaete* or *Phlebia* possess a branched metabolic pathway in which, in the presence of the phenylalanine lyase enzyme, phenolic compounds are formed from glucose

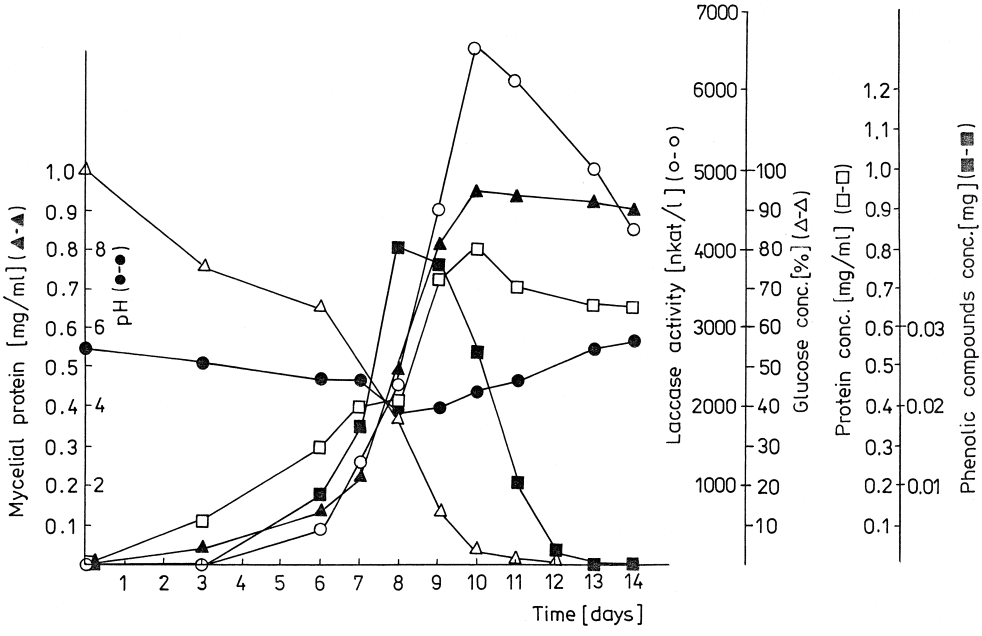

Fig. 1. The fermentation profile of *C. unicolor*. Conditions are as described in Section 2.

by de novo synthesis. In this respect, following the assumption of autoinduction of the mycelium, the concentration of phenolic compounds in the medium during the *C. unicolor* cultivation period was examined. The results are shown in Fig. 1. The typical correlation between the level of phenolic compounds and extracellular laccase activity can be observed.

3.2. Enzyme preparation

From an economic point of view, crude enzymatic preparations are used most readily in large-scale biocatalytic processes. The culture broth from a 10-day old *C. unicolor* fermentation was used as a source of crude laccase. This culture fluid was concentrated by means of such methods as ultrafiltration on AMICON TCF-10 (YM 10 membrane), lyophilization and vacuum evaporation at 34 or 40°C. The enzyme activities of these preparations are given in Table 2. Ultrafiltration, lyophilization and vacuum evaporation at 34°C all lead to a 20% loss of laccase activity. In the vacuum process any temperature higher than 40°C causes more than 65% activity decrease. The culture liquid was subsequently concentrated 10 times under vacuum at 34°C and lyophilized.

Electrophoretic analysis of the concentrate revealed the presence of four protein bands of laccase activity. The obtained preparation was subsequently applied during the next steps of our examination.

3.3. Enzyme immobilization

Since the stability and efficiency of the enzyme can be theoretically enhanced by immobilization, the preparation was examined not only in the native but also in the immobilized forms. Two types of support materials and two types of surface activation procedures were used for the enzyme immobilization. In consequence, three types of the immobilized preparations were obtained:

1. The enzyme coupled with CPG activated by APTES employing glutaraldehyde (material I-GA);
2. The enzyme coupled with CPG activated by APTES using carbodiimide (material I-CDI);
3. The enzyme coupled with CPG covered by a double layer of polysaccharide applying carbodiimide (material II-CDI)—see Experimental.

The first type of the immobilization process which employs the bonding ability of the amine groups in the immobilized protein was analogous to that used earlier for the immobilization of laccase from *Trametes*, *Phlebia* and *Fomes fomentarius* [22,25,44]. The application of carbodiimide instead of glutaraldehyde (the second type of immobilization) allows for bonding the enzyme via the carboxyl group presented in the bonded molecule. During the activation of a siliceous surface by only a part of the silanol groups reacts with the activator. A significant amount of silanols exists on the siliceous sur-

Table 2
The effect of various methods of precipitation and concentration of the *C. unicolor* culture fluid on laccase activity

Fraction	Protein concentration [mg/ml]	Amount of protein in relation to the initial content [%]	Laccase activity	
			[U/ml]	In relation to the initial content [%]
Crude after-culture fluid	0.32	100.0	8590.0	100.0
Evaporation				
34°C	5.12	98.6	38,285.7	80.9
40°C	4.03	97.8	20,986.2	33.7
Lyophilization[a]	3.96	99.1	51,379.5	76.9
Ultrafiltration	4.22	88.3	54,083.3	86.1

[a] The data were obtained after dissolving fully lyophilized preparation in 8 ml of water.

face of the modified support material. These groups of acidic character can influence the properties of the enzyme chemically anchored with the support material. CPG covered with the double layer of dextran (see Experimental) was the second support material applied in the immobilization of the prepared enzymatic preparation. The dextran layer was formed to screen the interaction between the bound enzyme and hydroxyl groups on the surface of CPG. CDI was employed to immobilize the enzyme with the second type of the support material.

3.4. Enzyme kinetics

The results of the examination of the pH influence on the laccase activity in the presence of eight different substrates are shown in Fig. 2. As it is evident, in most cases (besides MHQ and veratric acid) the immobilization process shifts the optimum of the enzyme activity towards the lower pH region. In the presence of veratric acid the optimal pH for the enzymatic preparation in the immobilized forms is identical to that for native ones. In the case of MHQ the shift of the optimal enzymatic activity towards higher pH values is observed for the immobilized forms as compared to the pH for the native form of the enzyme under consideration. A detailed analysis of the plots presented in Fig. 2 shows that the pH range of the maximal enzymatic activity is explicitly broader for the immobilized forms than for the native ones.

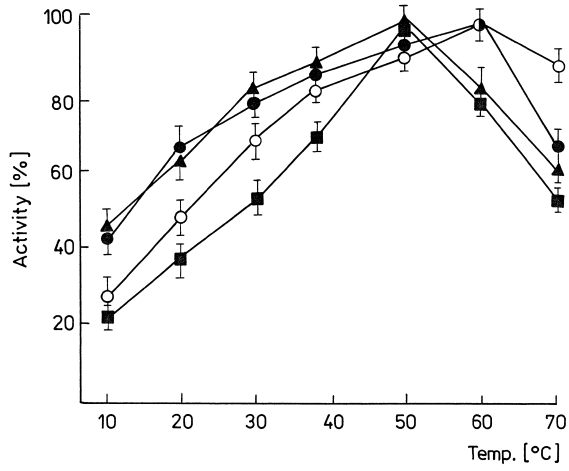

Fig. 3. The effect of temperature on the activity of (○) native and immobilized laccases from *C. unicolor* on (●) material I-GA, (▲) material I-CDI and (■) material II-CDI using 2,6-DMP as a substrate.

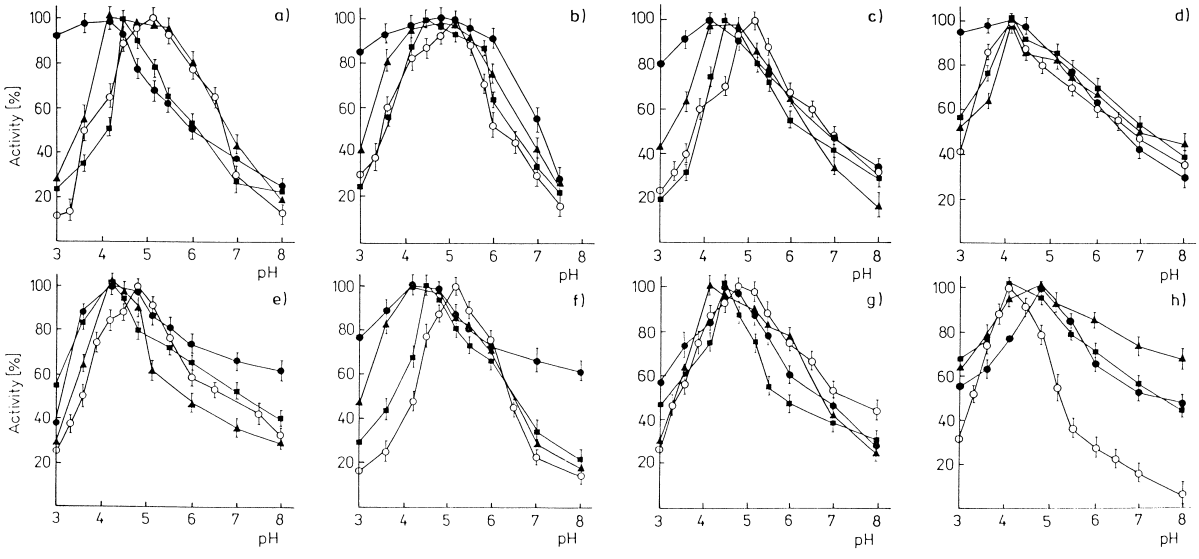

Fig. 2. The effect of pH on the activity of (○) native and immobilized laccases from *C. unicolor* on (●) material I-GA, (▲) material I-CDI and (■) material II-CDI using (a) 2,6-DMP; (b) syringic acid; (c) ferulic acid; (d) veratric acid; (e) vanillic acid; (f) caffeic acid; (g) guaiacol and (h) methoxyhydroquinone as a substrate.

Table 3
Kinetic data of native and immobilized laccases of *C. unicolor*

Substrate	Native laccase		Laccase immobilized on (carrier I)	
	K_m [M]	V_{max}	K_m [M]	V_{max}
Syringic acid	$8.03 \times 10^{-5} \pm 0.10 \times 10^{-5}$	34.43 ± 1.11	$1.24 \times 10^{-4} \pm 0.09 \times 10^{-4}$	25.54 ± 2.13
Caffeic acid	$3.64 \times 10^{-5} \pm 0.27 \times 10^{-5}$	38.49 ± 1.21	$1.04 \times 10^{-4} \pm 0.09 \times 10^{-4}$	31.22 ± 1.85
Vanillic acid	$1.72 \times 10^{-4} \pm 0.08 \times 10^{-4}$	15.85 ± 1.22	$1.23 \times 10^{-4} \pm 0.11 \times 10^{-4}$	21.65 ± 0.76
Veratric acid	$4.27 \times 10^{-5} \pm 0.16 \times 10^{-5}$	4.91 ± 0.29	$6.96 \times 10^{-5} \pm 0.12 \times 10^{-5}$	3.47 ± 0.13
Ferulic acid	$8.87 \times 10^{-5} \pm 0.18 \times 10^{-5}$	33.38 ± 1.25	$2.35 \times 10^{-4} \pm 0.16 \times 10^{-4}$	27.64 ± 0.75
2,6 DMP*	$7.80 \times 10^{-5} \pm 0.17 \times 10^{-5}$	46.69 ± 2.52	$8.20 \times 10^{-5} \pm 0.43 \times 10^{-5}$	27.58 ± 2.31
Guaiacol	$1.16 \times 10^{-4} \pm 0.19 \times 10^{-4}$	23.50 ± 0.80	$1.30 \times 10^{-4} \pm 0.09 \times 10^{-4}$	26.87 ± 1.89
MHQ**	$1.55 \times 10^{-4} \pm 0.14 \times 10^{-4}$	26.54 ± 1.70	$2.03 \times 10^{-4} \pm 0.18 \times 10^{-4}$	29.35 ± 1.03

2,6 DMP*, 2,6-Dimethoxyphenol.
MHQ**, Methoxyhydroquinone.

This feature applies in the case of the substrates which shift the pH of the maximal enzymatic activity to higher as well as to lower pH values as compared to the optimal pH for the native enzyme preparation. The relationship of enzymatic activity of the enzyme preparations (in the native and immobilized forms) and temperature is presented in Fig. 3. As it is evident from the curves, the optimal temperature for the material I-GA (the enzymes coupled with γ-APTES-CPG via glutaraldehyde) is similar to that of the native preparation. The application of CDI in the immobilization procedure of the preparation with supports I and II (γ-APTES-CPG or CPG covered with the dextran layers) lowers the optimal temperature to 50°C. In an immobilized form, laccase activity displays decreased activity in temperatures above the optimal temperature. Different results were obtained for the homogeneous laccase from *T. versicolor*, *P. radiata* or *F. fomentarius* [22,25,44].

The kinetic properties presented in Table 3 indicate that free laccase oxidizes phenolic substrates in the following order: caffeic acid > veratric acid > 2,6 DMP > syringic acid > ferulic acid > guaiacol > MHQ > vanillic acid. The immobilized enzyme shows a slightly lower affinity for the used substrates than the native preparation. Among the immobilized forms of laccase the one bonded with CPG containing dextran layers on its surface demonstrates the lowest affinity.

3.5. Effect of solutions

Recently, Milstein and coworkers [45–47] have shown that *C. versicolor* laccase immobilized on DEAE-Sephadex preserves its activity in a reaction medium in which most of the water is replaced by an organic solvent, including those solvents which completely solubilize lignins. Earlier, Dordick and coworkers [48] showed that lignin can be depolymerized with horseradish peroxidase in an organic solvent such as dioxane. Zaks and Klibanov [49] reported that enzymes, including laccase, added to hydrophobic solvents required substantially less water for the maximum activity than those suspended in hydrophilic ones. These authors concluded that the enzymatic activity in the presence of organic solvents was primarily determined by the interactions between water and the enzyme and not by those between the solvent and the enzyme [49]. Whether the laccase preparation from *C. unicolor* used in the native or immobilized form possesses analogous properties to those of the laccase from *P. radiata* [25] was tested subsequently. Table 4 illustrates the oxidizing activity of *C. unicolor* laccase (employed in native and immobilized forms in the presence of some organic solvents) against 2,6-DMP. Dioxane and formamide are the strongest denaturing solvents for the native laccase preparation. The activity of laccase towards 2,6-DMP decreases below 30% of its

Substrate	Laccase immobilized on (carrier II)		Laccase immobilized on (carrier III)	
	K_m [M]	V_{max}	K_m [M]	V_{max}
Syringic acid	$5.28 \times 10^{-5} \pm 0.43 \times 10^{-5}$	32.48 ± 0.91	$2.74 \times 10^{-5} \pm 0.12 \times 10^{-5}$	7.57 ± 0.62
Caffeic acid	$8.06 \times 10^{-5} \pm 0.16 \times 10^{-5}$	14.02 ± 0.59	$4.41 \times 10^{-4} \pm 0.19 \times 10^{-4}$	15.57 ± 1.23
Vanillic acid	$1.47 \times 10^{-4} \pm 0.11 \times 10^{-4}$	8.37 ± 0.78	$9.99 \times 10^{-4} \pm 0.56 \times 10^{-4}$	10.40 ± 0.93
Veratric acid	$3.42 \times 10^{-5} \pm 0.11 \times 10^{-5}$	4.62 ± 0.35	$4.21 \times 10^{-4} \pm 0.19 \times 10^{-4}$	7.48 ± 0.18
Ferulic acid	$8.34 \times 10^{-5} \pm 0.24 \times 10^{-5}$	25.43 ± 1.75	$1.12 \times 10^{-4} \pm 0.09 \times 10^{-4}$	8.09 ± 0.64
2,6 DMP *	$8.83 \times 10^{-5} \pm 0.23 \times 10^{-5}$	32.09 ± 2.04	$5.58 \times 10^{-5} \pm 0.19 \times 10^{-5}$	22.27 ± 0.90
Guaiacol	$5.48 \times 10^{-5} \pm 0.14 \times 10^{-5}$	8.12 ± 0.54	$1.38 \times 10^{-4} \pm 0.09 \times 10^{-4}$	9.87 ± 0.93
MHQ**	$2.03 \times 10^{-4} \pm 0.19 \times 10^{-4}$	16.14 ± 1.15	$2.91 \times 10^{-4} \pm 0.11 \times 10^{-4}$	10.74 ± 0.98

initial activity after the replacement of 20% of the buffer volume by the above mentioned solvents. The highest stability is observed for the native form when using ethanol as an organic solvent. It is worth noting that with most of the tested solvents, higher enzymatic activity towards 2,6-DMP is observed for the immobilized rather than for the native preparation, the system containing ethanol being an exception. In the latter case, the laccase preparation immobilized in the form of material I-GA and material I-CDI shows explicitly higher activity than the

Table 4
The effect of solvent type mixed with 0.1 M of citrate–phosphate buffer pH 4.5 (%; v/v) on the activity of native and immobilized laccases from *C. unicolor*

Solvent	Material	Laccase activities [in %] in various concentrations of solvent shown in the first column						
		0%	10%	20%	30%	50%	70%	90%
Ethanol	native	100.0 ± 2.3	–	96.0 ± 3.5	86.4 ± 3.8	62.2 ± 3.7	50.0 ± 3.0	11.5 ± 0.8
	I-GA	100.0 ± 1.9	–	98.1 ± 3.3	78.6 ± 2.2	62.5 ± 3.0	40.3 ± 3.1	11.4 ± 1.0
	I-CDI	100.0 ± 2.6	–	98.0 ± 1.9	62.2 ± 2.7	46.3 ± 2.2	22.6 ± 1.8	6.2 ± 0.5
	II-CDI	100.0 ± 1.9	–	99.2 ± 2.5	96.9 ± 5.1	69.9 ± 3.1	57.3 ± 4.5	21.7 ± 1.7
DMSO	native	100.0 ± 2.6	83.3 ± 2.2	35.1 ± 1.4	14.2 ± 0.9	0.0 ± 0.0	–	–
	I-GA	100.0 ± 2.3	92.3 ± 2.9	40.5 ± 1.7	18.6 ± 1.1	0.0 ± 0.0	–	–
	I-CDI	100.0 ± 3.1	97.1 ± 2.9	18.3 ± 1.1	9.2 ± 0.6	4.4 ± 0.2	–	–
	II-CDI	100.0 ± 2.1	100.0 ± 2.4	55.3 ± 2.3	25.9 ± 0.6	9.8 ± 0.6	–	–
Dioxane	native	100.0 ± 1.9	35.5 ± 1.4	20.3 ± 0.9	10.0 ± 0.4	0.0 ± 0.0	–	–
	I-GA	100.0 ± 2.4	97.7 ± 4.3	91.1 ± 3.8	36.1 ± 1.6	14.4 ± 1.0	–	–
	I-CDI	100.0 ± 2.2	96.1 ± 4.2	58.5 ± 2.3	22.4 ± 1.0	12.5 ± 0.9	–	–
	II-CDI	100.0 ± 2.4	100.0 ± 3.1	100.0 ± 2.7	45.2 ± 2.0	24.7 ± 1.8	–	–
Ethyl glycol	native	100.0 ± 1.7	70.1 ± 4.4	66.6 ± 2.6	43.3 ± 2.2	25.1 ± 1.7	–	–
	I-GA	100.0 ± 2.4	98.0 ± 3.9	91.0 ± 4.1	70.5 ± 4.3	45.2 ± 2.9	–	–
	I-CDI	100.0 ± 1.9	98.3 ± 4.4	83.4 ± 3.2	58.3 ± 3.1	31.5 ± 2.1	–	–
	II-CDI	100.0 ± 1.4	98.7 ± 5.1	98.6 ± 4.9	74.8 ± 4.8	51.9 ± 3.6	–	–
Acetone	native	100.0 ± 2.4	82.7 ± 3.6	40.2 ± 1.9	15.1 ± 0.9	6.6 ± 0.5	–	–
	I-GA	100.0 ± 3.0	100.0 ± 3.6	58.3 ± 4.2	26.2 ± 1.8	10.5 ± 0.6	–	–
	I-CDI	100.0 ± 2.9	89.6 ± 3.3	49.7 ± 3.2	20.2 ± 1.4	1.1 ± 0.1	–	–
	II-CDI	100.0 ± 2.6	100.0 ± 3.0	68.9 ± 3.4	35.5 ± 2.1	18.9 ± 1.4	–	–
Formamide	native	100.0 ± 1.9	50.5 ± 2.9	28.2 ± 1.9	8.2 ± 0.6	0.2 ± 0.0	–	–
	I-GA	100.0 ± 2.7	61.2 ± 2.8	35.5 ± 2.4	16.4 ± 1.1	14.4 ± 0.9	–	–
	I-CDI	100.0 ± 3.3	49.4 ± 3.2	31.1 ± 1.9	21.3 ± 1.5	18.5 ± 1.4	–	–
	II-CDI	100.0 ± 2.5	68.9 ± 4.2	49.8 ± 2.9	34.8 ± 2.1	24.9 ± 1.7	–	–

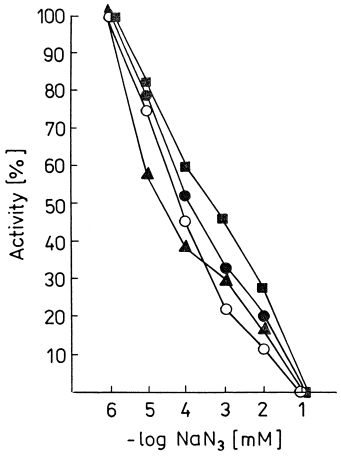

Fig. 4. The effect of NaN_3 on the activity of (○) native and immobilized laccases from *C. unicolor* on (●) material I-GA, (▲) material I-CDI and (■) material II-CDI.

native preparation containing only up to 20% of ethanol in the reaction mixture. The laccase preparation coupled with the second type of the support material, i.e., with CPG covered by dextran layers, shows the highest activity. The activity of the laccase preparation immobilized on support II (material II-CDI) equals 20% of its initial value even when using 50% dioxane or formamide (the strongest denaturing solvents) in the reaction mixture (when the native laccase preparation shows 0% activity).

Thioglycolic acid, sodium azide, thiourea or EDTA are known inhibitors of laccase from *T. versicolor*, *P. radiata*. Our preliminary examination indicates that thioglycolic acid, thiourea and EDTA at 1-mM concentration do not display inhibitory properties towards our laccase preparations.

The laccase preparation immobilized on the support with dextran layers showed the highest enzymatic activity within the range of used NaN_3 concentrations (see Fig. 4).

The results described here show that the laccase preparations from *C. unicolor* are very promising, from the scientific and practical point of view. The kinetics as well as the inhibitory characteristics show differences from the classic laccases prepared from *T. versicolor*, *P. radiata* or *F. fomentarius*.

Acknowledgements

Parts of this work were carried out with the support of the Environmental Research Programme of EC No. EV5V-0470 and Inter Faculty UMCS (BW/UMCS) project.

References

[1] N.G. Lewis, E. Yamamoto, Annu. Rev. Plant Physiol. Mol. Biol. 41 (1990) 455–496.
[2] K.-E. Eriksson, R.A. Blanchette, P. Ander, Microbial and Enzymatic Degradation of Wood and Wood Components, Springer, Berlin, 1990, pp. 1–407.
[3] A. Hatakka, A. Uusi-Rauva, Eur. J. Appl. Microbiol. Biotechnol. 17 (1983) 235–242.
[4] A. Hatakka, FEMS Microbiol. Rev. 13 (1994) 125–135.
[5] T.K. Kirk, R.L. Farrell, Annu. Rev. Microbiol. 41 (1987) 465–505.
[6] R.L. Crawford, Lignin Biodegradation and Transformation, Wiley-Interscience, New York, 1981, pp. 1–137.
[7] J.A. Buswell, E. Odier, CRC Crit. Rev. Biotechnol. 6 (1987) 1–60.
[8] K. Haider, J. Trojanowski, Arch. Microbiol. 105 (1975) 33–41.
[9] C.D. Boyle, B.R. Kropp, I.D. Reid, Appl. Environ. Microbiol. 58 (1992) 3217–3224.
[10] A. Hatakka, Degradation and conversion of lignin, lignin-related aromatic compounds and lignocellulose by selected white-rot fungi, PhD Thesis, Department of Microbiology, Univ. of Helsinki, Finland, 1986, pp. 1–99.
[11] A. Leonowicz, K. Grzywnowicz, M. Malinowska, Acta Biochim. Polon. 26 (1979) 431–434.
[12] K. Lunquist, P. Kristersson, Biochem. J. 229 (1985) 277–279.
[13] S. Kawai, T. Umezawa, M. Shimada, T. Higuchi, FEBS Lett. 236 (1988) 309–311.
[14] S. Kawai, T. Umezawa, T. Higuchi, Arch. Biochem. Biophys. 262 (1988) 99–110.
[15] J.-M. Bollag, M.J. Loll, Experientia 39 (1983) 1221–1231.
[16] K.L. Shuttleworth, J.-M. Bollag, Enzyme Microb. Technol. 8 (1983) 171–177.
[17] A. Lante, A. Crapisi, G. Pasini, A. Zamorani, P. Spettoli, in: D.S. Clark, D.A. Estell (Eds.), Enzyme Engineering XI, The New York Academy of Science, New York, 1992, pp. 558–565.
[18] J.-M. Bollag, A. Leonowicz, Appl. Environ. Microbiol. 48 (1984) 849–854.
[19] J. Rogalski, T. Lundell, A. Leonowicz, A. Hatakka, in: J.F. Kennedy, G.O. Philips, P.A. Williams (Eds.), Ligninocellulosics, Science, Technology, Development and Use, Ellis Horwood, New York, 1992, pp. 55–60.
[20] J. Rogalski, T. Lundell, A. Leonowicz, A. Hatakka, Phytochemistry 30 (1991) 2869–2872.
[21] M.-L. Niku-Paavola, E. Karhunen, A. Kantelinen, L. Viikari, T. Lundell, A. Hatakka, J. Biotechnol. 13 (1990) 211–221.

[22] J. Rogalski, A. Dawidowicz, A. Leonowicz, Acta Biotechnol. 10 (1990) 261–269.
[23] A. Leonowicz, L. Gianfreda, J. Rogalski, M. Jaszek, J. Luterek, M. Wojtaś-Wasilewska, E. Malarczyk, A. Dawidowicz, M. Fink-Boots, G. Ginalska, N.-S. Cho, Proc. Int. Sem. for Sci., Chungbuk Nat. Univ., Chengju, Korea, 1997, pp. 15–34.
[24] H.J. Doddema, Biotechnol. Bioeng. 32 (1988) 716–718.
[25] J. Rogalski, E. Jóźwik, A. Hatakka, A. Leonowicz, J. Mol. Catal. 95 (1995) 99–108.
[26] G. Lindenberg, G. Holm, Physiol. Plant. 5 (1952) 100–114.
[27] A. Leonowicz, K. Grzywnowicz, Enzyme Microb. Technol. 3 (1981) 55–58.
[28] M. Tien, T.K. Kirk, Appl. Environ. Microbiol. 54 (1984) 466–472.
[29] J.K. Glenn, M.H. Gold, Arch. Biochem. Biophys. 242 (1985) 329–341.
[30] T. Lundell, A. Leonowicz, J. Rogalski, A. Hatakka, Appl. Environ. Microbiol. 56 (1990) 2623–2629.
[31] A.J. Cornish-Bowden, Principles of Enzyme Kinetics, Butterworth, London, 1975, pp. 1–240.
[32] E. Malarczyk, J. Korszeń-Pilecka, A. Leonowicz, Phytochemistry 26 (1987) 1321–1324.
[33] J. Rogalski, J. Szczodrak, A. Dawidowicz, Z. Ilczuk, A. Leonowicz, Enzyme Microb. Technol. 7 (1985) 395–400.
[34] A. Dawidowicz, J. Rogalski, Polish Pat. No. 267182, 1991.
[35] A. Dawidowicz, T. Rauckyte, J. Rogalski, J. Liq. Chromatogr. 17 (1994) 817–831.
[36] A. Dawidowicz, T. Rauckyte, J. Rogalski, Chromatography 37 (1993) 168–172.
[37] D.A. Lappi, F.E. Stolzenbach, N.O. Kaplan, M.D. Kamen, Biochem. Biophys. Res. Commun. 69 (1976) 878–884.
[38] B.T. Kaufman, J.V. Pierce, Biochem. Biophys. Res. Commun. 44 (1971) 608–613.
[39] D.N. Salter, J.E. Ford, K.J. Scott, P. Andrewa, FEBS Lett. 20 (1972) 302–306.
[40] S.A. Enebak, R.A. Blanchette, Can. J. Forest Res. 19 (1989) 225–231.
[41] A. Leonowicz, Determination of laccase induction in some basidiomycetes fungi, PhD Thesis, Maria Curie-Skłodowska University, Poland, 1975, 1–102.
[42] K. Lunquist, T.K. Kirk, Phytochemistry 17 (1978) 1676.
[43] J. Rogalski, O. Niemenmaa, A. Uusi-Rauva, A. Hatakka, in: E. Srebotnik, K. Messner (Eds.), Biotechnology in the Pulp and Paper Industry WUV-Universitätsverlag, Wien, 1996, pp. 451–454.
[44] J. Rogalski, M. Wojtaś-Wasilewska, R. Apalovic, A. Leonowicz, Biotechnol. Bioeng. 37 (1991) 770–777.
[45] O. Milstein, B. Nicklas, A. Hüttermann, Appl. Microbiol. Biotechnol. 31 (1989) 70–74.
[46] O. Milstein, A. Hüttermann, H.-D. Lüdemann, A. Majcherczyk, B. Nicklas, in: T.K. Kirkand, H.-M. Chang (Eds.), Biotechnology in Pulp and Paper Manufacture, Butterworth-Heinemann, Boston, 1990, pp. 375–387.
[47] O. Milstein, A. Hüttermann, A. Majcherczyk, K. Schulze, R. Fründ, H.-D. Lüdemann, J. Biotechnol. 30 (1993) 37–43.
[48] J.S. Dordick, M.A. Marletta, A.M. Klibanov, Proc. Natl. Acad. Sci. U.S.A. 83 (1986) 6255–6257.
[49] A. Zaks, A.M. Klibanov, J. Biol. Chem. 263 (1988) 8017–8021.