Available online at www.sciencedirect.com
SCIENCE DIRECT®
Journal of Molecular Catalysis B: Enzymatic 31 (2004) 25–30
JOURNAL OF MOLECULAR CATALYSIS B: ENZYMATIC
www.elsevier.com/locate/molcatb

Determination of the activity of laccase, and mediated oxidation of a lignin model compound, in aqueous-organic mixed solvents

Francesca d'Acunzo, Anna Maria Barreca, Carlo Galli*

Dipartimento di Chimica, Università "La Sapienza", IMC-CNR Sezione Meccanismi di Reazione, P. le A. Moro 5, 00185 Roma, Italy

Received 19 April 2004; received in revised form 4 June 2004; accepted 1 July 2004
Available online 10 August 2004

Abstract

The activity and stability of laccase from *Poliporus pinsitus* in ten mixed solvents, consisting of 1:1 buffered-water/organic-cosolvent, is studied by resorting to the new spectrophotometric assay based on 3-hydroxyantranilic acid (HAA). The enzyme is found to initially retain at least 70% of its activity, for content as high as 60% (w/w) of cosolvents such as dioxane, isopropanol, ethylene glycol, and acetonitrile, and the results obtained by use of the HAA assay are compared with those obtained with other chromogens and laccases of different origin. The enzyme activity does not remain stable in the mixed solvents, but decreases as a function of time; the decrease becomes substantial over a 24-hours period in the case of dioxane, isopropanol, or acetonitrile, whereas ethylene glycol does not affect the enzymatic activity. The major result of the present study is the evaluation of the effect caused by the cosolvents on the efficiency of oxidation of an hydrophobic non-phenolic substrate when using four laccase-mediator (ABTS, HPI, VLA, TEMPO) systems. Some mediator-specific solvent effects emerge.

Keywords: Laccase activity; Mediators; Mixed solvents; Spectrophotometric assay; 3-Hydroxyantranilic acid

1. Introduction

In our preliminary investigation on the activity of laccase (EC 1.10.3.2) in mixed solvents [1], it has been demonstrated that the well-established spectrophotometric assay based on $ABTS^{\bullet+}$ as the indicator [2] is unreliable in the presence of dioxane. As a better alternative to ABTS or other chromogens, such as pyrocatechol, syringaldazine, and 2,6-dimethoxyphenol, a natural substrate of laccase such as 3-hydroxyantranilic acid (HAA) [3] has been proposed for use in aqueous-organic mixed solvents [1]. By means of this new assay, which is based on the oxidation/dimerisation reaction depicted in Fig. 1, it was shown that laccase from *Poliporus pinsitus* initially retains a significant fraction of its activity when exposed to aqueous buffer/dioxane 1:1 at 25 °C. However, the activity decays within few hours, and this inactivation is yet more pronounced at 45 °C. In the present study, the affinity of laccase for HAA is determined in buffered-water by means of a Lineweaver–Burke plot, and the spectrophotometric method based on HAA is used for a determination of laccase activity at 25 °C in a series of aqueous-organic mixed solvents, where the fraction of the organic component is varied in all proportions, from 0 to 100%. The results obtained by means of the HAA assay are compared with those obtained (with laccases of different origin) with other chromogens [4–7]. The time-dependence of laccase activity in 1:1 mixed solvents is monitored, and results qualitatively similar for the organic cosolvent tested; the only exceptions are ethylene glycol, which can be used in high concentration without affecting either the activity or stability of the enzyme, and 2,4-dimethoxyethane, which is surprisingly detrimental to the enzyme. Finally, the effect of selected cosolvents is evaluated on the mediating ability of ABTS, HPI, VLA and TEMPO in the

Abbreviations: HAA (3-hydroxy-antranilic acid); ABTS (2,2′-azinobis (3-ethylbenzothiazoline-6-sulfonate) salt); HPI (*N*-hydroxyphthalimide); VLA (violuric acid); TEMPO (2,2′,6,6′-tetramethyl-piperidine-*N*-oxyl).

* Corresponding author. Fax: +39 06 490421.
E-mail address: carlo.galli@uniroma1.it (C. Galli).

1381-1177/$ – see front matter © 2004 Elsevier B.V. All rights reserved.
doi:10.1016/j.molcatb.2004.07.001

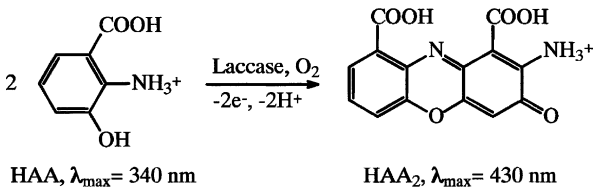


Fig. 1. Laccase catalysed oxidation–dimerisation reaction of HAA.

laccase-mediator catalysed oxidation of 4-methoxybenzyl alcohol.

2. Experimental procedures

2.1. Enzyme preparation

Laccase from *P. pinsitus* was kindly donated by Novo Nordisk Biotech and purified by ion-exchange chromatography [8,9] on Q-Sepharose by elution with phosphate buffer; laccase having an absorption ratio A_{280}/A_{610} of 20–30 was considered sufficiently pure [10]. It had an activity of 9000 U/mL according to the standard spectrophotometric assay with ABTS in buffered-water [2].

2.2. Materials

HAA, TEMPO, HPI, VLA, 4-methoxybenzyl alcohol (all purchased from Aldrich) were used as received. ABTS (also from Aldrich) was recrystallised from ethanol/water prior to use. All organic solvents were of the highest available grade (C. Erba). Buffers were prepared from citric or tartaric acid using ultrapure water obtained from a MilliQ apparatus; the pH was adjusted with a NaOH solution.

2.3. Laccase assay with HAA

Indicator solutions were prepared by adding 1 mg HAA (6.5 mmol) to 10 ml of solvent (either sodium tartrate buffer 0.1 M pH 3.5, or buffered-water-organic solvent mixtures, or neat organic solvent). HAA did not completely dissolve in buffer only (saturated solution), and the solution presented a tiny amount of precipitate (undissolved HAA) that was decanted prior to use; no solid residue was instead observed in the cosolvent-containing solutions. Consequently, a higher concentration of HAA (λ_{max} = 290, 340 nm) was present in the experiments carried out in mixed solvents. Laccase was added in the cuvette, so that its final concentration was 0.06–0.2 U/ml, and the absorption at 430 nm, corresponding to HAA_2 (cf. Fig. 1), was monitored over time (about 1 h). All data are corrected for differences in laccase concentrations. Laccase activity is expressed as the initial, linear increase in absorbance at 430 nm per unit time (AU/s).

2.4. Stability of laccase in mixed solvents

50 U/mL solutions of laccase in 1:1 buffered-water–organic mixed solvents were prepared by diluting the purified laccase stock solution into the desired solvent mixture, and kept at 25 °C. At the different time points, aliquots of this solution were used in the spectrophotometric assay with HAA.

2.5. Oxidation of 4-methoxybenzyl alcohol by laccase-mediator systems

The oxidation reactions were performed in air at room temperature in magnetically stirred buffered-water (0.1 M citrate buffer at pH 5.0) or 1:1 buffered-water–organic mixed solvent (3 mL). The concentration of the reagents was: [4-methoxybenzyl alcohol] = 20 mM, [mediator] = 6 mM, with 10 units of laccase. After a reaction time of 24 h, the internal standard 4-methoxyacetophenone was added, and the reaction crude extracted with ethyl acetate. The yields of oxidation were determined by GC analysis, suitable response factors being determined from authentic products. A Varian 3400 Star instrument, fitted with a 20 m × 0.25 mm methyl silicone gum capillary column, was employed in the GC analyses (N_2 carrier gas) with a ramp of oven temperature from 50 to 230 °C. The identity of the products was also confirmed by GC–MS analysis, run on a HP 5892 GC, equipped with a 12 m × 0.2 mm methyl silicone capillary column (He carrier gas), and coupled to a HP 5972 MSD instrument operating at 70 eV. Mass recovery was greater than 90% with all the cosolvents used.

3. Results

3.1. Affinity of laccase for HAA

The laccase-catalysed reaction delineated in Fig. 1 has been carried out on varying the concentration of HAA, and the initial rate of appearance of HAA_2 determined at 430 nm by spectrophotometry. In this way, a linear Lineweaver–Burke plot (Fig. 2) could be obtained, the slope of which, under the assumption of simple Michaelis–Menten kinetics, is related (see equation in Fig. 2 legend) to the Michaelis constant (K_M), i.e. the affinity of the enzyme for HAA. The K_M value (i.e., 3.3 × 10^{-4} M) is comparable to that obtained for the structurally similar 2,6-dimethoxyphenol with *P. radiata* laccase [6].

3.2. Dependence of laccase activity on organic cosolvent content

The initial laccase activity values, i.e. determined immediately after diluting the enzyme in the solvent system, in ten 1:1 buffered-water:organic mixed solvents are summarised in Table 1. In the vast majority of these solvent mixtures, the residual activity of the enzyme is quite high, being

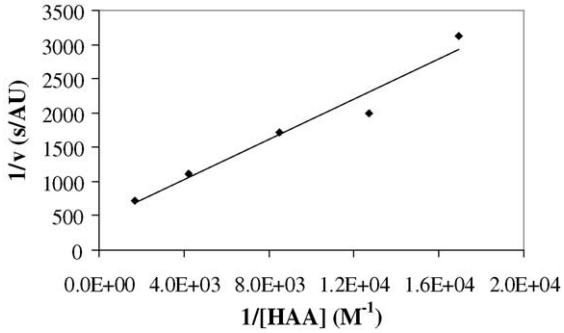


Fig. 2. Lineweaver–Burke plot for the laccase catalysed oxidation–dimerisation of HAA. A value of $K_M = 3.3 \times 10^{-4}$ M is obtained for the Michaelis constant through the following equation, assuming simple Michaelis-Menten kinetics: $(1/v) = (1/v_{max}) + (K_m/v_{max}) \times (1/[S])$, where $[S]$ is HAA concentration in mol $\times$ L^{-1} and v is the rate of increase in absorption at 430 nm, in AU $\times$ s^{-1}.

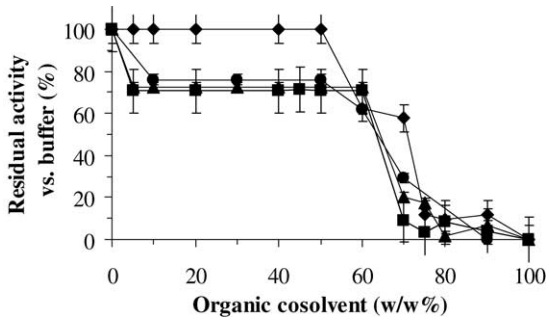


Fig. 3. Dependence of initial laccase activity on organic solvent concentration in miscible buffered-water–organic solvent mixtures. Cosolvents are ethylene glycol (♦), acetonitrile (●), isopropanol (▲), and dioxane (■). Each data point is the average of at least three measurements. The error bars visualise the spread of the values.

40–75% of the activity determined in buffer only. However, two of the cosolvents tested behave differently: ethylene glycol, which does not affect the enzymatic activity, and 2,4-dimethoxyethane, which inactivates the enzyme substantially, i.e. down to 10% residual activity. On varying over a wide range the content of the organic cosolvent, in the case of dioxane, isopropanol, ethylene glycol, and acetonitrile (Fig. 3), the cosolvent concentrations at which the activity of laccase is halved (C_{50}) come out in the 60–75% range, when determined with the HAA assay. With the exception of ethylene glycol, these values are significantly higher than those obtained by Khmelnitsky et al. [4] with laccase from *Poliporus versicolor*, and by Luterek et al. [11] with laccase from *Cerrena unicolor*, using other chromogens, thereby supporting the higher reliability of the HAA assay.

Of course, organic solvents may affect diverse laccases differently, but another point is perhaps more important.

Table 1
Initial rate of oxidation of HAA (AU/s) by laccase, determined immediately after diluting the enzyme in the mixed solvent

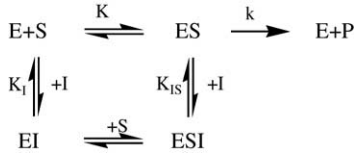
Mixed solvent (1/1 w/w)[a]	(AU/s) $\times 10^3$	Residual activity vs. buffer only (%)
Buffer only (reference)	4.4	100
Dioxane	3.3	75
Acetone	2.9	60
Ethanol	2.1	45
Acetonitrile	3.3	75
Isopropanol	3.2	70
Dimethylformamide	1.9	40
Dimethylsulfoxide	1.7	40
Glycerol	2.9	60
2,4-dimethoxyethane	0.4	10
Ethylene glycol	4.4	100

[a] In 2.5 mL tartrate buffer or tartrate buffer/cosolvent 1/1 (w/w); HAA 1.2–1.7 mg/mL; laccase 0.2 U/mL (as determined with the ABTS method [2] in citrate buffer at pH 5.0).

Haber and co-workers [6] have pointed out that the apparent solvent inhibitory effect depends on the indicator used for determining the enzymatic activity, since the organic cosolvent behaves as a competitive or mixed inhibitor, as shown in Scheme 1.

One should, therefore, consider that the residual activity of an enzyme could only be underestimated by indicators that suffer more strongly from competition by the organic solvent.

With respect to the initial laccase activity as a function of the content of cosolvents (Fig. 3), all plots (except that of ethylene glycol) exhibit three regions: first, an apparent sudden decrease of activity (15–20%) for a relatively small (5–10%) content of cosolvent, followed by a region where the enzymatic activity remains unaffected in spite of an increase of the content of organic cosolvent up to ca. 60% and, finally, a third region where the activity drops, to become almost negligible when the organic cosolvent exceedes 80%. We suspect that the first region is mostly an artefact, which begins to take place during the set-up of the spectrophotometric determination. In the second region, the water content of the mixed solvent clearly decreases and, consequently, the hydration of the enzyme does change [4,6,7,11,12], but not to the point of causing an immediate denaturation of laccase. This takes place in the third region, instead, as the activity drops down sharply [6], and independently on the nature of the cosolvent. The trends obtained show consistency with previous literature data and knowledge [4,6].



Scheme 1. Mixed inhibition by a cosolvent (I) in the laccase-catalysed oxidation of a substrate (*S*), such as HAA, or a mediator, such as HPI, VLA or TEMPO (Scheme taken from ref [6]).

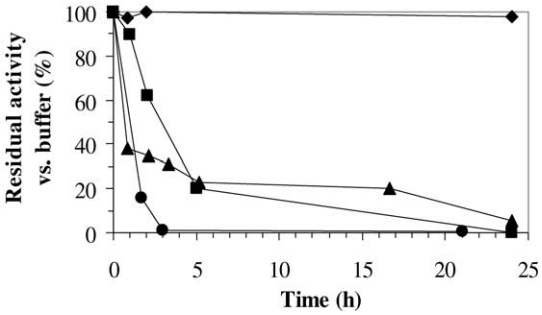
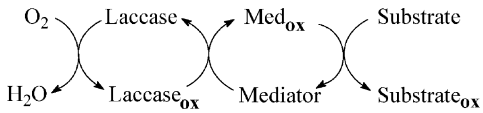


Fig. 4. Time-dependence of laccase activity in 1:1 buffered-water–organic solvent mixtures. Cosolvents are ethylene glycol (♦), acetonitrile (●), isopropanol (▲), and dioxane (■).

3.3. Stability of laccase in 1:aqueous-organic solvent mixtures

Fig. 4 shows the time-dependence of laccase activity in four significant aqueous-organic solvent mixtures at a 1:1 w:w composition. The presence of this content of ethylene glycol does not affect either laccase activity or stability at room temperature for 24 h. Set aside ethylene glycol, all organic cosolvents tested exert the same qualitative effect on laccase, i.e. the inactivation follows a roughly exponential time-profile. In fact, the solvent composition used in this case falls in the denaturing region of the enzyme [6], so that any differences among solvent mixtures are amenable to differences in the denaturation kinetics. The effect is likely to depend quantitatively on specific features of the cosolvent used, but attempts to correlate the effect with a thermodynamic parameter such as water activity (a_w) were not conclusive [6,13]. A better outcome had a correlation of the inhibitory effect with the Reichardt–Dimroth solvent polarity parameter (E_T) [6].

Rather than attempting to rationalise in depth the effect of the cosolvent on the denaturation of the enzyme, we were more interested in sorting out a cosolvent that induces a slower denaturation of laccase, in order to take advantage from an appropriate time window for the oxidation of fast-reacting but water-insoluble substrates. For example, phenols are natural substrates of laccase, and are rapidly oxidised if solubility problems are absent. With a sparingly soluble oligomeric phenol, the extent of oxidation could be enhanced by a factor of three when using a 1:1 buffer-dioxane mixture with respect to buffer alone [14], whereas acetonitrile as the co-solvent had a more detrimental effect on the oxidation of this oligomeric phenol. Looking more closely at the data in Fig. 4, it indeed emerges that acetonitrile inactivates laccase substantially within three hours whereas, in the presence of dioxane, the enzyme retains a significant part of its activity (60%) after 2 h. The same residual activity is detected in the buffered-water–isopropanol mixture after less than one hour. This is an useful and practical information.



Scheme 2. A laccase-mediated oxidation.

3.4. Laccase-mediated oxidation of 4-methoxybenzyl alcohol

Oxidation of non-phenolic substrates by laccase can occur only in the presence of mediators. The oxidation is not carried out by the enzyme directly, but rather by the oxidised form of the mediator (Med_{ox}, in Scheme 2).

Non-phenolic substrates are more likely to be water-insoluble, and clearly the problem of finding a good cosolvent becomes more stringent for their oxidation. A compromise must be reached between a moderate depression of laccase activity and a convenient increase of solubility of the substrate. Competitive binding of the cosolvent with respect to the mediator can now cause a mixed inhibition to the enzyme, thereby retarding the formation of the Med_{ox} species and, consequently, the ensuing non-enzymatic oxidation. Use of longer reaction times, if the the enzyme activity survives long enough, may enable laccase to catch up from this competitive binding. We have addressed here the oxidation of a simple non-phenolic lignin model compound (i.e., 4-methoxybenzylalcohol) by laccase, at room temperature for a 24 h reaction time, in four significant 1:1 buffered-water–organic solvent mixtures. Four mediators have been investigated, which are representative of the different non-enzymatic mechanisms of oxidation that can take place [15]. In fact, the Med_{ox} form of ABTS (i.e., $ABTS^{\bullet+}$ or $ABTS^{++}$) oxidises the substrate by electron transfer; in contrast, the Med_{ox} form of both HPI and VLA (i.e., the aminoxyl radical, $>N-O^{\bullet}$) follows a H-atom abstraction route [16], whereas the Med_{ox} form of TEMPO (i.e., the oxoammonium ion, $>^{+}N=O$) undertakes an ionic mechanism [17]. It is expected that the yield of the oxidised product (4-methoxybenzaldehyde) can be negatively affected not only by the decreased activity and stability of laccase in the mixed solvents, but also by any additional detrimental effect caused by the cosolvent to the Med_{ox} species. The data reported in Table 2 confirm this expectation, in general. More in detail, in the case of ABTS it has already been shown [1] that the stability of its radical cation is decreased in the presence of dioxane, and one could easily argue that other organic cosolvents exert a similar effect, so to explain the low oxidation yields obtained.

TEMPO is commonly used for the oxidation of aliphatic alcohols in combination with co-oxidants [18–21]; it is no wonder, then, that negligible yields of 4-methoxybenzaldehyde are obtained in the presence of isopropanol and ethylene glycol, since the cosolvent, which is in large molar excess, competes with the substrate to be oxidised by TEMPO-oxoammonium. In the case of HPI, on the contrary (Table 2), which in our experience has a low

Table 2
Oxidation of 4-MeO-benzyl alcohol in mixed solvents with laccase-mediator systems[a]

Mixed solvent 1/1 w/w	ABTS	HPI	VLA	TEMPO
Citrate buffer 0.1 M pH 5.0	20	70	90	100
Dioxane	<5 (>75)	10 (86)	30 (67)	20 (80)
Acetonitrile	<5 (>75)	5 (93)	10 (89)	10 (90)
Isopropanol	5 (75)	10 (86)	10 (89)	5 (95)
Ethylene glycol	<5 (>75)	30 (57)	60 (33)	<5 (>95)

Yields of 4-MeO-benzaldehyde (%) with respect to the initial amount of 4-MeO-benzyl alcohol are given. The percent decrease of such yield, as induced by the cosolvent, is reported in parentheses with respect to the yield in buffered-water only.

[a] Substrate 60 mM; Mediator 20 mM; laccase 3 U/ml (ABTS method [2]), 24 h reaction time at 25 °C. The GC yields of 4-MeO-benzaldehyde were calculated with the internal standard method; the rest of mass balance is recovered starting material.

tendency to oxidise aliphatic alcohols, a depression in the yield of 4-methoxybenzaldehyde should more closely reflect the lower stability of laccase at this content (i.e., 1:1) of co-solvents. In fact, no major effects from the polarity of the mixed solvents are expected in the radical oxidation route followed by the Med_{ox} (i.e., >N–O$^{\bullet}$) of this mediator [22]. The same qualitative trend is obtained with mediator VLA, which follows the same oxidation route [15,22]. We finally observe that, in the presence of ethylene glycol, the yield of 4-methoxybenzaldehyde under HPI and VLA mediation (i.e., 30 and 60%, respectively; Table 2) is lower than in buffer only (70 and 90%). Because laccase is stable (within this time-frame) in the presence of ethylene glycol, and since no specific depletion of the aminoxyl radical of the mediator is expected in ethylene glycol, the above decrease in substrate conversion may be attributed to an inhibition mechanism consistent with Scheme 1 [6], or to a specific mediator-induced suppression of the enzyme activity [23].

4. Conclusions

The spectrophotometric method based on HAA as an indicator allowed us to determine that, in a series of 1/1 (w/w) aqueous-organic solvent mixtures, laccase from *P. pinsitus* initially retains at least 40–75% of its activity, relative to the value determined in 100% buffered-water. Among the cosolvents tested, 1,2-dimethoxyethane, which causes an immediate 90% loss of enzymatic activity, and ethylene glycol, which does not affect the initial laccase activity, constitute an exception. In general, on a longer time-scale (24 h) the enzymatic activity falls to low values in the presence of co-solvents at room temperature, with the exception of ethylene glycol, where laccase is stable for at least 24 hours. These results are certainly dependent on the particular laccase employed [6], and different laccases might behave slightly differently; anyhow, *P. pinsitus* laccase is easily available and commonly used, besides displaying one of the highest redox capacity (0.8 V/SCE [24]), so that the information provided here is likely to be relevant to researchers in the field. As an original extension of this study, the effect caused by the cosolvents on the efficiency of oxidation of a non-phenolic lignin model compound by four laccase-mediator (ABTS, HPI, VLA or TEMPO) systems has been investigated. The depression in the extent of oxidation of 4-methoxybenzyl alcohol is in part to be attributed to the limited stability of laccase in the cosolvents. However, mediator-specific effects should also be taken into account, because the solvent can compete for the enzyme with the mediator (as the conversions obtained with HPI and VLA seem to indicate), or else it can undermine the stability of the oxidised form of the mediator (as in the case of ABTS), which is the direct oxidising species, thereby depressing the efficiency of the non-enzymatic oxidation route (cf. Scheme 2).

Acknowledgements

We thank the EU for financial support (grant QLK5-CT-1999-01277) to the OXYDELIGN project.

References

[1] G. Cantarella, F. d'Acunzo, C. Galli, Biotechnol. Bioeng. 82 (2003) 395–398.
[2] B.S. Wolfenden, R.L. Willson, J. Chem. Soc., Perkin Trans. II (1982) 805–812.
[3] C. Eggert, U. Temp, J.F.D. Dean, K.-E.L. Eriksson, FEBS Lett. 391 (1996) 144–148.
[4] Y.L. Khmelnitsky, V.V. Mozhaev, A.B. Belova, M.V. Sergeeva, K. Martinek, Eur. J. Biochem. 198 (1991) 31–41.
[5] V.V. Mozhaev, Y.L. Khmelnitsky, M.V. Sergeeva, A.B. Belova, N.L. Klyachko, A.V. Levashov, K. Martinek, Eur. J. Biochem. 184 (1989) 597–602.
[6] J. Rodakiewicz-Nowak, S.M. Kasure, B. Dudek, J. Haber, J. Mol. Cat. B: Enzyme 11 (2000) 1–11.
[7] S.H.M. Van Erp, E.O. Kamenskaya, Y.L. Khmelnitsky, Eur. J. Biochem. 202 (1991) 379–384.
[8] R. Bourbonnais, M.G. Paice, Appl. Microbiol. Biotechnol. 36 (1992) 823–827.
[9] A. Potthast, T. Rosenau, C.L. Chen, J.S. Gratzl, J. Org. Chem. 60 (1995) 4320–4321.
[10] F. Xu, Biochemistry 35 (1996) 7608–7614.
[11] J. Luterek, L. Gianfreda, M. Wojtas-Wasilewska, N.S. Cho, J. Rogalski, M. Jaszek, E.B. Malarczyk, M. Staszczak, M. Fink-Boots, A. Leonowicz, Holzforschung 52 (1998) 589–595.
[12] S.B. Lee, K.-J. Kim, M.G. Kim, Adv. Bioprocess Eng. (1994) 481–484.
[13] G. Bell, A.E.M. Janssen, P.J. Halling, Enzyme Microb. Technol. 20 (1997) 471–477.
[14] F. d'Acunzo, C. Galli, B. Masci, Eur. J. Biochem. 269 (2002) 5330–5335.
[15] M. Fabbrini, C. Galli, P. Gentili, J. Mol. Cat. B: Enzyme 16 (2002) 231–240.
[16] F. d'Acunzo, P. Baiocco, M. Fabbrini, C. Galli, P. Gentili, New J. Chem. 26 (2002) 1791–1794.
[17] F. d'Acunzo, P. Baiocco, M. Fabbrini, C. Galli, P. Gentili, Eur. J. Org. Chem. (2002) 4195–4201.
[18] A. Cecchetto, F. Fontana, F. Minisci, F. Recupero, Tetrahedron Lett. 42 (2001) 6651–6654.
[19] A. De Mico, R. Margarita, L. Parlanti, A. Vescovi, G. Piancatelli, J. Org. Chem. 62 (1997) 6974–6977.

[20] A.E.J. de Nooy, A.C. Besemer, H. van Bekkum, Synthesis 10 (1996) 1153–1174.
[21] M.F. Semmelhack, C.R. Schmid, D.A. Cortés, C.S. Chou, J. Am. Chem. Soc. 106 (1984) 3374–3376.
[22] P. Baiocco, A.M. Barreca, M. Fabbrini, C. Galli, P. Gentili, Org. Biomol. Chem. 1 (2003) 191–197.
[23] K. Li, F. Xu, K.-E.L. Eriksson, Appl. Environ. Microbiol. 65 (1999) 2654–2660.
[24] F. Xu, J.J. Kulys, K. Duke, K.L.K. Krikstopaitis, H.-J.W. Deussen, E. Abbate, V. Galinyte, P. Schneider, Appl. Environ. Microbiol. 66 (2000) 2052–2056.