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Immobilization of defined laccase combinations for enhanced oxidation of phenolic contaminants

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Abstract Immobilization is an important method to increase enzyme stability and allow enzyme reuse. One interesting application in the field of environmental biotechnology is the immobilization of laccase to eliminate phenolic contaminants via oxidation. Fumed silica nanoparticles have interesting potential as support material for laccase immobilization via sorption-assisted immobilization in the perspective of applications such as the elimination of micropollutants in aqueous phases. Based on these facts, the present work aimed to formulate laccase–nanoparticle conjugates with defined laccase combinations in order to obtain nanobiocatalysts, which are active over a broad range of pH values and possess a large substrate spectrum to suitably address pollution by multiple contaminants. A multi-enzymatic approach was investigated by immobilizing five different types of laccases originating from a *Thielavia* genus, *Coriolopsis polyzona*, *Cerrena unicolor*, *Pleurotus ostreatus*, and *Trametes versicolor* onto fumed silica nanoparticles, separately and in combinations. The laccases differed concerning their pH optima and substrate affinity. Exploiting their differences allowed the formulation of tailor-made nanobiocatalysts. In particular, the production of a nanobiocatalyst could be achieved that retained a higher percentage of its relative activity over the tested pH range (3–7) compared to the dissolved or separately immobilized enzymes. Furthermore, a nanobiocatalyst could be formulated able to oxidize a broader substrate range than the dissolved or separately immobilized enzymes. Thereby, the potential of the nanobiocatalyst for application in biochemical oxidation applications such as the elimination of multiple target pollutants in biologically treated wastewater has been illustrated.

Keywords Laccase · Fumed silica nanoparticles · Immobilization · Phenolic micropollutant · Wastewater treatment · Tailor-made nanobiocatalysts

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Introduction

The economy of biocatalytic processes can be improved by enzyme reuse and increase of enzyme stability by means of immobilization technologies. Implementing the capacity of enzyme retention or recovery in existing processes enables biocatalyst separation from products; thereby allowing continuous processes and preventing carry-through of biocatalysts to subsequent process steps (Polizzi et al. 2007).

Even if obvious advantages could be achieved by enzyme immobilization (Brady and Jordaan 2009), only approximately 20 % of biocatalytic processes make use of immobilized enzymes (Straathof et al. 2002). Interesting new developments in enzyme immobilization and applications thereof have been recently reported, particularly in the field of environmental technologies (i.e., wastewater treatment, water purification, biomass conversion, resource recovery, depollution, etc.). Thus, novel concepts for biocatalytic depollution have been developed (Corvini and Shahgaldian 2010), addressing the need to improve conventional wastewater treatment plants regarding their ability to cope with emerging organic contaminants (EOC).

In recent decades, EOCs such as pharmaceuticals, personal care products, and other hormonally active chemicals are often detected in wastewater treatment plant effluents, in surface waters, and even in groundwater (Cirja et al. 2008; Kuster

et al. 2008; Lapworth et al. 2012). Wastewater treatment technologies for the removal of EOCs are especially challenging because exhaustive removal is needed as these contaminants retain their adverse properties even at low concentrations. Initial concentrations are typically very low, and the remediation techniques should generally be applicable to a wide range of different chemical compounds possessing diverse physicochemical properties, thus making the treatment cost per unit mass prohibitively expensive (Klavarioti et al. 2009).

One promising approach to face the challenge of cost-efficient EOC removal consists of the use of immobilized biocatalysts, such as the four-copper containing metalloenzymes laccases (benzenediol: oxygen oxidoreductases, EC 1.10.3.2), which can oxidize and thereby transform phenolic or anthropogenic compounds into less toxic derivatives by one-electron abstraction reaction (Majeau et al. 2010). However, the use of free enzymes for wastewater treatment is still limited due to their instability over time caused by harsh conditions of the environment; inadequate pH, temperature, chemical denaturants, or salt concentration (Majeau et al. 2010; Cabana et al. 2011). The improvement of enzyme performance, such as activity even at environmentally relevant concentrations, well below K_M values and stability under application relevant conditions can be achieved by enzyme immobilization on solid surfaces, such as fumed silica nanoparticles (Hommes et al. 2012). Moreover, immobilization of the biocatalyst can reduce the enzyme loss and facilitate their use in continuous processes, such as bioreactors using membrane filtration technology to retain the immobilized enzymes (Sheldon 2007; Hu et al. 2009; Corvini and Shagaldian 2010; Hommes et al. 2012).

Presently, few data are available on the removal of EOC from treated wastewater by means of enzymatic systems in literature. Therefore, the main objectives of this study were to demonstrate the feasibility of combining defined enzyme combinations specially designed to eliminate several EOCs. In the present study, the removal of seven EOCs, i.e., carbamazepine (CBZ), diclofenac (DF), sulfamethoxazole (SMX), ibuprofen (IBU), gemfibrozil (GFZ), benzophenone-2 (BP-2), benzophenone-4 (BP-4), and bisphenol A (BPA) all occurring in effluents from wastewater treatment plants (Pal et al. 2010; Fent et al. 2010; Bertanza et al. 2011), have been addressed.

In general, laccases have a broad range regarding substrate specificity, but the latter can vary a lot depending on the chemical structure of the target substrates. Therefore, laccase combinations were immobilized on functionalized fumed silica nanoparticles (fsNP) in order to combine the beneficial characteristics of different laccases as nanobiocatalysts and to increase the efficiency of EOC removal.

Material and methods

Origins of laccases

Origin and characteristics of applied laccases are shown in Table 1. Laccases were precipitated with acetone (up to 80 % (v/v); on ice; 1 h) before application in order to remove possible impurities. Subsequently, they were centrifuged (21,000×*g*; 5 min; 4 °C), whereupon the supernatant was discarded, and the precipitate was dissolved in the respective buffer or NaCl (0.9 % (w/v)) for experiments with the extracellular Flux Analyzer XF-96-2 (CFA 96) and XF-24-2 (CFA 24) (Seahorse Bioscience, USA) respectively.

Immobilization procedure

Surface modification of fsNP (surface area: 390±40 $m^2\ g^{-1}$; aggregates of particles with an average size of 7 nm) from Sigma-Aldrich (Switzerland) were performed according to Hommes et al. (2012). Based on previous results (Hommes et al. 2012), optimal protein load, i.e., the protein load at which virtually all enzyme molecules applied could be immobilized, was calculated to be approximately 0.07 mg protein per milligram fsNP. Immobilization of single enzymes was conducted according to Hommes et al. (2012). Proteins were applied in amounts close to the optimal protein load and are summarized for each nanobiocatalyst formulation in Table 2.

Co-immobilization of two and simultaneous immobilization of five enzymes was conducted in similar fashion. The same weight of protein per laccase was applied, and it was assured that the total protein amount used corresponded to the optimal protein load. In case of co-immobilization of two enzymes, control experiments were conducted, immobilizing the respective enzymes separately. For each enzyme, the protein amount applied for the production of single enzyme-fsNP conjugate was equal to the protein amount of the corresponding enzyme applied for co-immobilization. In case of the simultaneous immobilization of five enzymes, four separate control experiments were conducted. The number of laccases to be immobilized was increased in each experiment from one to four. For each enzyme, the protein amount applied in control experiments was equal to the protein amount of the corresponding enzyme applied for the simultaneous immobilization of five enzymes.

Assays

Analytical isoelectric focusing was carried out using a Model 111 Mini IEF Cell (Bio-Rad) according to the instruction manual. Amersham calibration kits (low range (pH 3–10) and high range (pH 5–10.5)) for p*I* determinations (GE Healthcare) were used as p*I* marker proteins. Bio-Lyte ampholyte (3/10,

Table 1 Characteristics of the dissolved laccases

Laccase	Specific activity [U mg^{-1} protein]	Molecular activity [a] [GU mol^{-1} laccase]	Molecular Weight [b] [kDa]	Isoelectric point p*I* [pH]
Genus *Thielavia* (GTL) AB Enzymes GmbH (Germany)	14.2±0.3	3.3±0.1	75.6	4.2–7.6
Coriolopsis polyzona (CPL) Wetlands Engineering (Belgium)	22.1±1.0	26.0±0.6	59.8	3.9
Cerrena unicolor (CUL) Wetlands Engineering (Belgium)	6.0±0.1	7.2±0.6	58.1	3.8
Pleurotus ostreatus (POL) Sigma-Aldrich (Switzerland)	7.9±0.1	16.2±2.0	58.3	<3.0
Trametes versicolor (TVL) Sigma-Aldrich (Switzerland)	12.5±0.3	20.4±0.9	61.6	<3.0

[a] Molecular activity calculated based on the molecular weight and specific laccase activity (U mg^{-1} laccase) reported recently by Hommes et al. (2013)

[b] Molecular weight as reported recently by Hommes et al. (2013)

40 %) was used as carrier ampholyte for the pH gradient, and gels were stained with Coomassie brilliant blue R-250. The total protein content of laccase working solutions was quantified by the bicinchoninic acid assay (Smith et al. 1985). Bicinchoninic acid protein assay kits (Pierce®, Thermo Scientific, Germany) were used, and assays were conducted according to the instruction manual.

Standard activity measurements by means of colorimetric assays were performed as described elsewhere (Zimmerman et al. 2011; Hommes et al. 2013). One unit (U) has been defined as the amount of laccase able to oxidize 1 μmol 2, 2′-Azino-bis (3-ethylbenzthiazoline)-6-sulphonic acid (ABTS) per minute at pH 3 at 25 °C. Furthermore, the activities of both free and immobilized laccases were determined by

Table 2 Comparison of different laccase–fsNP conjugates produced with different laccases and laccase mixtures (n=3)

Applied laccase(s)	Washing Loss		Immobilization Yield [c]	Enzyme Load [d]
	[%][a]	[%][b]	[%]	[U mg^{-1} fsNP]
GTL (0.056 mg protein mg^{-1} fsNP)	4.8±3.9	6.0±0.5	126.1±9.6	0.91±0.05
CPL (0.088 mg protein mg^{-1} fsNP)	17.1±1.8	15.2±0.5	139.3±9.5	2.42±0.12
CUL (0.080 mg protein mg^{-1} fsNP)	17.6±6.5	18.0±0.8	161.3±17.3	0.70±0.07
POL (0.062 mg protein mg^{-1} fsNP)	2.1±3.1	8.5±1.3	228.3±16.4	1.05±0.06
TVL (0.050 mg protein mg^{-1} fsNP)	7.2±1.8	2.9±0.1	191.1±8.9	1.08±0.02
GTL (0.014 mg protein mg^{-1} fsNP)	7.4±4.5	0.5±0.0	180.0±8.7	0.30±0.01
GTL, CPL (0.028 mg protein mg^{-1} fsNP)	6.7±2.7	0.5±0.0	205.4±15.4	0.78±0.06
GTL, CPL, and CUL (0.042 mg protein mg^{-1} fsNP)	5.5±2.1	0.5±0.0	204.7±3.4	0.86±0.04
GTL, CPL, and CUL, POL (0.056 mg protein mg^{-1} fsNP)	9.1±0.5	2.6±0.1	201.9±13.1	1.01±0.02
GTL, CPL, CUL, POL, and TVL (0.070 mg protein mg^{-1} fsNP)	11.8±3.3	5.0±0.3	190.7±1.8	1.10±0.03
GTL (0.035 mg protein mg^{-1} fsNP)	5.7±0.6	4.3±0.3	166.1±3.1	0.69±0.00
POL (0.035 mg protein mg^{-1} fsNP)	5.1±3.5	3.4±0.3	227.4±3.8	0.55±0.02
GTL and POL (total 0.070 mg protein mg^{-1} fsNP)	10.0±2.9	9.8±0.8	155.7±1.7	1.03±0.02
CPL (0.035 mg protein mg^{-1} fsNP)	8.5±3.1	0.5±0.0	194.4±6.8	0.93±0.02
TVL (0.035 mg protein mg^{-1} fsNP)	9.7±2.2	2.3±0.2	212.0±4.7	0.75±0.04
CPL and TVL (total 0.070 mg protein mg^{-1} fsNP)	9.7±4.0	6.4±0.1	184.0±5.6	1.37±0.03

Washing loss was determined in two different ways, i.e., as [a] relative difference between the apparent (measured) laccase activity bound to the fsNP after exhaustive washing and the apparent laccase activity before washing (100 %) and [b] apparent laccase activity discarded during washing relative to the apparent laccase activity before washing (100 %), i.e., the relative laccase activity discarded during washing

[c] Immobilization yield was defined as apparent laccase activity of the completed nanobiocatalyst suspensions relative to the initially applied laccase activity (100 %)

[d] Enzyme load was defined as apparent laccase activity per mg fsNP of the completed nanobiocatalysts

oxygen consumption rate (OCR) measurements using a novel assay based on fluorimetric sensors in a multiwell plate format using the CFA 24 and CFA 96 as recently described (Hommes et al. 2013). All OCR measurements were performed in McIlvaine phosphate–citrate buffer (McIlvaine 1921). The method for determination of laccase activities towards target substances differed between immobilized and dissolved enzymes.

In case of dissolved enzymes, laccase was injected through the injection ports in order to start the enzymatic reaction. OCR measurements with dissolved laccases and target substances were conducted using the CFA 24. All additional experiments were performed with the CFA 96 in order to increase throughput. Substrate concentrations were usually 1 mM, except for 2, 6-dimethoxyphenol (2, 6-DMP), for which concentrations were 2 mM.

Applied enzymatic activities of dissolved laccases were varied depending on laccase origin, substrate, and pH and were between 200 and 13,000 U L^{-1} for the reference phenolic substrates, i.e., 2, 6-DMP and hydroquinone (HQ) and between 3,000 and 40,000 U L^{-1} for the target substances. OCR were calculated using the "Level (direct) Akos" algorithm that is part of the Seahorse CFA software package. The algorithm accounts for O_2 flux into the measurement volume through the walls of the microplate and the atmosphere and has been described before (Gerencser et al. 2009).

All experiments with immobilized laccases were conducted using the CFA 96. Contrary to the experiments using dissolved laccases, substrates were injected through the injection ports in order to start the enzymatic reaction because immobilized enzymes would have clogged the injection ports. For BP-2 and 2, 6-DMP, initial stock concentration was adjusted in order to reach a well concentration of 1 and 2 mM after injection, respectively. All other substances were applied at their solubility limit, and final well volume was reduced from 200 to 180 μL in order to reach substance concentrations as high as possible inside the wells.

Applied enzymatic activities of immobilized laccases for oxidation of the reference phenolic substrate 2, 6-DMP varied depending on laccase origin, substrate, and pH and were between 50 and 2,100 U L^{-1}. OCRs were calculated as for dissolved laccases.

Applied enzymatic activities of immobilized laccases for the oxidation of target substances at pH 7 varied depending on laccase origin and substrate and were between 1,000 and 42,000 U L^{-1}, corresponding to fsNP concentrations between 1 and 30 g L^{-1}.

Decrease in O_2 concentration after substrate injection was calculated by linear regression of the measured O_2 concentrations. In order to evaluate if significant O_2 consumption occurred, O_2 decreases in the samples were compared to O_2 decreases in controls containing immobilized enzymes, where only buffer and no substrate was injected by using analysis of variances (ANOVA). Prior to ANOVA, the dataset was tested on its normal distribution (Shapiro–Wilk normality test, chosen significance level $p<0.1$) and equal variances (Bartlett test of homogeneity of variances, chosen significance level $p<0.1$), respectively.

Stability assays to compare the stability of the immobilized and dissolved enzymes were conducted in filtered (11 μm cut-off, grade 1, Whatman, Germany) wastewater effluent (pH 8.3, COD 24.8 mg L^{-1}) from wastewater treatment plant Birsfelden, Switzerland, as described elsewhere (Zimmermann et al. 2011), with slight modifications. Initial applied enzymatic activity usually was 10,000 U L^{-1} in total, except for laccase combinations, and co-immobilized laccases initial activity was 1,000 U L^{-1}. In order to study the influence of initial laccase activity on stability, experiments with *Coriolopsis polyzona* (CPL) immobilized on fsNP (CPL–fsNP) were conducted with 1,000 U L^{-1} as well as with 10,000 U L^{-1}.

Results

Laccases were tested regarding their suitability for immobilization, their activity over a broad range of pH values, and their ability to transform selected pollutants. Furthermore, a strategy to design defined multi-enzymatic nanobiocatalyst for environmental applications has been investigated.

Immobilization of laccases and laccase combinations

In the present paper, five laccases of different origin have been successfully immobilized on surface-modified fumed silica nanoparticles as summarized in Table 2.

Coupling of single laccases

Losses of enzymatic activity during washing steps of the immobilization procedure (washing losses) of single laccases were below 10 % for genus *Thielavia* (GTL), *Pleurotus ostreatus* (POL), and *Trametes versicolor* (TVL), indicating that these biocatalysts were almost entirely immobilized on the support material. For CPL and *Cerrena unicolor* (CUL), washing losses were higher compared to the other three enzymes but still below 20 %. The slightly higher washing losses for CPL and CUL were most likely due to the fact that the protein loads (0.088 and 0.080 mg protein per milligram fsNP, respectively) actually used were slightly above the calculated optimal protein load (0.07 mg protein per milligram fsNP) for these two enzymes. The specific activities of the enzyme suspensions correlated nicely with the enzyme loads of the corresponding nanobiocatalysts, i.e., higher specific activities of the enzyme suspensions resulted in higher enzyme loads of the nanobiocatalysts. Particularly for CPL, the

enzyme load was quite high (2.42±0.12 U mg^{-1} fsNP) in comparison to the other nanobiocatalysts.

Coupling of multiple laccases

In order to test whether immobilization of all five investigated laccases onto the same particles was feasible, different experiments were conducted. The number of laccases to be immobilized was increased successively from one up to five. With each increase in the number of applied laccases, the resulting nanobiocatalysts showed an increase in total enzyme load. Herewith, it could be shown that all five laccases were successfully co-immobilized, and thereby a biocatalyst containing all five laccases (multi-fsNP) could be produced. Therefore, the suitability of the applied method to immobilize defined combinations of laccases was proven. This allowed selection of laccase combinations for immobilization on the same particle based on their capabilities in order to produce nanobiocatalysts with enhanced abilities compared to the single enzymes. Two pairs of laccases were selected for co-immobilization, i.e., CPL and TVL as well as GTL and POL. CPL and TVL were selected for co-immobilization onto the same particles, in order to produce nanobiocatalysts able to transform a broader range of compounds than single-enzyme nanoparticle conjugates. GTL and POL were chosen for co-immobilization in order to produce nanobiocatalysts that retain most of their maximal enzymatic activity over a broader pH range.

Co-immobilizations of two laccases were assessed similar to the immobilization of five laccases by running control experiments applying only one laccase. Enzyme load of both two-enzyme nanobiocatalysts, i.e., CPL and TVL co-immobilized on fsNP (CPL–TVL–fsNP) as well as for GTL and POL co-immobilized on fsNP (GTL–POL–fsNP), was higher after immobilization of both laccases compared to experiments where only one laccase was immobilized, indicating successful immobilization.

Stability of laccases and nanobiocatalysts thereof in conventionally treated wastewater

Long-term stability assays in conventionally treated wastewater showed a considerable stabilization of the enzymatic activity of the immobilized compared to the free enzymes. Immobilized laccases retained activity over the whole measurement period of 40 days, while soluble forms of CPL, POL, and TVL lost virtually all activity within 9 days, and CUL and GTL lost virtually all activity within 23 days. However, the stability of the single-laccase conjugates differed considerably depending on the laccase. Between 19.7±2.3 (GTL–fsNP) and 72.4±2.0 % (CUL-fsNP) of the initial enzymatic activity (10,000 U L^{-1}) remained after 40-days. Stability of the enzymatic activity of the conjugates depended on the initially applied enzymatic activity, as experiments conducted with CPL–fsNP applying two different initial enzymatic activities, i.e., 1,000 and 10,000 U L^{-1} showed. After 40 days of incubation, enzymatic activity decreased to 15.7±2.3 and 49.7±2.9 % for the experiment applying 1,000 and 10,000 U L^{-1}, respectively. Enzymatic activity of the double- and multi-fsNP conjugates remained at 17.8±0.4, 39.1±1.1, and 27.1±4.0 % for GTL–POL–fsNP, CPL–TVL–fsNP, and multi-fsNP conjugates, respectively, after 40 days of incubation and initial enzymatic activity of 1,000 U L^{-1}.

Substrate oxidation by dissolved and immobilized laccases

The ability of free laccases to oxidize selected organic contaminants in buffer solutions was investigated over a pH range of 3–7 by means of oxygen consumption measurements. For benchmarking 2, 6-DMP, a standard substrate to determine laccase activity was used.

Oxidation activity—benchmarking using standard substrate

Figure 1 shows the normalized activity of the different laccases (A) and immobilized laccases (B) at different pH values in the presence of 2, 6-DMP. All laccases reached their maximal activity at pH 4 with the exception of GTL, which reached its maximal activity at pH 5. Laccase activity was reduced below 20 % of the maximal activity at pH 6 for CUL, POL, and TVL. At pH 7, the measured activity for these three laccases was below the limit of detection. For CPL, the reduction in laccase activity at higher pH values was not as drastic and remained at approximately 31 and 16 % of the maximal activity at pH 6 and 7, respectively. GTL retained 80 % of its maximal activity at pH 6, and at pH 7, 50 % of the maximal activity remained.

For all investigated laccases, the immobilization of the enzymes did not lead to an observable shift of the optimal pH regarding enzymatic activity. However, immobilized enzymes retained a higher percentage of their maximal activity compared to free enzymes at pH 6 and 7. Especially, GTL–fsNP showed little losses in activity at higher pH values compared to other immobilized laccases, retaining 97 and 74 % of maximal enzymatic activity at pH 6 and 7, respectively. At pH 3, losses in enzymatic activity compared to the maximal enzymatic activity were also smaller for all enzymes after immobilization, except for GTL. Notably, TVL retained most of its maximal enzymatic activity after immobilization at pH 3, i.e., 88 %, while it only retained 28 % in its dissolved form.

The co-immobilization of GTL and POL led to the anticipated extension of the pH range, in which the nanobiocatalysts retained enzymatic activity. Enzymatic activities were always above 70 % of the activity maximum of the GTL–POL–fsNP

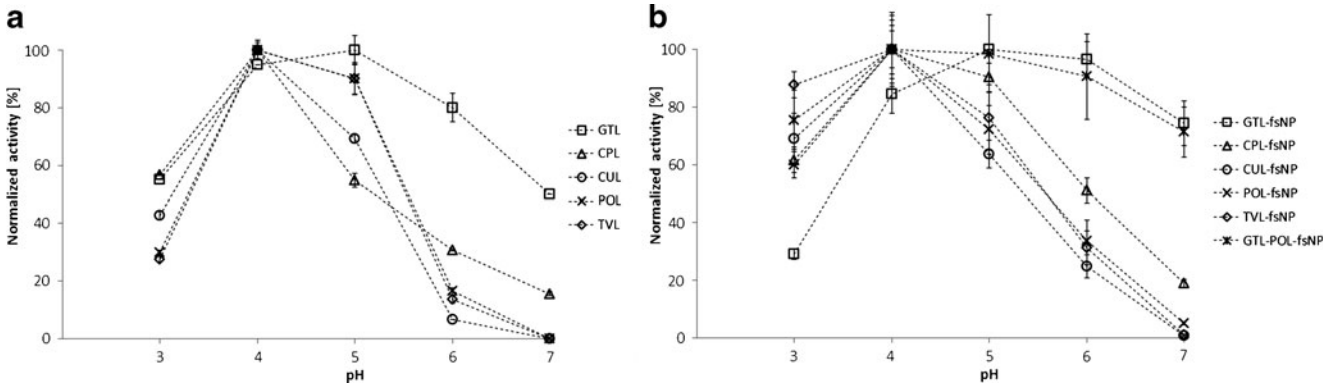

Fig. 1 Enzymatic activity of laccases (**a**) and immobilized laccases (**b**) at different pH values during the oxidation of 2, 6-DMP (2 mM). **a** Single laccase in its soluble form from genus *Thielavia* laccase (*GTL*), *Coriolopsis polyzona* (*CPL*), *Cerrena unicolor* (*CUL*), *Pleurotus ostreatus* (*POL*), and *Trametes versicolor* (*TVL*). **b** Single laccase-fsNP conjugates from genus *Thielavia* laccase (*GTL–fsNP*), *Coriolopsis polyzona* (*CPL–fsNP*), *Cerrena unicolor* (*CUL–fsNP*), *Pleurotus ostreatus* (*POL–fsNP*), and *Trametes versicolor* (*TVL–fsNP*) and two laccase-fsNP conjugates from GTL and POL (*GTL–POL–fsNP*). Laccase activity is normalized to the highest measured activity of the corresponding enzyme, *error bars* show the standard deviations (n=5)

over the tested pH range from 3 to 7. This constitutes a clear improvement at pH values above 4 compared to the nanobiocatalyst, in which POL was used as a sole enzyme and at pH 3 compared to the one-enzyme nanobiocatalyst, in which GTL was used.

Furthermore, an increase in enzymatic activity towards the substrate ABTS at pH 3 was observed for all laccase genera after immobilization, as is apparent by the immobilization yields above 100 % that were achieved for all laccases and laccase combinations (Table 2). Enzymatic activity after immobilization also increased, when 2, 6-DMP was used as a substrate for all laccases at all pH values investigated. One-enzyme nanobiocatalysts showed increased enzymatic activity towards 2, 6-DMP between 3.5- (pH 5, TVL–fsNP) and 9.4-fold (pH 6, TVL–fsNP) compared to the corresponding dissolved enzymes. Enzymatic activity towards 2, 6-DMP by co-immobilized enzymes increased between 4.9- (pH 4, GTL–POL–fsNP) and 11.6-fold (pH 7, GTL–POL–fsNP) compared to the corresponding mixture of dissolved enzymes at the same enzyme ratio. Immobilization of five different laccases onto one carrier led to an increase in enzymatic activity towards 2, 6-DMP between 10.3- (pH 3) and 15.5-fold (pH 7), in comparison to a mixture of the respective native laccases applied at the same enzyme ratio.

Oxidation of target compounds

Table 3 summarizes the results from the screening of the five laccases regarding their ability to oxidize different potential target compounds. No noticeable OCRs were measured with compounds SMX, CBZ, or IBU irrespective of the laccase used, indicating that these substrates are not or only slowly transformed by the studied enzymes.

HQ and BPA could be transformed by all laccases, while experiments with DF showed relevant OCRs for all laccases except CUL. GFZ, BP-2, and BP-4 seemed to be less prone to oxidation by laccases, since no remarkable OCRs were observed for three (in case of BP-2 and BP-4) or even four (in case of GFZ) of the tested laccases. Notable OCRs for GFZ were only measured with CPL; for BP-2, OCRs above LOD were detected in the presence of TVL and CUL, and for BP-4, noteworthy OCRs could be measured in the presence of TVL and CPL.

Regarding the pH activity profiles of the laccases concerning the oxidation of the selected target contaminants, it is clear that the pH activity optimum of the respective laccase is substrate dependent. CPL for example showed highest enzymatic activity at pH 4, 5, and 6 for 2, 6-DMP, HQ, and DF, respectively. Crude GTL was the only laccase showing high oxidation activity at neutral pH towards BPA and DF, indicating that the application of GTL in wastewater treatment is suitable, since domestic wastewater is usually neutral to alkaline (pH 7–9).

Apart from the compounds that are not transformed by all laccases, GFZ was the only compound that was not oxidized by TVL. Since CPL was the only laccase that measurably reduced oxygen concentration in the presence of GFZ, CPL and TVL were co-immobilized on fsNP in order to produce a nanobiocatalyst able to oxidize all five EOCs prone to oxidation by laccase. Oxidation of target substances with CPL–TVL–fsNP led only to considerable decreases in O_2 concentration, when particle concentrations were 1 g L^{-1} or higher. Since preliminary experiments have shown that O_2 flux into the measurement volume is measurably influenced by fsNP concentrations above ~1 g L^{-1}, OCR calculations using the "Level (direct) Akos" algorithm were not suitable for the experiments using CPL–TVL–fsNPs for the oxidation of target substances. Therefore, observed linear decreases in O_2 concentration were quantified by linear regression. Decreases in O_2 concentrations were 3.78±0.48, 0.74±0.14, 0.80±0.08, 1.86±0.30, and 0.49±0.08 mmHg min^{-1} for BPA, DF, GFZ, BP-2, and BP-4,

Table 3 Measured initial OCRs during treatment of different potential target compounds with dissolved laccases. Target compounds were applied with an initial concentration of 1 mM

Compound	Laccase	OCR [μmol min^{-1} mg^{-1} laccase]				
		pH 3	pH 4	pH 5	pH 6	pH 7
Hydroquinone (HQ)	Genus *Thielavia*	<LOD	<LOD	2.30±0.43	5.68±0.37	4.45±0.34
	Coriolopsis polyzona	9.56±1.58	43.51±7.42	70.00±7.70	40.22±4.04	9.09±0.50
	Cerrena unicolor	<0.69	2.75±0.30	12.80±1.73	3.61±0.79	<0.22
	Pleurotus ostreatus	3.28±0.45	27.15±1.68	55.59±13.72	22.62±2.73	3.53±0.21
	Trametes versicolor	1.41±0.38	8.92±2.53	41.24±6.97	15.30±4.11	<0.66
Bisphenol A (BPA)	Genus *Thielavia*	<LOD	<LOD	<0.12	0.14±0.04	0.23±0.03
	Coriolopsis polyzona	<0.95	<0.95	1.48±0.08	1.53±0.17	<0.95
	Cerrena unicolor	<0.43	<0.76	<0.76	0.85±0.15	<LOD
	Pleurotus ostreatus	<LOD	<LOD	<1.27	<1.27	<LOD
	Trametes versicolor	<LOD	<0.33	<0.66	<0.66	<0.34
Diclofenac (DF)	Genus *Thielavia*	–	–	<LOD	<LOD	<0.09
	Coriolopsis polyzona	–	–	0.44±0.05	0.63±0.08	0.38±0.06
	Pleurotus ostreatus	–	–	<LOD	<0.33	<LOD
	Trametes versicolor	–	–	<0.14	0.35±0.04	0.23±0.02
Gemfibrozil (GFZ)	*Coriolopsis polyzona*	<0.42	<0.42	1.04±0.05	0.78±0.10	0.49±0.06
Benzophenone-2 (BP-2)	*Cerrena unicolor*	<LOD	<0.11	<0.11	0.17±0.03	<0.11
	Trametes versicolor	<LOD	<0.28	0.50±0.18	0.98±0.11	0.29±0.02
Benzophenone-4 (BP-4)	*Coriolopsis polyzona*	<0.10	<0.10	0.16±0.04	0.09±0.03	0.11±0.02
	Trametes versicolor	<LOD	<0.20	0.22±0.02	0.32±0.04	0.25±0.03

Note that all laccases were tested at all pH values for all compounds. When for a particular laccase, no noteworthy enzymatic activity towards a defined compound was observed at all tested pH values, results corresponding to this laccase were omitted in this table

Less than sign (<) oxygen consumption could be detected but was below the limit of quantification (*LOQ*). The value given corresponds to the LOQ of the respective experiment, <LOD no oxygen consumption rate above the limit of detection (*LOD*) was measured under the applied conditions, *en dash* (–) solubility of target compound below 1 mM at applied pH conditions

respectively, while they were −1.27±0.59, 0.00±0.11, 0.25±0.11, −0.06±0.15, and 0.25±0.11 mmHg min^{-1} in the corresponding controls only containing the same CPL–TVL–fsNP concentration and no substrate. ANOVA results for BPA, DF, GFZ, BP-2, and BP-4 (p values were 5.3×10^{-7}, 2.1×10^{-7}, 1.5×10^{-7}, 6.5×10^{-9}, and 7.0×10^{-4}, respectively) using CPL–TVL–fsNP as biocatalyst showed that the measured O_2 decreases in the samples containing one of the target substrates were significantly higher compared to O_2 decreases in respective substrate-free controls. This suggests that the resulting nanobiocatalyst was able to oxidize GFZ, a compound that was not oxidized by TVL alone, as well as BP-2, a compound that was not oxidized by CPL alone, indicating that the goal of producing a nanobiocatalyst with a broader range of substrates compared to the one-laccase nanobiocatalysts could be achieved.

Discussion

Immobilizing different laccases on solid surfaces for industrial applications is of increasing interest. During the last years, laccases were immobilized successfully on several different support materials, e.g., magnetically separable mesoporous silica spheres (Zhu et al. 2007), nanoporous gold particles (Qiu et al. 2009), mesostructured cellular foams (Rekuć et al. 2009), and fumed silica nanoparticles (Zimmermann et al. 2011). In the present study, a previously developed and optimized immobilization strategy (Zimmermann et al. 2011; Hommes et al. 2012) has been applied for the successful immobilization of different laccases and combinations thereof. Furthermore, some possibilities to enhance the properties of nanobiocatalysts by immobilizing defined laccase combinations have been explored, and the enzymatic activity of the different laccases towards several EOCs at different pH values has been determined.

Immobilization—coupling of single catalyst

Successful immobilization of all single enzymes tested in this study could be demonstrated and led to nanobiocatalysts harboring between 0.70±0.07 and 2.42±0.12 U laccase activity per milligram fsNP depending on the laccase used. The resulting enzyme loads correlated with the specific activity

of the applied laccases, and the resulting washing losses correlated with the applied protein amounts, indicating that similar protein amounts were immobilized per milligram fsNP irrespective of the enzyme used. This is in accordance with our previous studies (Zimmermann et al. 2011; Hommes et al. 2012), where we found that for CPL and GTL, relative washing losses were similar for similar protein amounts used irrespective of the enzyme and increased with increasing applied protein amounts.

Immobilized enzymes retained a higher percentage of their maximal enzymatic activity towards 2, 6-DMP compared to free enzymes over the tested pH range (except for GTL at pH 3), indicating that the enzymatic activity is enhanced due to immobilization even at unfavorable pH. Similar favorable effects of immobilization of laccases on the relative enzymatic activity at different pH values have been reported before (e.g., Yang et al. 2006). A shift of the optimum pH due to immobilization could not be observed for any of the laccases. However, since enzymatic activities were determined at discrete pH values, small shifts of the pH optimum, as reported for the immobilization of CUL (Luterek et al. 1998), might have not been detected.

Regarding the stability of the resulting biocatalysts in conventionally treated wastewater, the enzymes remain active over considerably longer time periods, if they are immobilized, and these results of the present study are in accordance with our previous findings (Zimmermann et al. 2011; Hommes et al. 2012). Stability increases due to immobilization on solid surfaces are mainly due to a reduction in the number of points of attack for proteases, an increase in the conformational stability due to the covalent binding, leading to a decreased possibility of unfavorable enzyme folding, and increased stability against harsh pH (Brady and Jordaan 2009; Rekuć et al. 2010; Zimmermann et al. 2011). Enzymatic activity decreased slower in experiments where higher initial enzymatic activities were used. This might be due to the presence of wastewater components that can act as enzyme inhibitors. A higher initial enzymatic activity means lower specific inhibitor concentrations (gram inhibitor per gram laccase). This might explain why inhibition of the enzymes progresses slower if initial activity is higher.

Immobilization—multi-catalyst approach

Successful co-immobilization of the studied laccases on the same particles could be demonstrated by successive immobilization of the five different enzymes. Furthermore, the co-immobilization of specifically selected laccases allowed the production of biocatalysts with a broader pH-activity range, in case of the GTL–POL–fsNP, and with a broader substrate range, in case of the CPL–TVL–fsNP, compared to the corresponding singly immobilized laccases. This indicates that the characteristics of single laccases regarding pH activity profile and substrate specificity complemented one another after co-immobilization.

The amounts of enzyme that were immobilized on the fsNP were calculated based on the assumption that cross-linking with glutaraldehyde is in general nonspecific towards different proteins and consequently towards different enzymes, since cross-linking of proteins to a carrier with glutaraldehyde usually involves non-protonated ε-amino groups of lysin residues (Weetall 1974), and proteins usually exhibit many lysine residues on their surface, due to the polarity of the amine group (Migneault et al. 2004).

The experiments demonstrated that applying enzyme mixtures with total protein amounts equal or slightly below the protein amount that can be immobilized on the fsNP allows immobilizing defined combinations of enzymes. Whereas various laccases were immobilized in the present study, this method should be applicable to any combination of different classes of enzymes. The only issue to clarify would be whether the designated enzymes retain or lose their activity due to immobilization.

Oxidation of EOCs

Oxidation activity – benchmarking using standard substrates

An increase in enzymatic activity could be observed for all tested laccases towards the standard substrates ABTS and 2, 6-DMP after immobilization, indicating that the active sites of the enzymes were more readily accessible for these substrates due to conformational changes induced by immobilization, or that substrate concentrations near the particle surface were higher than in bulk solution due to interactions of the particle with the substrate. This is in accordance with our previous work, where immobilization as well as cross-linking with glutaraldehyde of GTL led to an increase in enzymatic activity towards ABTS (Hommes et al. 2012). Increased enzymatic activities due to cross-linking of laccases (Durán et al. 2002) and increased enzymatic activities towards 2, 6-DMP by approximately 10-fold due to encapsulation of laccase in sol–gel silica (Mohidem and Mat 2009) were reported before. Although, immobilization led to increased enzymatic activities towards the standard substrates for all five studied laccases, it has to be pointed out that this is not always valid. In a previous study, we found that enzymatic activity towards ABTS for laccase of *Phoma* sp. is reduced considerably after cross-linking with glutaraldehyde (Hommes et al. 2012).

Oxidation of target compounds

The results of the screening of the different laccases regarding their capability to oxidize different EOCs generally correspond well to results reported in literature. BPA and DF

transformation by laccases has been confirmed by several studies (e.g., Uchida et al. 2001; Cabana et al. 2009; Galliker et al. 2010; Marco-Urrea et al. 2010; Tran et al. 2010; Hommes et al. 2012; Hommes et al. 2013), which is consistent with the results of the present study, where high OCRs in the presence of BPA could be measured for all five and in the presence of DF for four of the studied laccases. Furthermore, it has been shown that sulfonamides by laccase alone, i.e., in the absence of redox mediators are only slowly transformed or even not at all (Bialk et al. 2005; Schwarz et al. 2010; Weng et al. 2012). This is in agreement with the present study, where no considerable OCRs could be measured with laccases in the presence of SMX.

Slow degradation of CBZ, IBU, and GFZ by commercial TVL has been previously reported (Tran et al. 2010). However, the authors of this study pointed out that degradation of these compounds might not have been due to laccase activity, since commercially prepared laccase might contain other enzymes and/or redox mediators (Tran et al. 2010). This might explain why no remarkable OCR with TVL in the presence of these three compounds could be measured in our study. Another explanation might be that transformation of these compounds occurred too slowly, and therefore not enough oxygen was consumed over time to detect a noteworthy OCR.

Results of the present study suggest that both BP-2 and BP-4 can be oxidized by some laccases. To the best of our knowledge, there are, to date, no reports on the transformation of these compounds by laccase. Therefore, the occurrence of oxygen consumption by laccases in the presence of these UV absorbers provides first evidence that their degradation is catalyzed by these enzymes.

Resulting OCRs obtained during the screening of the capabilities of the different laccases to oxidize the target substances showed that pH optima for the different substrates are between 5 and 7 and depend on the substrate as well as on the laccase. This is in agreement with what is reported in literature. It is well known that the pH optima for laccase activity are highly dependable on the substrate (Kunamneni et al. 2007). For substrates whose oxidation by laccases goes along with an H^+ dissociation, e.g., phenols or anilines, a bell-shaped pH-activity profile with optimal pH between 5 and 7 is usually reported (Xu et al. 2007).

Two laccases, i.e., CPL and TVL, were selected for the production of a biocatalyst able to oxidize a broad range of EOCs. Selection was based on the results of the screening of the different laccases regarding their ability to oxidize EOCs. The suitability of this approach was demonstrated, since the resulting CPL–TVL–fsNPs were able to transform a broader substrate range than the separate enzymes. Thus, the outlined immobilization method allows for facile formulation and production of biocatalysts tailored for specific applications. One application would be immobilization of laccases for the elimination of specific target pollutants in classical-treated wastewater but other applications in industries, where enzymes are applied can be envisaged like in textile processing, pulp and paper, or food applications.

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