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Direct immobilization of laccase on titania nanoparticles from crude enzyme extracts of *P. ostreatus* culture for micro-pollutant degradation

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Abstract

Enzymatic treatment can effectively degrade recalcitrant micro-pollutants in wastewater. However, its industrial application is constrained by the high cost of the purified enzyme preparations. This work introduces a novel technique to directly immobilize the in-house crude enzyme extracts (from *P. ostreatus*) onto the functionalized TiO₂ nanoparticle surface. Comprehensive investigations were carried out to understand the interactions between complex crude enzyme extracts and the immobilization support. By simple dilution of the crude enzyme extract, the immobilization efficiency can be significantly improved. The resultant biocatalytic nanoparticles had comparable performance to the immobilized purified commercial enzymes. Finally, the micropollutant degradation capability of the biocatalytic nanoparticles was demonstrated with two micro-pollutants, namely, bisphenol-A and carbamazepine, commonly detected in sewage. The efficient laccase extraction and immobilization on biocatalytic nanoparticles show great promise as a cost-effective alternative to conventional wastewater treatment processes for recalcitrant micro-pollutants.

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1 **Direct immobilization of laccase on titania nanoparticles from crude**
2 **enzyme extracts of *P. ostreatus* culture for micro-pollutant degradation**

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11 Graphical abstract

Crude laccase immobilization on functionalized TiO_2 nanoparticles

12

13

Abstract

Enzymatic treatment can effectively degrade recalcitrant micro-pollutants in wastewater. However, its industrial application is constrained by the high cost of the purified enzyme preparations. This work introduces a novel technique to directly immobilize the in-house crude enzyme extracts (from *P. ostreatus*) onto the functionalized TiO₂ nanoparticle surface. Comprehensive investigations were carried out to understand the interactions between complex crude enzyme extracts and the immobilization support. By simple dilution of the crude enzyme extract, the immobilization efficiency can be significantly improved. The resultant biocatalytic nanoparticles had comparable performance to the immobilized purified commercial enzymes. Finally, the micropollutant degradation capability of the biocatalytic nanoparticles was demonstrated with two micro-pollutants, namely, bisphenol-A and carbamazepine, commonly detected in sewage. The efficient laccase extraction and immobilization on biocatalytic nanoparticles show great promise as a cost-effective alternative to conventional wastewater treatment processes for recalcitrant micro-pollutants.

Keywords:

Crude enzyme extract, enzyme immobilization, TiO₂ nanoparticles, micropollutant degradation

33 1. Introduction

34 Enzymatic bioconversion technologies have attracted growing interests in the field of
35 wastewater treatment. Their advantages include a high activity on a broad range of substrates
36 at mild conditions, a high specificity that allows elimination of the selected and specific
37 compounds, as well as less toxic biodegradation by-products [1]. With these characteristics,
38 enzymatic systems are particularly beneficial for the selective removal of pollutants from
39 waste streams, especially the recalcitrant micro-pollutants which cannot be treated effectively
40 by the traditional methods (Yang et al., 2013b).

41 White-rot fungi can efficiently degrade a broad range of recalcitrant micro-pollutants such as
42 endocrine disruptor chemicals (EDCs), pharmaceutically active chemicals (PhACs), hormone
43 and pesticide via one or more extracellular enzymes including lignin peroxidases,
44 manganese-dependent peroxidases and laccase [2–6]. Among these enzymes, laccase uses
45 dissolved oxygen as the final electron acceptor, and possesses relatively good stability. As a
46 result, it is considered as the most promising candidate for industrial wastewater treatment
47 [7–9]. However, rapid denaturation of the free enzyme and its difficulty in reuse restrict their
48 wider applications for industrial processes [10,11].

49 For practical application, the immobilization of enzymes is desirable for the improved
50 operational stability under various industrial conditions. It also facilitates the design of the
51 biocatalytic reactors with easier enzyme reuse and process control [9]. Recently, the TiO₂
52 based enzyme immobilization technique has attracted increasing attention, due to its low
53 price, good stability, coordination ability with amine and carboxyl groups, as well as good
54 biocompatibility. In our previous research, we sequentially functionalized TiO₂ nanoparticles
55 with 3-aminopropyltriethoxysilane (APTES) and glutaraldehyde (GLU) to allow its covalent
56 bonding with purified commercial laccase [12,13]. The immobilized laccase retained most of

its original activity and the biocatalytic nanoparticles exhibited good operational stability for micro-pollutant degradation.

The enzymatic wastewater treatment process is still relatively expensive, mainly due to the cost of commercial enzymes. One potential solution is the application of crude enzyme extracts [14]. However, for practical application, crude enzyme extracts have to be purified to improve their apparent activity and catalytic efficiency [15]. The reported enzyme purification processes such as multi-step solvent partitioning [16], the aqueous two-phase system [17], ammonium sulfate precipitation [18] and sequential chromatographic process [19,20] are tedious and time-consuming. In addition, these steps also incur extra downstream processing costs. Ideally, the enzyme purification and stabilization should be achieved simultaneously, and recently a few related techniques have been reported, such as support surface activation [16,21,22], protein-inorganic composite formation [23], and enzyme self-crosslinking [24–26]. As discussed above, the functionalized TiO₂ nanoparticle can be a promising candidate for enzyme immobilization. However, the complex components within the crude enzyme extracts can cover the immobilization support surface and degrade the enzyme performance, and this aspect has not been fully investigated. In addition, it remains to be elucidated whether the biocatalytic nanoparticles can be used for the treatment of emerging micro-pollutants of concern.

In contrast to the conventional biocatalytic wastewater treatment processes, our strategy follows a bottom-up enzyme synthesis approach using *P. ostreatus* and then the application of the functionalized TiO₂ nanoparticle to realize a one-step purification/immobilization of the crude enzyme extracts. This work systematically investigated the interaction between the immobilization supports and the non-protein components within the crude enzyme extracts, and then the immobilization process was optimized for the highest apparent activity. The

biocatalytic nanoparticle stability within a wide range of pH, under different temperature, and after long-term storage was also examined. Finally, the biocatalytic nanoparticles were studied for the degradation of two representative micro-pollutants (bisphenol-A and carbamazepine) within a hybrid membrane reactor. The selection of these two micro-pollutants was based on their frequent presence in water bodies, ecotoxicological impact on aquatic organisms, as well as their high resistance to conventional biodegradation [27,28].

2. Materials and methods

2.1 Chemical and materials

Crude enzyme extract from *P. ostreatus* was prepared using malt extract broth (Merck, Germany). The pH of the fungus growth medium was adjusted to 4.5 with HCl. The stock fungal suspension was grown on a rotary shaker at 28 °C in sterilized growth medium for a week. More details on the crude enzyme preparation can be found in our previous work [29]. Commercially purified laccase from *P. ostreatus*, 2,2'-azino-bis-(3-ethyl benzothiazoline-6-sulfonic acid) (ABTS), 3-aminopropyltriethoxysilane (APTES), glutaraldehyde (GLU), citric acid, Na₂HPO₄ and NaH₂PO₄ were purchased from Sigma-Aldrich. TiO₂ nanoparticles (Degussa, P25) were supplied by Degussa. Hydrophilized polyvinylidene fluoride (PVDF) microfiltration (0.45 μm) membranes were supplied by Millipore Pty. Ltd. All chemicals were of analytical grade and used without further purification.

2.2 SDS-PAGE and Native-PAGE

To identify the protein component of the crude enzyme extract, sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed on a 13% resolving gel and a 4% stacking gel [30,31]. Protein bands were visualized by Coomassie Brilliant Blue R 230 staining overnight. To detect specifically the band with laccase activity, native-PAGE was

performed using the same gels without adding SDS and then stained with 0.5 mM ABTS in 0.1 M pH 5 citric acid-phosphate buffer for 30 min, where the laccase active bands were highlighted in dark green.

2.3 Immobilization of the crude enzyme

Crude enzyme was covalently immobilized on TiO₂ nanoparticles via a sequential technique developed previously [32]. Briefly, TiO₂ nanoparticles were modified by APTES in pure ethanol for 24 h and then dispersed in 4% (v:v) GLU in 100 mM pH 7 phosphate buffer solution for 12 h. After being washed with centrifugation and re-dispersion processes, the functionalized particles (typically 50 mg) were immersed in 10 mL enzyme solution (pH 7) with continuous shaking for 48 h under 4 °C. Finally, the biocatalytic nanoparticles were centrifuged and re-dispensed three times to remove the loosely attached enzymes and other non-protein components. For efficient immobilization of the enzyme, the crude extract was diluted, and the diluted samples were designated as CE-T (i.e. CE-10), where T represents the dilution times.

Crude enzyme content was determined using the Lowry method [32]. The activities of both free and immobilized enzymes were determined by the oxidation of ABTS. The reaction was conducted at 25 °C in a volume of 20 ml. The reaction mixture consisted of 19.5 ml of 0.5 mM ABTS in 0.1 M pH 3 citric acid-phosphate buffer and 0.5 ml of enzyme solution (free enzyme solution or nanoparticle with immobilized enzyme suspension solution). The change in absorbance, corresponding to the oxidation of ABTS, was monitored at 420 nm over a period of 5 min with Cary 100 Bio UV-Vis Spectrophotometer. Enzyme activity was expressed in units per ml crude extract (U/ml) for free enzyme and units per mg nanoparticles (U/mg) for the immobilized enzyme, respectively. One unit of enzyme catalyzes the

conversion of 1 μmol of ABTS per min. More details can be found in our previous publication [32].

The activity recovery η_{ar} is defined as

$$\eta_{AR} = \frac{A_{E,NP}}{A_{E,Initial} - A_{E,Supernatant}} \quad (1)$$

where $A_{E,NP}$ is the apparent activity of the biocatalytic nanoparticles, and the denominator stands for the activity theoretically bound to the nanoparticles (i.e. the difference between the activity of the initial enzyme solution $A_{E,Initial}$ and the activity of the supernatant after immobilization $A_{E,Supernatant}$).

The loading efficiency of enzyme is defined as the ratio of the amount of enzyme immobilized on the nanoparticles and the initial enzyme content before immobilization. The amount of enzyme immobilized on the nanoparticles was estimated from the difference of the initial supernatant enzyme content before immobilization, ($m_{E, Initial}$) and residual enzyme content in the supernatant after immobilization ($m_{E, Supernatant}$),.

$$\eta_{loading} = \frac{m_{E, Initial} - m_{E, Supernatant}}{m_{E, Initial}} \quad (2)$$

A more rigorous term “specific activity of crude laccase” was defined to better reflect the efficiency of crude enzyme immobilization, which was the ratio between the actual activity of the immobilized enzyme and the enzyme loading on the nanoparticles. Kinetic constants (K_m and k_{cat}) of free and immobilized enzymes were determined by non-linear curve fitting of the oxidation rate of ABTS versus ABTS concentration based on the Michaelis-Menten equation [32]. Oxidation of ABTS was initiated with an injection of 0.05 ml enzyme solution (free or immobilized enzyme) into a 3 ml vial containing 2.95 ml ABTS solution (0.1 M pH 3 citric

acid-phosphate buffer), and the corresponding change in absorbance was measured at 420 nm over a period of 10 min.

Total organic carbon (TOC) and total nitrogen (TN) of the enzyme solution were measured using a TOC analyser (SHIMADZU, Japan). The loading of TOC (or TN) on nanoparticles was estimated based on the difference of TOC (or TN) of enzyme solution supernatant before and after enzyme immobilization. Samples were filtered through the filter (0.45 μ m PVDF membrane) prior to the analysis. Background TOC and TN values caused by the leakage of cross-linkers from nanoparticles and the loss of TOC and TN due to sample preparation (i.e. membrane filtration) were considered and deducted. The average values and standard deviations were reported based on triple measurements.

2.4 The effect of pH and temperature on the activity and stability of the crude enzyme

The effect of pH and temperature on the activity of the free and immobilized enzymes was determined using ABTS assay at different pH (2.5-7) and temperatures (25-70 °C). pH conditions were maintained using a citric acid-phosphate buffer (0.1 M, pH 2.5-5) or a phosphate buffer (0.1 M, pH 6-7). The stability was determined by comparing the activity of enzyme before and after 12-hour incubation in different pH buffer at 25 °C. Thermal stability tests of the free and immobilized enzyme were performed by incubating enzyme samples at different temperature (20-70 °C) in pH 3 buffer for 2 h. To determine the long-term stability, both free and immobilized enzymes were stored at 25 °C in pH 5 buffer solution for 30 days. For all the stability tests, the enzyme activity was determined with ABTS assay under standard conditions (0.5 mM ABTS, pH 3, 25 °C).

2.5 Micro-pollutants degradation with the biocatalytic nanoparticles

170 Micro-pollutants degradation experiments were carried out in a hybrid membrane reactor,
171 where biocatalytic TiO₂ nanoparticles were suspended in the membrane feed solution and a
172 0.45 µm hydrophilic PVDF membrane was used to retain the particles within the feed side.
173 The details of the experimental rig can be found in our previous publication [12]. Two
174 frequently detected micro-pollutants in wastewater, namely bisphenol-A and carbamazepine
175 were selected as the targeted compounds. In a typical test, a 50 ml solution of single-
176 compound (bisphenol-A or carbamazepine at 10 µM) or their mixture (bisphenol-A and
177 carbamazepine, 10 µM each) was poured into the membrane cell and a magnetic stirrer was
178 applied to completely mix the solution (150 rpm). The pH of the test solution was 5.5. A
179 certain amount of biocatalytic nanoparticles was then added into the reactor to initiate the
180 reaction. A peristaltic pump equipped with Masterflex C-flex tubing was used to recirculate
181 the permeate solution back to the membrane cell with an operation flux of 5 L/m² h. The
182 degradation experiments lasted at least 24 h at the lab with constant temperature control (25 ±
183 1 °C). The trans-membrane pressure and membrane flux were monitored during the
184 degradation experiment. Quantitative analysis of the micro-pollutants from permeate was
185 performed using high-performance liquid chromatography (HPLC) coupled with a UV-
186 visible detector (Shimadzu LC-20AT). A detailed procedure is available elsewhere [33]. The
187 reusability of the biocatalytic nanoparticles was investigated by running 5 consecutive
188 micropollutant degradation cycles in the hybrid membrane reactor under identical operating
189 conditions. Each cycle lasted 24 h, and freshly prepared 50 ml of 10 µM micro-pollutant
190 mixture (bisphenol-A and carbamazepine, pH 5.5) was used at the beginning of each cycle.
191 At the end of each cycle, all the remaining solution was drained, and biocatalytic
192 nanoparticles were washed with Milli-Q water for three times to remove all the remaining
193 micro-pollutants or oxidation products. To monitor the activity of the immobilized enzyme

during the reaction, a certain amount of nanoparticles was collected at the end of each cycle to perform ABTS assay.

3. Results and discussion

3.1 Crude enzyme immobilization on TiO₂ nanoparticles

Prior to the attempt of immobilizing crude enzyme onto nanoparticles, the culture supernatant from fungus *P. ostreatus* was electrophoretically analysed using the SDS-PAGE to identify the protein components. Crude enzyme extract from *P. ostreatus* is shown to be homogeneous with a single band according to the result of SDS-PAGE (Fig. 1a). The relative molecular mass for the band is in the range between 50 and 75 kD as estimated by the protein markers, which aligns the molecular mass of the purified commercial laccases secreted from the same white-rot fungus [34] (Fig. 1a). This observation suggests that laccase is the primary enzyme component present in the crude enzyme extract. Fungus *P. ostreatus* has been reported to primarily secrete laccase, together with barely detectable amounts of ligninolytic enzymes including lignin peroxidase and manganese peroxidase within most culture medium applied for fungus growth [35,36]. To verify the crude laccase activity, no-denaturing gel (Native-PAGE) was performed using ABTS as a substrate to stain the protein band. In the absence of SDS, proteins migration rate in the gels is based on their isoelectric point and mass. As demonstrated in Fig. 1b, a single green-colored protein band is detected for the crude enzyme sample after the ABTS staining, and the band position is in good agreement with the result of the purified commercial laccase [34]. Therefore, laccase was confirmed as the main protein component within the crude enzyme extract.

Fig. 1. SDS-PAGE (a) and Native-PAGE (b) of the crude enzyme extract and purified laccase from fungus *P. ostreatus*.

In our previous research, the APTES-functionalized TiO_2 nanoparticles have been demonstrated to be effective candidates to covalently immobilize commercial laccase originated from *T. versicolor* [12]. In the current work, both the purified commercial laccase and crude enzyme extract from *P. ostreatus* were immobilized onto the TiO_2 nanoparticles and their immobilization performance is summarized in Table 1. For the purified commercial laccase, its loading and activity on the nanoparticles are $7.4 \mu\text{g}/\text{mg}_{\text{NP}}$ and $0.15 \text{ U}/\text{mg}_{\text{NP}}$, which are consistent with the results reported in our previous work using laccase from *T. versicolor* [12]. However, dramatically different results are observed with the crude enzyme: the direct crude enzyme immobilization leads to much lower laccase loading ($0.8 \mu\text{g}/\text{mg}_{\text{NP}}$) as well as an apparent activity ($0.019 \text{ U}/\text{mg}_{\text{NP}}$) for the biocatalytic nanoparticles. Given the fact that the laccase from crude enzyme extract has similar molecular mass and isoelectric point with the purified commercial counterpart (Fig. 1), the undesirable performance of the biocatalytic nanoparticles with the crude enzyme can be mainly attributed to the complex composition of the extract. Indeed, the crude enzyme extract is a cocktail of laccase, growth medium (malt extract broth in this work), and some other unidentified minor cofactors. As a result, the

functionalized nanoparticles could be covered or buried by the non-enzyme components during the direct immobilization process, which leads to a reduction of the surface area available for enzyme anchoring and weaker interaction between the enzyme and the cross-linkers on the TiO₂ surface. Even though the apparent activity is quite low, an unexpectedly high activity recovery rate is observed for the crude enzyme after immobilization (i.e. ~125%), indicating that the enzyme is more active after immobilization than in its free form, possibly due to the reduced unfavorable interaction (e.g. aggregation) of the enzyme in solution.

Table 1. Performance of the immobilized laccase on TiO₂ nanoparticles using different enzyme sources.

Enzyme source	Laccase loading ($\mu\text{g}/\text{mg}_{\text{NP}}$)	Apparent activity ($\text{U}/\text{mg}_{\text{NP}}$)	Activity recovery (%)	Reference
Crude enzyme extract (<i>P. ostreatus</i>)	0.8 ± 0.03	0.019 ± 0.001	125.8 ± 15.2	Current work
Purified commercial laccase (<i>P. ostreatus</i>)	7.4 ± 0.7	0.150 ± 0.018	63.7 ± 7.4	Current work
Purified commercial laccase (<i>T. versicolour</i>)	5.1 ± 0.5	0.135 ± 0.015	79.0 ± 6.0	[12]

Fig. 2. The comparison of enzyme loading (a) and activity (b) for the crude enzyme immobilization with different diluted samples (ABTS assay).

Although the apparent activity achieved was rather low, initial immobilization results highlighted the advantage of enzyme immobilization in terms of activity recovery rate. In order to reduce the attachment of the non-enzyme components onto the TiO_2 nanoparticle surface, the original crude enzyme extract was diluted prior to immobilization. As shown in Fig. 2, with the initial dilution of the crude enzyme, significant improvements in the laccase loading and nanoparticle apparent activity are achieved, and their highest values with CE-10 (i.e. 10 times dilution) are 5.0 $\mu\text{g}/\text{mg}_{\text{NP}}$ (loading) and 0.053 $\text{U}/\text{mg}_{\text{NP}}$ (apparent activity), respectively. Furthermore, as revealed by the enzyme loading efficiency, over 80% of the

laccase within the original crude extract is immobilized onto the nanoparticles when the dilution time is higher than 10, and this value improves slightly with further dilution of the original crude enzyme extracts. In other words, diluting the crude enzyme extract can effectively reduce the aforementioned competitive attachment effect of the non-protein components onto the TiO₂ nanoparticle surface, thus making the surface of nanoparticles more accessible to enzymes. The enzyme immobilization involves covalent bonding between amine groups on protein and aldehyde groups on the functionalized nanoparticle surface, while the unfavorable attachment between non-protein items onto nanoparticle surface is mainly based on hydrophilic interaction and van der Waals' force. With the dilution of the crude enzyme, the covalent bonding of enzyme appears to dominate, thus leading to the improved immobilization performance. To further confirm this assumption, the amounts of proteins and all organic compounds (including malt growth substrate, protein and other unidentified cofactors) contained in the crude enzyme extract were estimated with the total nitrogen and total organic carbon. The nitrogen to carbon ratio (N/C) of free and immobilized enzymes was compared. As demonstrated in Fig. 3, free crude enzymes with different dilution ratios show stable N/C ratios of around 10.5 %. In contrast, for the immobilized crude enzymes, the N/C ratios are strongly dependent on the dilution ratio, and the N/C ratios increase significantly with higher dilution ratios applied. The biocatalytic nanoparticles prepared using the original undiluted crude enzyme result in the lowest N/C ratio of 2 %, while the biocatalytic particles obtained with 100-time diluted crude enzyme (CE-100) give a much higher N/C ratio over 25%. Given the fact that protein molecules are the major contributor to total nitrogen, this observation revealed that the proportion of enzymes immobilized on the particles increase when the diluted crude enzyme is used for immobilization. This is consistent with the enzyme loading results demonstrated in Fig. 2a. Furthermore, with a dilution of the original crude enzyme (e.g. CE-10 to CE-100), the N/C

ratios of immobilized enzymes were much higher than that of free crude enzymes and close to that of purified enzymes, thus further highlighting the ‘purification’ effect of the crude enzyme after immobilization.

Fig. 3 Comparison of nitrogen to carbon ratio (N/C) of enzyme solution before and after immobilization. N/C ratio was determined according to the values of total nitrogen and total organic carbon. The pure laccase results are from commercial laccase (*P. ostreatus*)

The activity recovery exceeded 100% in nearly all cases (except for the crude enzyme with 5 times dilution) and generally increased with more dilution. Corresponding with this observation is the improvement in the specific activity of crude laccase ($\text{U/mg}_{\text{laccase}}$) after immobilization. As indicated in Table S1, the specific activities of immobilized enzymes are generally higher than that of the free enzyme, and peaked at $20.8 \text{ U/mg}_{\text{laccase}}$ for the biocatalytic particles prepared using 100 times diluted crude enzyme (CE-100). It is usually reported that an enzyme loses its activity after being immobilized on supports due to the potential conformational change of protein structure and the increased mass transport resistance between the active site of the enzyme and its substrate. However, a few studies

have also observed increased activity after immobilization, mainly due to the partial unfolding of the enzyme leading to more exposure of the active sites [37,38]. However, in this work, the immobilization purified commercial laccase still experienced activity loss, indicating that the improved activity of the crude enzyme is not originated from the conformational change of the proteins. Considering the complex composition of the crude enzyme extract, the free enzyme can have unfavorable interactions with the non-protein components, leading to aggregation and less accessible active sites. In comparison, after immobilization, laccase is covalently immobilized on nanoparticles, while other components are loosely attached to the support surface via physical interactions. With this regard, the immobilization reduces the unfavorable interactions for the laccase within free crude enzyme extract, and the active site of immobilized laccase would be more accessible to substrates, thus leading to a higher apparent activity.

In order to further understand the effect of dilution on the biocatalytic nanoparticle performance, we conducted particle size analysis to understand the particle aggregation during the crude enzyme immobilization process. As shown in Fig. 4, the mean diameter of nanoparticle clusters increases significantly after crude enzyme immobilization, indicating the bonding of protein and non-protein components onto the particles. It should be noted that the original undiluted crude enzyme leads to a significant aggregation of the particles, while the biocatalytic nanoparticles fabricated with the diluted enzyme have smaller particle sizes. Such an observation well aligns our previous results. By using the undiluted enzymes, the nanoparticles can be covered by a large amount of the non-protein components, leading to nanoparticle aggregation and increased mass transfer resistance. This can also compromise the large surface area of the biocatalytic nanoparticles, thus leading to much lower enzyme loadings and activities.

Fig. 4. Particle size distribution of biocatalytic nanoparticles prepared with different times diluted crude enzyme extract. (CE-10 stands for 10 times diluted crude enzyme).

It has been demonstrated that the dilution of the crude enzyme can effectively improve the immobilization performance and the 10-time diluted sample (CE-10) exhibits the highest laccase loading and activity. We further investigated the effect of the enzyme amount on the immobilization performance by soaking a fixed amount of nanoparticles into different volumes of the 10-time diluted crude enzyme extracts. As shown in Fig. 5a, the increase of the enzyme amount results in an improvement in laccase loading but a reduction in the laccase loading efficiency. In terms of the biocatalytic nanoparticle activity, it increases with enzyme amount and reached 0.11 U/mg_{NP} at an enzyme amount of ~850 μg (Fig. 5b). However, further increase of the initial crude enzyme volume led to a significant loss of the activity. Such an observation indicates that all the enzyme anchoring sites on the nanoparticle surface have been occupied by the laccase at this condition, and a further increase in the initial crude enzyme amount lead to the undesired lateral interaction between the over-crowded laccase and unfavorable interactions between immobilized laccase and non-protein

components, thus causing the loss in activity. The activity recovery rate drops gradually with a higher initial enzyme amount (Fig. 5b). In general, laccase from the crude extract mixture can be effectively immobilized onto the TiO_2 nanoparticles, and the immobilization performance (i.e. laccase loading and activity) is comparable to that of the purified commercial enzymes (Table 1). In the following sections, the immobilized crude laccase is further characterized in terms of kinetic constants, pH and temperature stability. Then the performance of the biocatalytic particles is compared with that of the free crude enzyme.

Fig. 5. Effect of the initial enzyme to nanoparticles ratio on the enzyme loading (a) and activity (b) at fixed crude extract concentration (10 times diluted)

350

351 3.2 Kinetics of the immobilized crude enzyme on TiO₂ nanoparticles

352 Kinetic experiments of both the free and immobilized crude enzymes were conducted for the
 353 oxidation of ABTS at pH 3 under 25 °C and the kinetic constants are summarized in Table 2.
 354 For the free crude enzyme, the substrate affinity (K_m) and catalytic activity (k_{cat}) are 37.3 μ M
 355 and 101.3 μ mol min⁻¹ mg⁻¹, respectively. These values fall into the same range as those
 356 reported for laccase from *P. ostreatus* and well align with our previous results on laccase
 357 from *T. versicolor* [12,32,39]. After immobilization, the K_m value is relatively unchanged,
 358 indicating a good preservation of the affinity between immobilized enzyme to substrates.
 359 Correspondingly, the efficiency of laccase to convert substrate (k_{cat}/K_m) also decreases
 360 slightly after immobilization (i.e. 2.8 L min⁻¹ mg⁻¹ for free enzyme vs 1.8 L min⁻¹ mg⁻¹ for the
 361 immobilized enzyme). These changes are commonly observed in enzyme immobilization
 362 studies [9,21,22]. The increase of K_m after immobilization can be mainly ascribed to the
 363 internal and external limitations, such as mass transfer resistance and limited accessibility of
 364 active sites after immobilization. In addition, the conformational changes of the enzyme
 365 molecule and steric hindrance could also lead to an increase in K_m . However, compared with
 366 the previous reports on laccase immobilization, the increase of K_m observed in the current
 367 work is relatively minor, suggesting that immobilizing crude enzyme on TiO₂ nanoparticles
 368 can be a promising solution to retain the catalytic efficiency of the enzyme.

369 Table 2. Kinetic parameters of the crude enzyme immobilized on TiO₂ nanoparticles (ABTS
 370 assay).

	K_m (μ M)	k_{cat} (μ mol min ⁻¹ mg ⁻¹)	$k_{cat} K_m^{-1}$ (L min ⁻¹ mg ⁻¹)
Free crude enzyme	37.3 $\pm$ 2.5	101.3 $\pm$ 11.8	2.8 $\pm$ 0.8
Immobilized crude enzyme	42.9 $\pm$ 3.3	75.5 $\pm$ 9.4	1.8 $\pm$ 0.3

371

372

373 **3.3 Effect of pH and temperature on the activity and stability of crude enzyme**

374 The activity of both free and immobilized enzymes on pH was determined via the ABTS
375 assay and the results were plotted in Fig. 6a. Typical monotonic activity curves are observed
376 for both free and immobilized enzymes as the pH increases from 3 to 7. Such an observation
377 is consistent with the reported impact of pH on fungal laccase activity: this has been
378 attributed to the OH⁻ inhibition to T2/T3 copper at higher pH [30,40]. As demonstrated in Fig.
379 6a, the activity of free enzyme drops more significantly along with higher pH, and less than
380 20% of the relative activity is observed at neutral pH (pH 7). In comparison with the free
381 enzyme, the immobilized enzyme performed significantly better in the pH range of 3.0 to 6.0.
382 This is further demonstrated by the pH-stability plot (Fig. 6b), which shows that the
383 immobilized enzyme generally retained higher relative activity.

384

385

386 Fig. 6. Effect of pH on the activity (a) and stability (b) of the free and immobilized crude
 387 enzyme. (a) Activity of the crude enzyme was assayed in the pH range of 2.5-7 at 25 °C. The
 388 relative activity was determined using the maximum activity obtained at pH 3 as the
 389 benchmark. (b) Stability of the crude enzyme under different pH conditions was determined
 390 by incubating the samples at pH 2.5-7 at room temperature for 12 h. After incubation, the
 391 enzyme activity was assayed at standard conditions (pH 3, 25 °C) and the relative activity
 392 after incubation was determined using the reaction velocity at pH 3 before incubation as the
 393 benchmark.

394 In terms of the effect of temperature, both the free and immobilized enzyme achieve the
395 maximum activity at 50 °C, while a more stable performance profile over the tested
396 temperature range is observed for the immobilized enzyme (Fig. 7a). For example, the
397 immobilized enzyme retains 85% of its initial activity after being incubated at 50 °C for 2 h,
398 whereas the free enzyme only has 50% of its initial activity (Fig. 7b). At higher incubation
399 temperature (70 °C), the free enzyme almost completely loses its activity while the
400 immobilized enzyme still retains 20% of relative activity. These observations suggest the
401 improved resistance to the thermal-induced denaturation of the immobilized enzyme
402 compared to the free enzyme. The long-term stability of the immobilized enzyme was further
403 investigated by storing the enzyme samples in citric acid-phosphate buffer (0.1 M, pH 5) at
404 25 °C for 30 days. As shown in Fig. S1, the immobilized enzyme retains most of its activity
405 (over 75%) after the 30-day storage period. In contrast, the free enzyme loses its activity
406 rapidly along with time and is completely inactivated after 23 days. Overall, according to the
407 aforementioned results, both the pH and temperature resistance and stability of the crude
408 enzyme are effectively improved after immobilization. While this finding is consistent with
409 previous studies on enzyme immobilization [41–44], our study demonstrates in the case of
410 the crude enzyme extraction for the first time. The increased stability can be mainly attributed
411 to the stabilization of immobilized enzyme on supporting materials via the formation of
412 covalent bonds, which reduces the occurrence of drastic conformational changes of enzyme
413 molecule under extreme conditions [45].

414

415 Fig. 7. Effect of temperature on the activity (a) and stability (b) of the free and immobilized
 416 crude enzyme. (a) Activity of the enzyme was assayed at different temperatures (25-70 °C) in
 417 pH 3 buffer solution. (b) Thermal stability of the enzyme was determined under standard
 418 conditions after incubation at different temperatures (25-70 °C) for 2 h. Enzyme activity after
 419 incubation was determined at standard condition (pH 3, 25 °C).

420

421 3.4 Micro-pollutants degradation by the biocatalytic nanoparticles

422 The removal of micro-pollutants bisphenol-A and carbamazepine was investigated in a
 423 hybrid membrane reactor, where the biocatalytic nanoparticles were suspended in the micro-

pollutant feed solution and the permeate was recirculated at a flow rate of 5 L/m² h. Fig. 8 presents the removal efficiencies of bisphenol-A and carbamazepine at pH 5.5, 25 °C and with 0.4 U/ml immobilized enzyme. Rapid removal is observed for the feed solution only containing bisphenol-A: 90 % of the bisphenol-A is degraded within the first 6 h of the reaction using the immobilized enzyme (Fig. 8a), while the more recalcitrant carbamazepine was hardly degraded and its degradation efficiency is only ~10% in a single-compound solution even after 48 h reaction (Fig. 8b). The poor removal of carbamazepine can be mainly attributed to the high redox potential of carbamazepine that impedes its oxidation by laccase [6,13,46,47]. However, it is noteworthy that carbamazepine degradation can be dramatically improved in the presence of bisphenol-A, and 40% removal of carbamazepine is achieved after a 24 h treatment (Fig 8b), which is comparable to the removal achieved using p-coumaric acid as a redox mediator [13]. This observation indicates that the oxidization products of bisphenol-A have a redox-mediator effect on carbamazepine degradation. In fact, phenolic compounds oxidation by laccase involves deprotonation of the hydroxyl group to generate phenoxy radicals that can break down to form smaller molecules, polymerize or react with other organic pollutants [2,48]. As bisphenol-A can be rapidly oxidized by the immobilized enzymes, the highly reactive radicals could react with carbamazepine, either as a mediator before returning to its initial stage, or by cross-linking to form high molecular weight products that are more prone to precipitate. These side reactions occurring in the presence of carbamazepine, on the other hand, may have a competitive effect on the bisphenol-A degradation, as evidenced by the slightly reduced removal of bisphenol-A compared with its single-compound solution (Fig. 8a). Further investigation is required to offer insight into the aforementioned process, but this finding highlights the cocktail effect of micro-pollutants mixture in the laccase-based biocatalytic system: the presence of more reactive micro-pollutant can promote the removal of the more recalcitrant pollutants [49].

449 Such an observation is preferable for practical applications where the micro-pollutants are
450 expected to be in the presence of a mixture. As a control experiment, bisphenol-A and
451 carbamazepine (mixture solution) degradation by free crude enzyme was performed with the
452 identical operation conditions. Compared to the biocatalytic nanoparticles suspension system,
453 a relatively rapid removal of bisphenol-A was achieved with the free enzyme (Fig. S2).
454 However, 75% of the activity of the free enzyme was lost at the end of the degradation
455 experiment, compared to that of 18% for the immobilized enzyme activity (Fig. S3). The
456 inactivation of the free enzyme mainly resulted from the interactions between the enzyme and
457 the phenoxy radicals and precipitating polymeric reactions products formed through the
458 catalytic reaction [50]. Furthermore, the hydraulic shear force near the membrane surface
459 may also lead to gradual loss of enzyme structure, thus resulting in the loss of activity.

460 In terms of membrane fouling performance, the increase of trans-membrane pressure (TMP)
461 during degradation process was negligible, suggesting that the formation of the TiO₂ particle
462 cake layer was relatively loose and did not cause irreversible membrane fouling [13].

Fig. 8. Removal of bisphenol-A (a) and carbamazepine (b) by biocatalytic nanoparticles in the hybrid membrane reactor (pH 5.5, 25 °C, 5 L/m²h with 0.4 U/ml of immobilized enzyme).
BPA: bisphenol-A; CBZ: carbamazepine; PCA: p-coumaric acid.

3.5 Reusability of the biocatalytic nanoparticles

As noted above, the biocatalytic nanoparticles show the substantial removal of micro-pollutants within the initial 24 h without significant loss of the enzyme activity. To investigate the reusability of the biocatalytic nanoparticles, five consecutive degradation cycles (bisphenol-A and carbamazepine mixture solution) were performed in the hybrid

membrane reactor. Each cycle lasted 24 h and was conducted with identical operation conditions (pH 5.5, 25 °C and 5 L/m² h of flux). As shown in Fig., the removals of both bisphenol-A and carbamazepine decrease gradually: for the 5th cycle, only 50% of bisphenol-A and 18% of carbamazepine are removed after 24 h incubation with the biocatalytic particles. As a comparison, the activity assay using ABTS as substrate shows a more significant activity loss (over 75%) of the biocatalytic particles after five cycles. As indicated previously, the formation of high molecular weight product after bisphenol-A oxidation can potentially bury the immobilized enzyme, which would decrease the mass transfer efficiency and make the enzyme less accessible by its substrates, especially for substrates with large molecular size such as ABTS. This leads to a significant loss in the catalytic competence of the biocatalytic particles on repeated use [51]. Therefore, further improvement of the reusability of the immobilized enzyme is still required by minimizing the bury effect of the oxidation products, potentially via optimizing the hydraulic condition within the hybrid reactor, however, that is beyond the scope of the current study.

Fig. 9. Micropollutant removal efficiency after 24 h in repeated degradation cycles in the hybrid membrane reactor (pH 5.5, 25 °C, 5 L/m²h with 0.4 U/ml of initially immobilized enzyme). BPA: bisphenol-A; CBZ: carbamazepine.

491 **4. Conclusion**

492 This work applied surface functionalized TiO₂ nanoparticles for the direct immobilization of
493 the crude enzyme extracts. Simply by diluting the original crude enzyme extracts, the
494 undesired interaction between the non-protein components and immobilization support
495 surface could be alleviated. The resultant nanoparticles exhibited comparable performance to
496 the purified commercial enzymes. The feasibility of using the biocatalytic nanoparticle for
497 wastewater treatment was further exemplified by two representative micro-pollutants. In
498 general, the biocatalytic nanoparticles in this work show a significant potential to immobilize
499 crude enzyme extracts for cost-effective practical applications.

500 Reference

- 501 [1] Y.-J. Kim, J.A. Nicell, Impact of reaction conditions on the laccase-catalyzed
502 conversion of bisphenol A, *Bioresour. Technol.* 97 (2006) 1431–1442.
503 doi:10.1016/j.biortech.2005.06.017.
- 504 [2] H. Cabana, J.P. Jones, S.N. Agathos, Elimination of Endocrine Disrupting Chemicals
505 using White Rot Fungi and their Lignin Modifying Enzymes: A Review, *Eng. Life Sci.*
506 7 (2007) 429–456. doi:10.1002/elsc.200700017.
- 507 [3] F.I. Hai, O. Modin, K. Yamamoto, K. Fukushi, F. Nakajima, L.D. Nghiem, Pesticide
508 removal by a mixed culture of bacteria and white-rot fungi, *J. Taiwan Inst. Chem. Eng.*
509 43 (2012) 459–462. doi:10.1016/j.jtice.2011.11.002.
- 510 [4] C. Nicolucci, S. Rossi, C. Menale, T. Godjevargova, Y. Ivanov, M. Bianco, L. Mita, U.
511 Bencivenga, D.G. Mita, N. Diano, Biodegradation of bisphenols with immobilized
512 laccase or tyrosinase on polyacrylonitrile beads, *Biodegradation*. 22 (2010) 673–683.
513 doi:10.1007/s10532-010-9440-2.
- 514 [5] S. Yang, F.I. Hai, L.D. Nghiem, L.N. Nguyen, F. Roddick, W.E. Price, Removal of
515 bisphenol A and diclofenac by a novel fungal membrane bioreactor operated under non-
516 sterile conditions, *Int. Biodeterior. Biodegrad.* 85 (2013) 483–490.
517 doi:10.1016/j.ibiod.2013.03.012.
- 518 [6] S. Yang, F.I. Hai, L.D. Nghiem, W.E. Price, F. Roddick, M.T. Moreira, S.F. Magram,
519 Understanding the factors controlling the removal of trace organic contaminants by
520 white-rot fungi and their lignin modifying enzymes: A critical review, *Bioresour.*
521 *Technol.* 141 (2013) 97–108. doi:10.1016/j.biortech.2013.01.173.
- 522 [7] M. Asif, T.C. Bhalla, Hydroxynitrile Lyase of Wild Apricot (*Prunus armeniaca* L.):
523 Purification, Characterization and Application in Synthesis of Enantiopure
524 Mandelonitrile, *Catal. Lett.* 146 (2016) 1118–1127. doi:10.1007/s10562-016-1725-6.
- 525 [8] P.F.X. Corvini, P. Shahgaldian, LANCE: Laccase-nanoparticle conjugates for the
526 elimination of micropollutants (endocrine disrupting chemicals) from wastewater in
527 bioreactors, *Rev. Environ. Sci. Biotechnol.* 9 (2009) 23–27. doi:10.1007/s11157-009-
528 9182-y.
- 529 [9] L. Lloret, G. Eibes, G. Feijoo, M.T. Moreira, J.M. Lema, F. Hollmann, Immobilization
530 of laccase by encapsulation in a sol–gel matrix and its characterization and use for the
531 removal of estrogens, *Biotechnol. Prog.* 27 (2011) 1570–1579. doi:10.1002/btpr.694.
- 532 [10] R.A. Sheldon, Enzyme Immobilization: The Quest for Optimum Performance, *Adv.*
533 *Synth. Catal.* 349 (2007) 1289–1307. doi:10.1002/adsc.200700082.
- 534 [11] R. Singh, M. Tiwari, R. Singh, J.-K. Lee, From Protein Engineering to Immobilization:
535 Promising Strategies for the Upgrade of Industrial Enzymes, *Int. J. Mol. Sci.* 14 (2013)
536 1232–1277. doi:10.3390/ijms14011232.
- 537 [12] J. Hou, G. Dong, B. Luu, R.G. Sengpiel, Y. Ye, M. Wessling, V. Chen, Hybrid
538 membrane with TiO₂ based bio-catalytic nanoparticle suspension system for the
539 degradation of bisphenol-A, *Bioresour. Technol.* 169 (2014) 475–483.
540 doi:10.1016/j.biortech.2014.07.031.
- 541 [13] C. Ji, J. Hou, K. Wang, Y. Zhang, V. Chen, Biocatalytic degradation of carbamazepine
542 with immobilized laccase-mediator membrane hybrid reactor, *J. Membr. Sci.* 502 (2016)
543 11–20. doi:10.1016/j.memsci.2015.12.043.
- 544 [14] L.N. Nguyen, F.I. Hai, J. Kang, F.D.L. Leusch, F. Roddick, S.F. Magram, W.E. Price,
545 L.D. Nghiem, Enhancement of trace organic contaminant degradation by crude enzyme
546 extract from *Trametes versicolor* culture: Effect of mediator type and concentration, *J.*
547 *Taiwan Inst. Chem. Eng.* 45 (2014) 1855–1862. doi:10.1016/j.jtice.2014.03.021.

- [15] L.A. Dahili, I. Kelemen-Horváth, T. Feczko, 2,4-Dichlorophenol removal by purified horseradish peroxidase enzyme and crude extract from horseradish immobilized to nano spray dried ethyl cellulose particles, *Process Biochem.* 50 (2015) 1835–1842. doi:10.1016/j.procbio.2015.08.008.
- [16] S.T. Sagu, E.J. Nso, T. Homann, C. Kapseu, H.M. Rawel, Extraction and purification of beta-amylase from stems of *Abrus precatorius* by three phase partitioning, *Food Chem.* 183 (2015) 144–153. doi:10.1016/j.foodchem.2015.03.028.
- [17] C.J. Mazzeu, E.Z. Ramos, M.H. da Silva Cavalcanti, D.B. Hirata, L.S. Virtuoso, Partitioning of *Geotrichum candidum* Lipase from fermentative crude extract by aqueous two-phase system of polyethylene glycol and sodium citrate, *Sep. Purif. Technol.* 156, Part 2 (2015) 158–164. doi:10.1016/j.seppur.2015.09.069.
- [18] Z. Dadshahi, A. Homaei, F. Zeinali, R.H. Sajedi, K. Khajeh, Extraction and purification of a highly thermostable alkaline caseinolytic protease from wastes *Penaeus vannamei* suitable for food and detergent industries, *Food Chem.* 202 (2016) 110–115. doi:10.1016/j.foodchem.2016.01.104.
- [19] R. Kumari Narwal, B. Bhushan, A. Pal, A. Panwar, S. Malhotra, Purification, physico-chemico-kinetic characterization and thermal inactivation thermodynamics of milk clotting enzyme from *Bacillus subtilis* MTCC 10422, *LWT - Food Sci. Technol.* 65 (2016) 652–660. doi:10.1016/j.lwt.2015.08.065.
- [20] M. Mahesh, K.V. Arivizhivendhan, P. Maharaja, R. Boopathy, V. Hamsavathani, G. Sekaran, Production, purification and immobilization of pectinase from *Aspergillus ibericus* onto functionalized nanoporous activated carbon (FNAC) and its application on treatment of pectin containing wastewater, *J. Mol. Catal. B Enzym.* 133 (2016) 43–54. doi:10.1016/j.molcatb.2016.07.012.
- [21] B. Kopka, M. Diener, A. Wirtz, M. Pohl, K.-E. Jaeger, U. Krauss, Purification and simultaneous immobilization of *Arabidopsis thaliana* hydroxynitrile lyase using a family 2 carbohydrate-binding module, *Biotechnol. J.* 10 (2015) 811–819. doi:10.1002/biot.201400786.
- [22] K. Matsushima, H. Kaneda, K. Harada, H. Matsuura, K. Hirata, Immobilization of enzymatic extracts of *Portulaca oleracea* cv. roots for oxidizing aqueous bisphenol A, *Biotechnol. Lett.* 37 (2015) 1037–1042. doi:10.1007/s10529-014-1761-5.
- [23] Y. Yu, X. Fei, J. Tian, L. Xu, X. Wang, Y. Wang, Self-assembled enzyme–inorganic hybrid nanoflowers and their application to enzyme purification, *Colloids Surf. B Biointerfaces.* 130 (2015) 299–304. doi:10.1016/j.colsurfb.2015.04.033.
- [24] K. Gupta, A.K. Jana, S. Kumar, M.M. Jana, Solid state fermentation with recovery of Amyloglucosidase from extract by direct immobilization in cross linked enzyme aggregate for starch hydrolysis, *Biocatal. Agric. Biotechnol.* 4 (2015) 486–492. doi:10.1016/j.bcab.2015.07.007.
- [25] S. Talekar, V. Ghodake, T. Ghotage, P. Rathod, P. Deshmukh, S. Nadar, M. Mulla, M. Ladole, Novel magnetic cross-linked enzyme aggregates (magnetic CLEAs) of alpha amylase, *Bioresour. Technol.* 123 (2012) 542–547. doi:10.1016/j.biortech.2012.07.044.
- [26] S. Talekar, V. Shah, S. Patil, M. Nimbalkar, Porous cross linked enzyme aggregates (p-CLEAs) of *Saccharomyces cerevisiae* invertase, *Catal. Sci. Technol.* 2 (2012) 1575–1579. doi:10.1039/C2CY20304A.
- [27] M. Carballa, F. Omil, J.M. Lema, M. Llompart, C. García-Jares, I. Rodríguez, M. Gómez, T. Ternes, Behavior of pharmaceuticals, cosmetics and hormones in a sewage treatment plant, *Water Res.* 38 (2004) 2918–2926. doi:10.1016/j.watres.2004.03.029.
- [28] B. Kasprzyk-Hordern, R.M. Dinsdale, A.J. Guwy, The removal of pharmaceuticals, personal care products, endocrine disruptors and illicit drugs during wastewater

- treatment and its impact on the quality of receiving waters, *Water Res.* 43 (2009) 363–380. doi:10.1016/j.watres.2008.10.047.
- [29] B. Ashe, L.N. Nguyen, F.I. Hai, D.-J. Lee, J.P. van de Merwe, F.D.L. Leusch, W.E. Price, L.D. Nghiem, Impacts of redox-mediator type on trace organic contaminants degradation by laccase: Degradation efficiency, laccase stability and effluent toxicity, *Int. Biodeterior. Biodegrad.* 113 (2016) 169–176. doi:10.1016/j.ibiod.2016.04.027.
- [30] O. Fokina, J. Eipper, L. Winandy, S. Kerzenmacher, R. Fischer, Improving the performance of a biofuel cell cathode with laccase-containing culture supernatant from *Pycnoporus sanguineus*, *Bioresour. Technol.* 175 (2015) 445–453. doi:10.1016/j.biortech.2014.10.127.
- [31] H. Zouari-Mechichi, T. Mechichi, A. Dhouib, S. Sayadi, A.T. Martínez, M.J. Martínez, Laccase purification and characterization from *Trametes trogii* isolated in Tunisia: decolorization of textile dyes by the purified enzyme, *Enzyme Microb. Technol.* 39 (2006) 141–148. doi:10.1016/j.enzmictec.2005.11.027.
- [32] J. Hou, G. Dong, Y. Ye, V. Chen, Laccase immobilization on titania nanoparticles and titania-functionalized membranes, *J. Membr. Sci.* 452 (2014) 229–240. doi:10.1016/j.memsci.2013.10.019.
- [33] C. Ji, J. Hou, V. Chen, Cross-linked carbon nanotubes-based biocatalytic membranes for micro-pollutants degradation: Performance, stability, and regeneration, *J. Membr. Sci.* 520 (2016) 869–880. doi:10.1016/j.memsci.2016.08.056.
- [34] O.V. Morozova, G.P. Shumakovich, M.A. Gorbacheva, S.V. Shleev, A.I. Yaropolov, “Blue” laccases, *Biochem. Mosc.* 72 (2007) 1136–1150. doi:10.1134/S0006297907100112.
- [35] E. Fernández-Fueyo, R. Castanera, F.J. Ruiz-Dueñas, M.F. López-Lucendo, L. Ramírez, A.G. Pisabarro, A.T. Martínez, Ligninolytic peroxidase gene expression by *Pleurotus ostreatus*: Differential regulation in lignocellulose medium and effect of temperature and pH, *Fungal Genet. Biol.* 72 (2014) 150–161. doi:10.1016/j.fgb.2014.02.003.
- [36] M. Mansur, M.E. Arias, J.L. Copa-Patiño, M. Flärdh, A.E. González, The white-rot fungus *Pleurotus ostreatus* secretes laccase isozymes with different substrate specificities, *Mycologia.* 95 (2003) 1013–1020.
- [37] Y. Cao, Z. Wu, T. Wang, Y. Xiao, Q. Huo, Y. Liu, Immobilization of *Bacillus subtilis* lipase on a Cu-BTC based hierarchically porous metal–organic framework material: a biocatalyst for esterification, *Dalton Trans.* 45 (2016) 6998–7003. doi:10.1039/C6DT00677A.
- [38] F. Lyu, Y. Zhang, R.N. Zare, J. Ge, Z. Liu, One-Pot Synthesis of Protein-Embedded Metal–Organic Frameworks with Enhanced Biological Activities, *Nano Lett.* 14 (2014) 5761–5765. doi:10.1021/nl5026419.
- [39] Tinoco, Pickard, Vazquez-Duhalt, Kinetic differences of purified laccases from six *Pleurotus ostreatus* strains, *Lett. Appl. Microbiol.* 32 (2001) 331–335. doi:10.1046/j.1472-765X.2001.00913.x.
- [40] F. Xu, Effects of Redox Potential and Hydroxide Inhibition on the pH Activity Profile of Fungal Laccases, *J. Biol. Chem.* 272 (1997) 924–928. doi:10.1074/jbc.272.2.924.
- [41] L. Fernando Bautista, G. Morales, R. Sanz, Immobilization strategies for laccase from *Trametes versicolor* on mesostructured silica materials and the application to the degradation of naphthalene, *Bioresour. Technol.* 101 (2010) 8541–8548. doi:10.1016/j.biortech.2010.06.042.
- [42] J. Mehta, N. Bhardwaj, S.K. Bhardwaj, K.-H. Kim, A. Deep, Recent advances in enzyme immobilization techniques: Metal-organic frameworks as novel substrates, *Coord. Chem. Rev.* 322 (2016) 30–40. doi:10.1016/j.ccr.2016.05.007.

- [43] N.A. Mohidem, H.B. Mat, Catalytic activity and stability of laccase entrapped in sol–gel silica with additives, *J. Sol-Gel Sci. Technol.* 61 (2011) 96–103. doi:10.1007/s10971-011-2596-3.
- [44] J. Shi, X. Wang, Z. Jiang, Y. Liang, Y. Zhu, C. Zhang, Constructing spatially separated multienzyme system through bioadhesion-assisted bio-inspired mineralization for efficient carbon dioxide conversion, *Bioresour. Technol.* 118 (2012) 359–366. doi:10.1016/j.biortech.2012.04.099.
- [45] J.N. Talbert, J.M. Goddard, Enzymes on material surfaces, *Colloids Surf. B Biointerfaces*. 93 (2012) 8–19. doi:10.1016/j.colsurfb.2012.01.003.
- [46] T. Hata, H. Shintate, S. Kawai, H. Okamura, T. Nishida, Elimination of carbamazepine by repeated treatment with laccase in the presence of 1-hydroxybenzotriazole, *J. Hazard. Mater.* 181 (2010) 1175–1178. doi:10.1016/j.jhazmat.2010.05.103.
- [47] N.H. Tran, T. Urase, O. Kusakabe, Biodegradation Characteristics of Pharmaceutical Substances by Whole Fungal Culture *Trametes versicolor* and its Laccase, *J. Water Environ. Technol.* 8 (2010) 125–140. doi:10.2965/jwet.2010.125.
- [48] H. Claus, Laccases: structure, reactions, distribution, *Micron*. 35 (2004) 93–96. doi:10.1016/j.micron.2003.10.029.
- [49] J. Margot, J. Maillard, L. Rossi, D.A. Barry, C. Holliger, Influence of treatment conditions on the oxidation of micropollutants by *Trametes versicolor* laccase, *New Biotechnol.* 30 (2013) 803–813. doi:10.1016/j.nbt.2013.06.004.
- [50] M.A. Tarr, ed., *Chemical degradation methods for wastes and pollutants: environmental and industrial applications*, M. Dekker, New York, 2003.
- [51] J. Hou, G. Dong, Y. Ye, V. Chen, Enzymatic degradation of bisphenol-A with immobilized laccase on TiO₂ sol–gel coated PVDF membrane, *J. Membr. Sci.* 469 (2014) 19–30. doi:10.1016/j.memsci.2014.06.027.

Supporting information

Table S1

Fig S1 to S3

Table S1. Activity recovery of the crude enzyme after immobilization TiO₂ nanoparticles.

Diluted crude enzyme (CE-T) ^a	Activity of free enzyme (U/mg _{laccase})	Activity of immobilized enzyme (U/mg _{laccase})	Activity recovery rate (%)
CE-0	10.8±0.5	13.8±1.4	126.8±15.2
CE-2.5	12.6±0.9	14.6±0.8	109.2±16.5
CE-5	12.2±1.4	10.8±0.6	83.4±11.9
CE-10	10.7±1.2	13.8±0.9	113.4±10.9
CE-25	11.3±0.5	14.7±1.4	112.9±14.2
CE-50	12.4±1.0	15.3±1.9	119.2±47.0

CE-100	11.9±1.1	20.8±3.0	150.6±24.8
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676 ^aDiluted enzyme samples were designated as CE-T, where T stands for the dilution time.

677

678

679 Fig. S1. The long-term stability of free and immobilized enzyme after 30 days of incubation
 680 in citric acid-phosphate buffer (0.1 M, pH 5) at 25 °C. The relative enzyme activity was
 681 determined using the activity at 0 day as 100%.

682

683 Fig. S2. Removal of the micro-pollutant mixture by free crude enzyme in the hybrid
 684 membrane reactor. (pH 5.5, 25 °C, 5 L/m² h with 0.4 U/ml of free enzyme). BPA: bisphenol-
 685 A; CBZ: carbamazepine.

686

687

688 Fig. S3. Activity of the free and immobilized crude enzyme during micro-pollutants

689 (bisphenol-A and carbamazepine mixture) degradation.

690