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Continuous adsorption and biotransformation of micropollutants by granular activated carbon-bound laccase in a packed-bed enzyme reactor

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Abstract

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Keywords

enzyme, bed, packed, laccase, bound, reactor, carbon, biotransformation, activated, continuous, granular, micropollutants, adsorption

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Abstract

Laccase was immobilized on granular activated carbon (GAC) and the resulting GAC-bound laccase was used to degrade four micropollutants in a packed-bed column. Compared to the free enzyme, the immobilized laccase showed high residual activities over a broad range of pH and temperature. The GAC-bound laccase efficiently removed four micropollutants, namely, sulfamethoxazole, carbamazepine, diclofenac and bisphenol A, commonly detected in raw wastewater and wastewater-impacted water sources. Mass balance analysis showed that these micropollutants were enzymatically degraded following adsorption onto GAC. Higher degradation efficiency of micropollutants by the immobilized compared to free laccase was possibly due to better electron transfer between laccase and substrate molecules once they have adsorbed onto the GAC surface. Results here highlight the complementary effects of adsorption and enzymatic degradation on micropollutant removal by GAC-bound laccase. Indeed laccase-immobilized GAC outperformed regular GAC during continuous operation of packed-bed columns over two months (a throughput of 12,000 bed volumes).

Keywords: Laccase; Granular activated carbon; Micropollutants; Biotransformation, Immobilization

1. Introduction

Conventional wastewater treatment processes have been specifically designed for the removal of basic water quality parameters such as suspended solids and chemical oxygen demand. Their removal efficiency for micropollutants is low and highly variable. As indicated by the term ‘micropollutants’, these contaminants occur in wastewater at very low concentrations (*i.e.*, as low as a few ng/L). They can induce certain therapeutic or toxic effects and thus can pose a considerable risk to the aquatic environment and even human health (Alexander et al., 2012; Schwarzenbach et al., 2006).

Laccases (E.C. 1.10.3.2) are oxidoreductive enzymes that can catalyze the oxidation of especially phenolic and certain non-phenolic compounds. Recent studies have demonstrated that laccase can efficiently degrade a broad spectrum of micropollutants that are hardly degradable by conventional biological processes (Tran et al., 2010; Yang et al., 2013b). However, major drawbacks of using freely suspended laccase are loss of enzymatic activity and continuous loss of laccase with treated effluent. These drawbacks hinder the application of laccase to remediation and increase the operational cost.

A potential approach to overcome the above mentioned drawbacks is to immobilize laccase onto solid supports. The advantages of laccase immobilization have been evaluated mainly using phenol as a model toxic compound (Davis and Burns, 1992). Several previous studies have also investigated degradation of dyes by immobilized laccase (Modin et al., 2014; Sathishkumar et al., 2014). By contrast, only a few studies have investigated the removal of micropollutants by immobilized laccase (Cabana et al., 2009; Hou et al., 2014; Lloret et al., 2012).

Immobilization of laccase has been demonstrated on different kinds of inert (e.g., alginate beads and aluminum oxide pellets) as well as active (e.g. silica gel, zeolite and activated carbon)

support materials. These materials were chosen due to their high surface area, mechanical strength, and non-toxic nature (Daâssi et al., 2014). Several methods have been developed for the immobilization of laccase such as entrapment, ionic interaction, covalent attachment, encapsulation and adsorption. The immobilization methods are primarily based on physical, covalent and affinity interactions between laccase and supports (Cristóvão et al., 2011; Daâssi et al., 2014). Among these methods, physical adsorption is a simple and economical process. However, the choice of inert supports requires that their surfaces are properly modified in order to offer functional groups for protein binding (Hou et al., 2014; Lloret et al., 2012). In this context, activated carbon, which is a well-known adsorbent, could be beneficial for laccase immobilization without the use of bonding reagents.

Granular activated carbon (GAC) is characterized with high specific surface area, high adsorption capacity, porous structure and commercial availability in high purity standards. These characteristics make GAC a potential support for enzyme immobilization. For example, Daoud et al. (2010) showed activated carbon as an effective adsorbent for cellulase immobilization. The structure of laccase is unlikely to be perturbed during the adsorption process which is important to maintain laccase activity. However, there are only a few reports in the literature regarding laccase immobilization on activated carbon (Davis and Burns, 1992; Liu et al., 2012).

Furthermore, previous studies using GAC-bound laccase have only tested its performance in batch or semi-continuous mode (Cristóvão et al., 2011; Hou et al., 2014). The performance of GAC as a support material during continuous operation over an extended period is unknown. This is a significant research gap that hinders the use of immobilized laccase for continuous treatment of micropollutants. Therefore, in this study the performance of GAC-bound laccase is assessed in both batch and continuous operation modes.

Notably, activated carbon such as GAC can efficiently adsorb a wide range of micropollutants (Nguyen et al., 2013a; Skouteris et al., 2015), but as with all adsorbents, micropollutant adsorption onto GAC decreases with the operation time due to surface area saturation (Hernández-Leal et al., 2011; Nguyen et al., 2013a). Therefore, replenishment and/or regeneration of GAC is required to maintain the system performance. In this context, pre-adsorption of laccase on GAC can be used as a strategy for *in-situ* regeneration of GAC. Immobilized laccase could degrade the adsorbed micropollutants and liberate sorption sites. Moreover, co-adsorption of laccase and micropollutants on GAC may improve the biodegradation by laccase due to the enhancement of electron transfer between laccase and micropollutants after adsorption (Zille et al., 2003). For example, Zille et al. (2003) observed an improvement in the removal of dyes due to their adsorption and subsequent laccase-mediated degradation. Nevertheless, the implications of co-adsorption of laccase and micropollutants on GAC remains poorly understood.

The aim of this study is to investigate immobilization of laccase on GAC and consequently use it for the removal of micropollutants in a continuous flow packed-bed reactor. The performance of immobilized and freely suspended laccase was systematically compared to highlight the benefit of laccase immobilization on GAC, particularly focusing on the added advantages of using an adsorbent as the support. With critical analysis of the data related to enzymatic activity, coverage of GAC surface area by the enzyme and the micropollutants, and overall micropollutant removal, this work provides unique insight into the behavior of immobilized laccase in a continuous system.

2. Methods

2.1 Micropollutants, granular activated carbon and laccase

Four micropollutants namely bisphenol A (BPA), diclofenac (DCF), carbamazepine (CBZ) and sulfamethoxazole (SMX) were selected based on their widespread occurrence in water and wastewater and their different degree of removal by laccase in previous studies (Nguyen et al., 2014a; Yang et al., 2013b). Bisphenol A is well degraded by laccase, while the removal of diclofenac is moderate (Yang et al., 2013a). On the other hand, carbamazepine and sulfamethoxazole have been reported to be resistant to degradation by laccase (Nguyen et al., 2013b; Yang et al., 2013b). These compounds were selected to facilitate a systematic investigation of the effect of GAC-bound laccase on their removal. The physicochemical properties of these micropollutants are summarized in Supplementary Data Table S1.

GAC-1300 (Activated Carbon Technologies Pty. Ltd., Victoria, Australia) with a specific surface area of 1180 m²/g was chosen as the laccase support. The GAC was sieved to obtain 0.4 - 0.7 mm particles, washed with Milli-Q water to remove fine particles, and dried at 100 °C for 24 h. The GAC was then modified by washing with hydrochloric acid (HCl) because it has been reported that such washing enhances laccase immobilization by increasing the number of surface carboxylic acid groups (Alkan et al., 2009; Cho and Bailey, 1979). The acid-wash procedure involved treating 50 g of GAC in 400 mL of HCl (1 M) for 12 h. The GAC was mixed thoroughly and heated at 60 °C using a magnetic stirrer hot plate. It was then rinsed with Milli-Q water to remove chloride ion until the conductivity of the supernatant was 25 mS/cm or less and then dried overnight at a temperature of 100 °C.

A commercially available laccase (Novozyme 51003) supplied by Novozymes, Australia was used in this study. This enzyme was purified from genetically modified *Aspergillus oryzae*. The molecular weight of this enzyme is 56 kDa. The enzyme stock solution had a density, purity and activity of 1.12 g/mL, approximately 3% (w/w) and 150000 mM_(DMP)/min (measured using 2,6-dimethoxy phenol (DMP) as substrate), respectively.

2.2 Laccase immobilization

Acid-washed GAC was used directly for laccase immobilization without any further modifications. Two grams of acid-washed GAC was suspended in 50 mL laccase solution in 400 mL beakers. The laccase solution was prepared by diluting 3 mL of stock laccase solution into 50 mL Milli-Q water. The solution showed an activity of 8100 μ M_(DMP)/min, which is equivalent to a laccase dose of 0.108 g. The laccase immobilization process was carried out in a rotary shaker at a shaking speed of 70 rpm and temperature of 25 °C for 24 h. It was confirmed via prior investigations that laccase adsorption on GAC reached equilibrium by 24 h. The supernatant of the solution was decanted and the residual laccase activity was measured (6000 μ M_(DMP)/min). Additionally, partially saturated GAC samples (30 % of the maximal laccase adsorption capacity of GAC) were prepared. The micropollutant removal performance of these two GAC samples differently loaded with laccase was compared (see Section 2.4.1). Additional samples were prepared to assess the immobilization of laccase on non-acid washed GAC.

2.3 Assessment of laccase stability

2.3.1 pH and temperature tolerance

The effect of solution pH on the stability of laccase preparations was studied by incubating free and immobilized laccase in sodium citrate buffer solutions over a pH range of 3 to 9 on a rotary

shaker at 25 °C. Free laccase was added to Milli-Q water to obtain a test solution with an initial laccase activity of 35 $\mu\text{M}_{(\text{DMP})}/\text{min}$, and 50 mg of laccase-immobilized GAC was added to separate containers to match that enzymatic activity. The residual laccase activity was measured after 2, 4 and 8 h of incubation. The thermal stability was assessed by incubating free and immobilized laccase at different temperatures (*i.e.*, 30-70 °C) for 8 h.

2.3.2 Reusability

The reusability of immobilized laccase was first assessed by repeatedly incubating a 50 mg sample of GAC with the enzymatic activity measurement reagent (see Section 2.5.1). In each run, laccase activity of the solution was measured after 2 min, the spent solution decanted, the GAC washed with Milli-Q water, and then fresh reagent was added to start the next run. The same process was repeated for 20 cycles. As described in Section 2.4.1, reusability was also assessed in terms of micropollutant degradation during repeated cycles.

2.4 Micropollutant degradation by GAC-bound laccase

2.4.1 Repeated use of immobilized laccase

The micropollutant removal performance of GAC and laccase-immobilized GAC (partial and full saturation) was first evaluated in batch tests. The reaction mixture contained 50 mg GAC or laccase-immobilized GAC and 100 mL of micropollutant solution (each at 2.5 mg/L) in a 400 mL beaker. The initial concentrations of the micropollutants and GAC were selected such that the micropollutant loading exceeded the maximum adsorption capacity of GAC, thus allowing the effect of laccase-catalyzed degradation to be clearly observed. In order to demonstrate that the improved micropollutant removal was due to biodegradation and not merely due to adsorption on the support, a similar protocol using elevated concentrations of the micropollutants

was proposed by Nguyen et al. (2014b). The reaction mixture was incubated in a rotary shaker at 70 rpm and 25 °C for 2 h. Micropollutant removal efficiency was measured based on their initial and final aqueous phase concentrations (Equation 1):

$$R = \frac{C_{initial} - C_{final}}{C_{initial}} \quad \text{Equation 1}$$

Where $C_{initial}$ and C_{final} are initial and final concentrations. After each run, the supernatant was decanted and fresh micropollutant solution was added to start the next cycle.

2.4.2 Contribution of biodegradation to overall removal

The removal of micropollutants by the immobilized laccase system is due to a combination of micropollutant adsorption onto the GAC and their degradation by laccase. In order to reveal the role of biodegradation in micropollutant removal, a series of experiments was carried out with GAC and free and immobilized laccase. The test solution (100 mL) included each micropollutant at a concentration of 2.5 mg/L. The free laccase solution possessed an activity of 37 $\mu\text{M}_{(\text{DMP})}/\text{min}$. To match this activity, 50 mg of laccase-immobilized GAC was added separately to test solution. Additional containers with 50 mg GAC was prepared to assess the extent of micropollutant adsorption. At the end of the incubation period, the micropollutant concentration in the aqueous phase and on GAC was measured (see Section 2.5.3).

2.4.3 Packed-bed column set up

Two borosilicate glass columns (Omnifit, Danbury, CT, USA) were filled with 7.5 g of GAC and laccase-immobilized GAC. The columns had an internal diameter of 1 cm and an active length of 22 cm, resulting in a bed volume (BV) of 17 mL (Supplementary Data Figure S2). The bottom and top ends of the column were plugged with glass fiber to prevent any loss of GAC. The feed

solution was pumped through the column in an up-flow mode at a flow rate of 2.4 mL/min (equivalent to 8.5 BV/h) via a peristaltic pump (Masterflex L/S, USA), resulting in an empty bed contact time (EBCT) of 7 min in each column. The feed was prepared daily in Milli-Q water using micropollutant stock solution to get the final concentration of 0.5 mg/L of each compound. Given that an adsorbent was used as a support for enzyme immobilization, the micropollutant concentration was selected to be higher than the reported concentration in real wastewater samples to accelerate the breakthrough of the micropollutants. This helped clearly demonstrating the advantage of laccase immobilization on GAC during continuous operations. The column temperature was maintained at 28°C by a temperature controller (Julabo, Germany). The columns were operated for 60 days (12000 BV) and the concentration of feed and effluent was measured every three days. At the end of experiment, the residual micropollutant concentrations on GAC in each column were measured and mass balance was conducted.

2.5 Analytical methods

2.5.1 Enzymatic activity assays

Laccase activity was determined by monitoring the oxidation of 10 mM DMP in 100 mM sodium citrate buffer (pH 4.5) over 2 min at room temperature. The reaction was started by the addition of DMP to the sample in presence of the sodium citrate buffer solution. The measurement was based on the change in absorbance at 468 nm. Laccase activity was then calculated from the molar absorptivity, $\epsilon_{468\text{ nm}} = 49.6/(\text{mM cm})$ and expressed in $\mu\text{M}_{(\text{DMP})}/\text{min}$. For the assay of the immobilized laccase, a sample of 50 mg was used. The reaction condition used were the same as those described in the free laccase assay. The final activity of laccase-immobilized GAC was expressed in $\mu\text{M}_{(\text{DMP})}/\text{min g}_{\text{GAC}}$.

2.5.2 Surface area analysis of GAC preparations

The surface area and pore volume of the GAC samples before and after laccase immobilization and after use in micropollutant removal tests were investigated. Gas sorption studies were carried out at the Wollongong Isotope and Geochronology Laboratory using a Quantachrome Autosorb MP instrument. Samples were degassed under dynamic vacuum at 80 °C for 12 h before collecting isotherms at 77 K up to 1 bar using high purity nitrogen (99.999 %) gas. The adsorption-desorption isotherms have been presented in Supplementary Data Table S3. Surface areas were determined using Brunauer-Emmett-Teller (BET) calculations. Micropore (< 2 nm) calculations were performed using the MP method within the Quantachrome ASiQWin 3.0 software. Quenched solid density functional theory analysis was performed based on slit/cylindrical pores for carbon.

2.5.3 Micropollutant analysis

A HPLC system (Shimadzu, Kyoto, Japan) was used to measure the micropollutant concentrations. It was equipped with a 300 × 4.6 mm (5 µm pore size) C-18 column (Supelco Drug Discovery, Sigma–Aldrich, Australia) and an UV–vis detector. The detection wavelength, column temperature, and sample injection volume were 280 nm, 20 °C, and 50 µL, respectively. The mobile phase comprised of acetonitrile and Milli-Q water buffered with 25 mM KH₂PO₄. Two eluents, A (80% acetonitrile and 20% buffer, v/v) and B (20% acetonitrile and 80% buffer, v/v) were delivered at 0.7 mL/min through the column for 30 min. The eluents were added in time-dependent gradient proportions [Time (min), B (%]): [0, 85], [5, 40], [8, 0], [22, 85]). The limit of quantification for the micropollutants under investigation using these conditions was approximately 10 µg/L.

Micropollutant adsorption on GAC and laccase-immobilized GAC was measured following a solvent extraction method (Wijekoon et al., 2013). Freeze-dried samples were thoroughly mixed with 5 mL of methanol. The mixture was then sonicated for 10 min and the supernatant was collected. The remaining solid mass was subjected to further extraction using 5 mL methanol and dichloromethane (1:1 v/v) and the supernatant was collected. The extracted micropollutant in the solution was measured by HPLC analysis as described above. The extraction efficiency of bisphenol A, diclofenac, carbamazepine and sulfamethoxazole was 84, 98, 82 and 79%, respectively.

3. Results and discussion

3.1 Immobilization efficiency

In this study, laccase was immobilized onto GAC by direct adsorption in the absence of any coupling reagents to promote bonding of the enzyme on GAC. Prior to laccase immobilization, the GAC was subjected to pre-treatment by acid washing. The acid-washed GAC achieved a significantly higher proportion of adsorbed laccase (an activity of $750 \mu\text{M}_{(\text{DMP})}/\text{min g}_{\text{GAC}}$, equivalent to 10 mg laccase/g_{GAC}) in comparison to non-acid washed GAC ($92 \mu\text{M}_{(\text{DMP})}/\text{min g}_{\text{GAC}}$). The superior adsorption of laccase on acid-washed GAC could be due to the removal of debris on the GAC during the acid-wash process. Indeed, BET surface area calculations based on the adsorption isotherms of GAC and acid-washed GAC indicate that acid-washing reveals a further 8% of N₂-accessible surface (Table 1). However, another possible reason for the better adsorption of laccase is that the acid washing process has been shown to develop more carboxylic groups on the GAC surface, which could enhance laccase adsorption. Davis and Burns (1992) observed an increase in carboxylic groups on surface of GAC after acid washing.

Overall, due to its superior activity, acid-washed GAC was used for laccase immobilization in the rest of the study.

According to the analysis of the surface area and pore volume data (Table 1 and Supplementary Data Figure S4), the surface of the GAC used in this study predominantly (*ca.* 80%) comprised micropores. In the case of the lower enzyme loading (i.e., 30% saturation), the enzyme appeared to be distributed/immobilized across all sites over the GAC, perhaps more at sites with pore-width greater than 3 nm (Supplementary Data Figure S5). A significant reduction in pore volume in the 2 to 5 nm pore-width region was noted when the enzyme loading was increased (i.e., ‘full’ saturation), which evidenced that additional enzyme was distributed mostly in those pores (Supplementary Data Figure S5). Nevertheless, it was confirmed that, even at saturation coverage, laccase might occupy as much as 36% of the GAC surface (laccase-immobilized GAC 806 m²/g vs GAC without laccase 1279 m²/g). The remaining surface area on GAC after laccase adsorption can be utilized for the removal of micropollutants via co-adsorption as further discussed in Section 3.3.

[TABLE 1]

3.2 Stability of immobilized laccase

3.2.1 pH tolerance and thermostability

The pH of a solution can significantly affect the structure and activity of enzymes. It is thought that pH influences the state of ionization of amino acids in a protein (Jordaan et al., 2009). If the state of ionization of amino acids is altered then the ionic bonds that help to determine the 3-D shape of the protein can be altered and may lead to inactivation of the enzyme. The stability of free and GAC-bound laccase was studied in the pH range 3.5 to 9 in solution. Both free and

GAC-bound laccase exhibited high levels of stability at neutral and basic pH (< 10% denaturation after 24 h of incubation). However, the GAC-bound laccase showed higher stability at acidic pH (3.5 and 4) (Figure 1). This observation suggests that GAC may confer some kind of protection to the bound laccase in the acidic pH region. Similar benefit of laccase immobilization (i.e., applicability over a broader pH range) was reported in a few studies, which however, used other supports than GAC such as magnetic chitosan (Jiang et al., 2005), polyethyleneimine coated spheres (Jordaan et al., 2009) and coconut fiber (Cristóvão et al., 2011). It is thought that the multi-point attachment on the support could improve the rigidification of the protein and protect it from denaturation. The increase in stability of GAC-bound laccase is a valuable point because it offers a greater opportunity for its use in the treatment of acidic wastewater, a common problem for many industries. Furthermore, in a previous study (Nguyen et al., 2014a), solution pH significantly influenced the micropollutant removal performance by free laccase: diclofenac was significantly degraded at the acidic pH only. However, the free laccase was significantly deactivated at acidic pH, which is problematic if a continuous treatment process were to be developed. The results in the current study confirm that GAC-bound laccase, which enhances stability of laccase at acidic pH, could be particularly suitable for the removal of some micropollutants. Further discussion regarding micropollutant removal is provided in Section 3.3.

[FIGURE 1]

The stability of the free and immobilized laccase was compared over a temperature range of 30 to 70 °C. This study confirms the increase in temperature resistance of laccase due to immobilization on GAC. As can be seen in Figure 2, the activity of both free and GAC-bound laccase was stable at 30 and 40 °C. However, the activity of the GAC-bound laccase was greater at temperatures between 60 and 70 °C: at 60 °C, the GAC-bound laccase retained more than 85%

of its activity as compared to 43% in case of free laccase (Figure 2). Immobilization can increase enzyme rigidity, thus improving stability against denaturation due to temperature rise (Hu et al., 2007). Nicolucci et al. (2011) reported that laccase immobilization on polyacrylonitrile beads maintained 80% of its initial activity, while free laccase was inactivated completely at 70 °C. Alkan et al. (2009) also reported similar findings. Evidently, the solid supports have a stabilizing effect that raises the temperature at which enzyme inactivation occurs. The physical characteristics of tolerance to a broad pH and temperature range make GAC-bound laccase a promising system for treating wastewater.

[FIGURE 2]

3.2.2 Reusability of GAC-bound laccase

The reusability of laccase is an essential feature for its industrial application. In this study, the GAC-bound laccase maintained activity completely for up to 8 cycles of continuous application (Figure 3). Afterwards, the residual laccase activity gradually decreased and reached 55% after 20 cycles. This observation is in line with other available reports where laccase immobilization has been shown to improve the reusability (Keerti et al., 2014; Sathishkumar et al., 2014). The results of this study are comparable or better than those reported in the literature using inert supports (Keerti et al., 2014; Spinelli et al., 2013). For example, Spinelli et al. (2013) stated that the activity of amberlite-bound laccase reduced to 30% within 7 cycles. It is possible that due to the weak physical bonds of the laccase and inert support, the operational stability is often lower. On the other hand, the surface of GAC may contain carboxylic groups and other surface oxides which could potentially bond with amino groups of laccase. Thus, despite using a method designed for the physical immobilization of laccase to the GAC support, it is conceivable that some covalent bonds may have formed between laccase and the activated GAC surface in this

study. The decrease in laccase activity in the later cycles could be due to leaching and/or denaturation of the enzyme during the reactions as further discussed in Section 3.3.1.

[FIGURE 3]

3.3 Micropollutant removal by GAC-bound laccase

3.3.1 Reuse of the immobilized enzyme

The removal of micropollutants by GAC-bound laccase was investigated in this work by repeated addition of fresh micropollutant solution for 20 cycles. A similar degree of micropollutant removal efficiency by GAC and laccase-immobilized GAC (partial/full saturation) was achieved in the first three cycles (Figure 4), demonstrating that the immobilization of laccase on GAC did not affect micropollutant adsorption on GAC. In fact, large portions of GAC surface (65%) remained available after laccase immobilization (Table 1). The laccase-catalyzed degradation was evident in the following cycles in that the removal efficiency of the GAC-bound laccase was higher. After biodegradation, the adsorption sites on GAC are liberated and cycle of sorption-biodegradation could begin anew. Therefore, the immobilization of laccase in GAC enables the regeneration of GAC sorption sites, allowing reuse. Additionally, it prevents the total saturation of GAC, which is a necessary feature if operation at continuous mode is desired.

It is also important to note that the better removal of micropollutants by GAC-bound laccase compared to GAC was compound-specific. Significant difference in the removal of bisphenol A and diclofenac and to some extent for carbamazepine and sulfamethoxazole, by GAC-bound laccase and GAC was observed (Figure 4). These results could be explained by the difference in laccase-catalyzed degradation capacity and affinity of the micropollutants for adsorption on

GAC. Laccase could achieve high biodegradation of bisphenol A and moderate biodegradation of diclofenac possibly due to the presence of the phenolic and aniline groups in their structures, respectively (Nguyen et al., 2014a; Yang et al., 2013b). Thus the degradation of bisphenol A and diclofenac by laccase regenerated adsorption sites for the next cycle. On the other hand, the immobilized laccase showed only minimal (10%) improvement in the removal of carbamazepine and sulfamethoxazole. This can be attributed to their resistance to biodegradation (Hai et al., 2011; Yang et al., 2013b). The presence of strong electron withdrawing groups in these compounds (carbamate in carbamazepine and sulfone in sulfamethoxazole) renders them less susceptible to degradation by laccase.

A unique aspect revealed in the current study is the impact of the amount of laccase immobilized on GAC on micropollutant removal (Figure 4). Overall, the higher laccase loading ('full' saturation) achieved the highest removal of all compounds in all cycles. However, for carbamazepine and sulfamethoxazole, the removals achieved by the immobilized laccase preparations (i.e., 'full' and 30% saturation) were close, possibly because of the inherent resistance of these compounds to laccase-catalyzed degradation.

Interestingly, a significant increase in pore-volume in the 2 to 5 nm pore-width region was noticed after the laccase-immobilized GAC was used to remove micropollutants from test solutions (Supplementary Data Figure S5). Increase in surface area and pore volume on GAC after the micropollutant test (Table 1) indicates dislodgement of enzyme from the GAC surface in the course of the batch test. Because no enzymatic activity was detected in the test solution, this possibly indicates that the dislodged enzyme molecules were also denatured. This can explain why the laccase activity and micropollutant removal efficiency by GAC-bound laccase gradually decreased (Figure 4). As noted in Section 3.2.2, previous studies have also reported

about the gradual diminution of laccase activity during reuse of immobilized enzyme preparations (Daâssi et al., 2014; Modin et al., 2014), but the current study offers additional insights into this aspect by presenting the micropollutant removal data and illustrating the dynamics of surface area of the immobilization support (i.e., GAC) during reuse.

[FIGURE 4]

3.3.2 Role of laccase-catalyzed degradation

Removal of micropollutants by GAC was due to adsorption, whereas the removal of micropollutants by GAC-bound laccase was via simultaneous adsorption and degradation. In order to distinguish between adsorption and laccase-catalyzed biodegradation, the fate of the micropollutants (*i.e.*, residual amount in liquid phase, adsorbed onto GAC and biodegraded) was assessed by extracting residual micropollutants from the GAC at the end of the incubation period. The adsorbed amount onto GAC was calculated taking the extraction efficiency into account (Section 2.5.3). The micropollutant removal efficiency of free laccase was compared with the biodegraded portion by the GAC-bound laccase (Figure 5). In comparison to the micropollutant degradation by free laccase, the GAC-bound laccase achieved higher degradation (10 - 50% difference). Apparently co-adsorption of laccase and micropollutant onto GAC facilitated enhanced micropollutant degradation by laccase. Zille et al. (2003) reported that immobilization of enzyme on alumina led to a more consistent performance for dye removal due to combined adsorption and biodegradation. The co-adsorption of dyes and laccase could promote the interaction of dyes with active sites of laccase (Hai et al., 2008; Zille et al., 2003). In this study, the benefit of laccase immobilization in micropollutant degradation was significant for compounds which are often resistant to laccase degradation such as diclofenac and carbamazepine. Inadequate oxidative efficiency of the laccase and the steric hindrance or

obstructed transfer of electrons between laccase and the substrate are two main reasons for the resistance of micropollutants (d'Acunzo and Galli, 2003). In this study, the enhancement in the degradation of micropollutants is possibly due to the enhancement of electron transfer between laccase and substrate molecules after adsorption on the GAC surface. Further studies on the specific mode of degradation improvement would be interesting but that is beyond the scope of this study.

Previous studies have used inert supports that showed low or no adsorption of micropollutant, such as TiO₂, silica or polyacrylonitrile beads (Cabana et al., 2007; Hou et al., 2014; Lloret et al., 2012). However, compared to the free enzyme, lower micropollutant removal efficiencies were reported using these inert supports. For example, free laccase could achieve 90% removal of bisphenol A, whereas only 25% was achieved by cross-linked laccase aggregates (Cabana et al., 2007). By contrast, in the current study, the immobilized enzyme consistently showed better performance than free enzyme. Overall, the results here highlight the benefit of using an active support such as GAC.

[FIGURE 5]

3.3.3 Continuous flow packed-bed enzymatic reactor

Two GAC-packed reactors (with and without laccase immobilization) were operated in parallel continuously for 60 d for the investigation of micropollutant removal. A complete removal of bisphenol A, diclofenac, sulfamethoxazole and carbamazepine was achieved by both columns until a BV of 6000, 8000, 4000 and 5000, respectively, following which the removal of micropollutants by the GAC-only column gradually decreased (Figure 6). The column with laccase-immobilized GAC maintained steady removal of all micropollutants for further 2000 to 3000 BV, but the removal efficiency eventually started to decline. The gradual reduction of the

micropollutant removal by the GAC column can be attributed to the saturation of adsorption sites (Nguyen et al., 2013a). Unlike in the GAC column without laccase, the removal of micropollutant by the GAC-bound laccase can occur in three stages: (i) extensive adsorption on GAC and negligible degradation by laccase at the initial phase (Russo et al., 2008) , (ii) adsorption on GAC and degradation by laccase at equilibrium phase (see Supplementary Data Figure S6), and (iii) decline of laccase-catalyzed degradation due to the dislodgement of enzyme from GAC surface and denaturation (as noted in Section 3.3.1). Similar results were reported by Cabana et al. (2009) who investigated the elimination of nonylphenol, bisphenol A and triclosan from five repeated batch treatment cycles in a packed –bed reactor by laccase covalently immobilized on diatomaceous earth. The current study demonstrates some advantages of laccase immobilization on GAC including better stability of laccase and regeneration of GAC through enzymatic degradation of adsorbed micropollutants (Supplementary Data Figure S6). Overall, simultaneous adsorption and laccase degradation prolonged the lifetime of the GAC-bound laccase column.

[FIGURE 6]

To date, the removal of micropollutants by immobilized laccase has been reported only for semi-continuous systems (Cabana et al., 2009; Hou et al., 2014). Cabana et al. (2009) achieved a complete removal of bisphenol A, triclosan and nonylphenol by laccase immobilized through the formation of cross-linked aggregates. Hou et al. (2014) evaluated the degradation of bisphenol A by laccase immobilized on TiO₂. However, the long-term operation of GAC-packed enzyme reactors remains a challenge, and the current study, reporting observations made over a period of 12000 BV, fills a critical knowledge gap in this context.

4. Conclusions

Data presented in this study confirm that GAC-bound laccase could overcome some of the associated problems in using free laccase for the catalytic degradation of micropollutants. The immobilization of laccase improved laccase reusability and stability over a broad range of pH and temperature. Compared to GAC and free laccase, the GAC-bound laccase removed micropollutants more effectively. This study made clear the complementary effects of enzyme immobilization and micropollutant adsorption on GAC on the degradation of four commonly detected micropollutants in wastewater via investigations in batch and continuous flow packed-bed reactors.

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Figure 1: Effect of solution pH on the stability of (a) free laccase and (b) GAC-bound laccase (full saturation). 50 mg of laccase-immobilized GAC was used to obtain an initial activity comparable to that of the free laccase solution. The error bars represent standard deviation of duplicate samples.

Figure 2: Effect of temperature on the stability of free laccase and GAC-bound laccase (full saturation). 50 mg of laccase-immobilized GAC was used to obtain an initial activity comparable to that of the free laccase solution. The error bars represent standard deviation of duplicate samples.

Figure 3: Reusability of the GAC-bound laccase as indicated by the stability of enzymatic activity. The error bars represent standard deviation of duplicate samples.

Figure 4: Removal of micropollutants during reuse of the immobilized-laccase preparation. The error bars represent standard deviation of duplicate samples. BPA, DCF, SMX and CBZ stands for bisphenol A, diclofenac, sulfamethoxazole, and carbamazepine, respectively.

Figure 5: Overall fate of micropollutants following treatment (24h) via GAC, free laccase and GAC-bound laccase. BPA, DCF, SMX and CBZ stands for bisphenol A, diclofenac, sulfamethoxazole, and carbamazepine, respectively.

Figure 6: Removal efficiency of micropollutants by continuous flow columns packed with GAC with and without laccase immobilization as a function of throughput (bed volumes). BPA, DCF, SMX and CBZ stands for bisphenol A, diclofenac, sulfamethoxazole, and carbamazepine, respectively.

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583 Figure 1 Effect of solution pH on the stability of (a) free laccase and (b) GAC-bound laccase

584 (full saturation). 50 mg of laccase-immobilized GAC was used to obtain an initial activity

585 comparable to that of the free laccase solution. The error bars represent standard deviation of

586 duplicate samples.

587

588 Figure 2 Effect of temperature on the stability of (a) free laccase and (b) GAC-bound laccase

589 (full saturation). 50 mg of laccase-immobilized GAC was used to obtain an initial activity

590 comparable to that of the free laccase solution. The error bars represent standard deviation of

591 duplicate samples.

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 594 Figure 3 Reusability of the GAC-bound laccase as indicated by the stability of enzymatic
 595 activity. The error bars represent standard deviation of duplicate samples.

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Figure 4 Removal of micropollutants during reuse of the immobilized-laccase preparation. The error bars represent standard deviation of duplicate samples. BPA, DCF, SMX and CBZ stands for bisphenol A, diclofenac, sulfamethoxazole, and carbamazepine, respectively.

Figure 5 Overall fate of micropollutants following treatment (24h) via GAC, free laccase and GAC-bound laccase. BPA, DCF, SMX and CBZ stands for bisphenol A, diclofenac, sulfamethoxazole, and carbamazepine, respectively.

List of Tables

Table 1: Surface area and pore volume of different GAC preparations

Parameter	Unmodified GAC	Acid-washed GAC	Laccase-immobilized GAC (partial, 30%) ^a	Laccase-immobilized GAC (full) ^a	Laccase-immobilized GAC (full) + micropollutant ^b
Total BET-Surface area (m ² /g)	1182	1279	1035	806	892
Micropore surface area (m ² /g)	927	1010	826	651	696
Total pore volume (cc/g)	0.889	0.912	0.756	0.629	0.693
Micropore pore volume (cc/g)	0.513	0.532	0.444	0.370	0.389

^a Laccase was immobilized on acid-washed GAC; ^bAfter reusing the immobilized laccase preparation for removal of micropollutants in batch tests

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Supplementary Data

617 Table S1: Physicochemical properties of the selected micropollutants

Compounds (CAS number)	Molecular weight (g/mol)	Log K _{OW} ^a	Log <i>D</i> at pH 7 ^a	Dissociation constant (pK _a) ^a	Chemical structure
Carbamazepine (C ₁₅ H ₁₂ N ₂ O) (298-46-4)	236.27	1.89 ± 0.59	1.89	13.94 ± 0.20 -0.49 ± 0.20
Diclofenac (C ₁₄ H ₁₁ Cl ₂ NO ₂) (15307-86-5)	296.15	4.55 ± 0.57	2.72	4.18 ± 0.10 -2.26 ± 0.50
Sulfamethoxazole (C ₁₀ H ₁₁ N ₃ O ₃ S) (723-46-6)	253.28	0.66 ± 0.41	0.43	5.81 ± 0.50 1.39 ± 1.10
Bisphenol A (C ₁₅ H ₁₆ O ₂) (80-05-7)	228.29	2.63±0.20	3.64	10.29 ± 0.10

618 ^a Source: SciFinder database: <https://origin-scifinder.cas.org>

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Figure S2: Schematic diagram of the GAC columns

Figure S3: Adsorption-desorption isotherms of GAC samples

Figure S4: Cumulative pore volume of the GAC over pore width

Figure S5: Distribution of volume over pore width of the GAC

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669 **Figure S6:** Fate of micropollutants in the GAC and laccase-immobilized GAC columns

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