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Keywords

bisphenol, continuous, diclofenac, biotransformation, laccase, enzymatic, membrane, reactor

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Research Highlights:

- Removal of BPA and DCF was investigated by an enzymatic membrane reactor (EMR)
- Purified laccase achieved greater degradation of BPA than of DCF in batch tests
- Despite complete membrane retention denaturation necessitated enzyme reinjection
- Stable removal of BPA and DCF was obtained by the continuous flow EMR
- Redox-mediator dosing improved removal of resistant compound DCF by EMR

Abstract

A novel enzymatic membrane reactor (EMR) was explored for continuous removal of two trace organic contaminants (TrOCs) ubiquitously detected in wastewater - namely bisphenol A (BPA) and diclofenac (DCF) - by a commercially available laccase from *Aspergillus oryzae*.. An ultrafiltration membrane prevented washout of the enzyme and allowed continuous removal of BPA and DCF (>85% and >60%, respectively) under a loading rate of $570 \pm 70 \mu\text{g/L.d}$ and $480 \pm 40 \mu\text{g/L.d}$ of each compound, respectively. The BPA and DCF removal could be further improved to >95% and >80%, respectively, by dosing to the EMR a natural redox-mediator compound - syringaldehyde ($5 \mu\text{M}$) – believed to act as an electron shuttle between laccase and the target pollutants. Of particular interest was the significant retention of the TrOCs on the gel layer of enzyme on the membrane surface, and their subsequent biodegradation.

Keywords: trace organic contaminant; enzymatic membrane reactor; laccase; biotransformation.

1. Introduction

Trace organic contaminants (TrOCs) can be classified into several groups including steroid hormones, phytoestrogens, other endocrine disrupting chemicals, pharmaceuticals and personal care products, disinfection by-products, and industrial chemicals. TrOCs are routinely detected in raw and treated municipal wastewater and wastewater-impacted natural water bodies at trace levels ranging from a few nanogram per litre (ng/L) to several microgram per litre (µg/L) (Alexander et al., 2012). Certain TrOCs have been found to cause adverse physiological changes in aquatic organisms, and can potentially affect human health on prolonged exposure (Schwarzenbach et al., 2006). Much effort has been devoted to improving current processes or developing new technologies for the removal of these compounds from wastewater.

Enzymatic transformation of resistant pollutants is a promising eco-friendly alternative to the conventional physicochemical methods (Hai et al., 2013). Enzymatic treatment is very attractive as it can be accomplished under mild conditions, achieves high reaction specificity and rates, and requires relatively small dosage even at an industrial scale. Compared with most chemical catalysis processes, enzymatic treatment also consumes less chemicals, water and energy, and produces less waste. Laccase (EC 1.10.3.2, p-diphenol: dioxygen oxidoreductases) has been reported to be a powerful biocatalyst for the degradation of resistant compounds such as dyes and aromatic hydrocarbons, and for industrial processes such as pulp delignification and denim bleaching (Hai et al., 2012; Rios et al., 2004). Recent studies have also demonstrated that laccase effectively degrades a wide variety of TrOCs that are inefficiently degraded by conventional biological processes (Marco-Urrea et al., 2009; Tran et al., 2010; Yang et al., 2013b). TrOC removal by an enzyme system is dependent on a range of factors including the molecular structure of the TrOCs, pH, temperature and ionic strength of the media, and the property of the specific enzyme (Yang et al., 2013b).

The application of enzymes in continuous systems such as wastewater treatment plants remains a challenge since enzymes are easily washed out with the treated effluent (Hai et al., 2012). The recovery of enzymes and their reusability for the continuous operation of an enzymatic reactor are key prerequisites for its large-scale application (Lloret et al., 2012). Recent studies have focused on mitigating this problem. For example, immobilization is a suitable approach to retain enzyme in the system. Different support materials including polyacrylonitrile, polystyrene, and

SiO₂ (celite), and sol-gel entrapment have been used to immobilise laccase (Yang et al., 2013b). Cabana et al. (2009) immobilized laccase on SiO₂ and demonstrated degradation of nonylphenol, bisphenol A, and triclosan in a packed bed reactor. The use of membranes with pore size smaller than the enzyme molecule is another approach to prevent enzyme washout (Lloret et al., 2012; López et al., 2004). This approach offers several advantages including more effective retention of enzymes, operation with free enzymes avoiding limitations of mass transfer typically associated with attachment on supports, and easy replenishment of fresh enzymes during long term operation (Lloret et al., 2012). An enzymatic membrane reactor (EMR) facilitates separation of enzyme from products and substrates by a semi-permeable membrane. The enzyme remains within the system (reactor) allowing continuous operation, with feeding and treated effluent withdrawal without significant loss of the enzyme. Recent studies have explored EMRs for the treatment of pollutants such as dyes (Chhabra et al., 2009; Mendoza et al., 2011). To date only one study has investigated continuous TrOC degradation by EMR (Lloret et al., 2012) where high removal of estradiol and estrone was demonstrated, albeit over a limited observation period of 8 h.

Catalysis by laccase can be enhanced by addition of low molecular weight mediators that act as an ‘electron shuttle’ between the oxidizing enzyme and target compounds (Kim and Nicell, 2006b). The degree of enhancement depends predominantly on the type of mediator and the TrOC structure. Most of the investigations on TrOC degradation by mediator-enhanced laccase systems used batch tests. Hata et al. (2010) reported that compared with a single addition of laccase and a mediator, their repeated addition resulted in better degradation of a resistant TrOC (carbamazepine) over the same incubation period. In this context, continuous dosing of a mediator to EMR may achieve an enhanced removal of TrOCs. However, no work investigating this could be identified in the literature.

The aim of this study was to develop an EMR for continuous degradation of TrOCs. Batch tests were first conducted to assess the effect of operating pH and mediator addition on the TrOC degradation efficiency by a commercially available laccase. In discussing the removal efficiency by the EMR, the molecular structure of the TrOCs and the role of membrane were taken into account. Particularly, the effect of mediator addition to the EMR on continuous TrOC removal was studied for the first time.

2. Materials and methods

2.1 Trace organic contaminants

Two TrOCs, namely, bisphenol A (BPA) and diclofenac (DCF) (Sigma-Aldrich, NSW, Australia) were selected in this study. Key properties of these compounds are summarised in Supplementary Data Table S1. BPA and DCF were selected as model compounds based on their widespread occurrence in raw sewage and sewage-impacted water bodies (Yang et al., 2013a). A stock solution of the TrOCs was prepared at a concentration of 1 g/L of each compound in pure methanol, stored at -18 °C and used within one month.

2.2 Enzyme solution and mediator

A commercially available laccase (Novozym 51003) purified from the culture of genetically modified *Aspergillus oryzae* was supplied by Novozymes (Novozymes Australia Pty. Ltd). A genomic DNA segment encoding an extracellular laccase was isolated from the thermophilic fungus *Myceliophthora thermophila*, and the nucleotide sequence of this gene was determined. A vector containing the *M. thermophila* laccase coding region was constructed for heterologous expression in *A. oryzae* (Berka et al., 1997). The enzyme had a molecular weight of 56 kDa, and the enzyme stock solution had a density, purity and activity of 1.12 g/mL, approximately 10% (w/w) and 150,000 $\mu\text{M}/\text{min}$ (measured using 2,6-dimethoxy phenol (DMP) as substrate), respectively.

Syringaldehyde (SA) (Sigma-Aldrich, NSW, Australia) was used as a natural redox mediator. The radicals generated due to oxidation of SA by laccase have been reported to be relatively more stable than those of other common mediators (González Arzola et al., 2009). A stock solution of SA was prepared in Milli-Q water at a concentration of 50 mM and stored at 4 °C.

2.3 Batch test description

2.3.1 Effect of pH

In this study, enzymatic degradation of the TrOCs was investigated under a range of pH from acidic to basic (i.e., 3, 4.5, 6, 7 and 9). The experiments were conducted in screw-capped test tubes (13 mL). All laboratory apparatus was sterilized by autoclaving before use to maintain aseptic conditions. The concentrated stock solution (3 μL) of laccase was diluted to a final

volume of 5 mL in the test tubes by Milli-Q water to obtain an initial enzymatic activity of approximately 90 $\mu\text{M}/\text{min}$ (measured using DMP as substrate). The TrOCs were added at an initial nominal concentration of 1000 $\mu\text{g}/\text{L}$ into test tubes (actual concentrations of 1100 ± 10 and 860 ± 10 $\mu\text{g}/\text{L}$ ($n=3$) for BPA and DCF, respectively). Sodium citrate at a concentration of 100 mM was used to control the pH of the reaction media. All test tubes were incubated in a rotary shaker at 70 rpm and 28 °C (Bioline Shaker Incubator BL 8600, Edwards Group Pty. Ltd, NSW, Australia), and samples were collected at 2, 4, 8 and 22 h for TrOC analysis and enzymatic activity measurement. To verify that degradation took place only due to enzymatic oxidation, control samples containing only TrOCs in Milli-Q water (without enzyme) were incubated in parallel.

2.3.2 Effect of redox-mediator addition

The effect of addition of the redox-mediator SA on the degradation of BPA and DCF as well as the stability of enzymatic activity was tested. Based on the results from the tests described in Section 2.3.1, the effect of SA concentration was studied at a pH of 7. SA concentrations of 5, 100, 500, and 1000 μM were selected based on the concentration-range used in other batch test studies (Mendoza et al., 2011; Mizuno et al., 2009). All other steps were similar to those described in Section 2.3.1.

2.4 EMR system and operation protocol

A laboratory scale EMR system consisting of a 1.5 L (active volume) glass reactor was used (Supplementary Data Figure S2). An ultrafiltration (UF), hollow fiber membrane module made of polyacrylonitrile was submerged in the reactor. The membrane was supplied by Microza Membranes (Pall Corporation, NSW, Australia). It had a molecular weight cut off (MWCO), surface area and clean water flux of 6 kDa, 0.19 m^2 , and 10 L/h.bar, respectively.

The EMR was first operated to confirm retention of the enzyme by the UF membrane. Stock solution (1 mL) of laccase was diluted to a final volume of 1.5 L in the reactor by Milli-Q water to obtain an initial enzymatic activity of approximately 90 $\mu\text{M}/\text{min}$. The membrane was operated at a flux of 1.1 $\text{L}/\text{m}^2\cdot\text{h}$ via a peristaltic pump (Masterflex L/S, USA) with a 8 min on and 1 min off cycle, and the permeate was returned to the reactor. The reactor was placed in a water bath with a temperature control unit (Julabo, Germany) to maintain the temperature at 28

°C. Dissolved oxygen (DO) concentration was maintained at above 3 mg/L via an air pump (ACO-002, Zhejiang Sensen Industry Co. Ltd, Zhejiang, China) connected to a diffuser placed at the bottom of the reactor. The pH of the reactor contents was 6.8 ± 0.2 . Transmembrane pressure (TMP) was continuously monitored using a high-resolution (± 0.1 kPa) pressure sensor (SPER scientific 840064, Extech equipment Pty. Ltd., Victoria, Australia) connected to a computer for data logging. Permeate and reactor supernatant samples were taken every 2 h for enzymatic activity measurement over a period of 24 h for this specific part of the experiment.

Once enzyme retention by the membrane was confirmed, the stability of enzymatic activity under the applied hydraulic conditions was tested. Feed containing TrOCs in Milli-Q water (4.5 L) was prepared daily. The EMR was operated at a hydraulic retention time (HRT) of 8 h, and the TrOC concentration in the feed water was maintained such that a TrOC loading rate of approximately 500 $\mu\text{g/L.d}$ (for each TrOC) was applied. Operation was initiated with an enzymatic activity of 90 $\mu\text{M/min}$. Permeate and reactor supernatant samples were taken every 5 h for TrOC removal and enzymatic activity measurement until no enzymatic activity was detected in the reactor (72 h). Following this, another dose of enzyme was added to the EMR to reinstate the enzymatic activity to the initial level (90 $\mu\text{M/min}$), and the above run was repeated.

The EMR was then continuously operated to investigate TrOC degradation. Laccase solution (200 μL laccase/ L reactor volume) was re-injected every 12 h into the EMR to maintain the enzymatic activity within a range of 70 to 100 $\mu\text{M/min}$. This run was conducted at an HRT of 8 h, and the TrOC loading rates were 570 ± 70 $\mu\text{g/L.d}$ and 480 ± 40 $\mu\text{g/L.d}$ for BPA and DCF, respectively. Permeate and reactor supernatant samples were taken every 5 h for TrOC concentration and enzymatic activity measurements over a period of 132 h.

To study the effect of mediator (SA) addition on TrOC removal, SA was added continuously via a peristaltic pump at a flow rate of 1.0 mL/min to achieve a mediator concentration of 5 μM in the reactor. To avoid probable interaction between SA and TrOCs during storage in the feed tank, feed containing TrOCs and SA was prepared daily and added separately by two different pumps. Permeate and reactor supernatant samples were taken every 4 h for TrOC concentration and enzymatic activity measurements over a period of 60 h.

After each test, ex-situ cleaning was performed to restore the membrane to its original condition. Ex-situ backwash was performed first with 1 L of Milli-Q water and then with 1 L of NaOCl

(500 mg/L active chloride) at a flux of 5 L/m²h. After each Milli-Q water cleaning, the enzymatic activity of the cleaning solution was measured to detect any attachment of enzyme on the membrane surface.

2.5 Analytical methods

2.5.1 TrOC analysis

A HPLC system (Shimadzu, Kyoto, Japan), equipped with a 300 x 4.6 mm (5 µm pore size) C-18 column (Supelco Drug Discovery, Sigma-Aldrich, Australia) and a UV-vis detector, was used to measure the TrOC concentrations. The detection wavelength and sample injection volume were 280 nm 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 in the following time-dependent gradient proportions [Time (min), B (%): [0, 80], [12, 80], [20, 0], [25, 80] (Hai et al., 2011). The limit of quantification for the analytes under investigation using these conditions was approximately 10 µg/L. The samples were diluted two-fold in methanol to immediately stop any residual enzymatic activity. The accuracy of the analysis was confirmed by always including standard TrOC solutions. The removal efficiency

was calculated as $R = 100 \times \left(1 - \frac{C_{Eff}}{C_{Inf}} \right)$, where C_{Inf} and C_{Eff} are influent and effluent (permeate) concentrations of the TrOCs, respectively.

2.5.2 Enzymatic and toxicity assay

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 buffer solution. The measurement was based on the change in absorbance at 468 nm by a spectrophotometer (UV-Visible UV-1700, Shimadzu, Kyoto, Japan). Laccase activity was then calculated from the molar extinction coefficient $\epsilon = 49.6 \text{ / (mM.cm)}$ at $\lambda = 468 \text{ nm}$ and expressed in µM/min (Hai et al., 2012). The bacterial toxicity of feed and permeate was analysed in duplicate by measuring bioluminescence inhibition in *Photobacterium leiognathi* (ToxScreen3 assay; CheckLight Ltd, Israel) as described

previously (Nguyen et al., 2013). Toxicity was expressed as relative Toxic Unit (rTU), calculated as $rTU=1/IC_{20}$, with IC_{20} (concentration of the sample required to kill 20% of the bacteria) determined by linear regression of the toxicity response vs. relative sample enrichment within the linear range of the concentration-effect curve (up to IC_{40}).

3. Results and discussion

3.1 TrOC removal in batch tests

3.1.1 Effect of pH

BPA was degraded almost completely (96%) within 4 h of incubation at a pH of 7, while over the same period a degradation of 56%, 61%, 88% and 74% was achieved at a pH of 3, 4.5, 6 and 9, respectively (Table 1). However, irrespective of the media pH, at least 80% removal of BPA was observed after 22 h (Figure 1a). The overall high degradation of BPA can be attributed to the strong electron donating group (EDG) –OH in its structure which renders it a good substrate of laccase (Yang et al., 2013b). This observation is consistent with that in the available literature. For example, a complete degradation of three different bisphenols (bisphenol A, bisphenol B and bisphenol F) by laccase within 1.5 h of incubation was reported by Nicolucci et al. (2011). Furthermore, the slightly lower removal of BPA at highly acidic (pH 3) and alkaline (pH 9) conditions may be attributed to the significant (*i.e.*, 46% and 53%) inactivation of the laccase activity within 2 h of incubation (Figure 1b). Kim and Nicell (2006b) also reported a complete removal of BPA in the pH range of 5 - 7, which decreased only slightly in the acidic regime (pH 3 - 4).

[TABLE 1]

[FIGURE 1]

Unlike BPA, DCF degradation was significantly better at the acidic pH (3 and 4.5), and the removal consistently decreased irrespective of the incubation period with the increase in media pH beyond a value of 4.5 (Table 1). For example, compared to a complete removal after 22 h of incubation at a pH of 4.5, DCF removal reached only 61% at pH 7 over the same period (Figure 1a). Lloret et al. (2010) also reported high removal of DCF by laccase at acidic pH. According to Sathishkumar et al. (2014), the drop in laccase mediated degradation at alkaline pH is probably due to the combination of hydroxide ion inhibition and its binding with the T2/T3 site

of laccase (Sathishkumar et al., 2014). Instances of significant effect of pH on the degradation of DCF by other enzymes can also be found in the literature. For example, Zhang and Geissen (2010) reported that DCF was completely degraded by lignin peroxidase (LiP) in the pH range of 3 - 4.5. However, its degradation by LiP significantly decreased from complete removal at a pH of 4.5 to 10% at a pH of 6.

Not only the extent of removal but also the stability of enzymatic activity is important, particularly in case of a continuous flow reactor. In this study, significant inactivation of laccase activity at acidic pH was observed. In contrast, almost no enzyme inactivation was found at the neutral pH (Figure 1b). As a compromise between treatment efficiency and stability of enzymatic activity, and considering the ease of operation under neutral pH (avoiding the requirement of pH adjustment), further investigation was conducted at a pH of 7.

3.1.2 Effect of redox-mediator addition

BPA was completely removed within 4 h of incubation irrespective of the mediator concentration (Figure 2a). This was expected as BPA was completely removed even without mediator addition (Section 3.1.1). However, DCF removal was significantly improved after SA addition. For example, over 80% removal was achieved within 8 h at all mediator concentrations tested (Figure 2a). This compares favourably with a 40% removal of DCF in the absence of SA over the same period. Furthermore, the addition of SA (at all concentrations) achieved a complete removal of DCF within 22 h (end of observation), while the removal in the absence of SA was only 60%. This observation is in line with the literature. For example, Lloret et al. (2010) observed an enhancement of DCF removal from less than 5% to complete removal due to the addition of SA at a concentration of 100 μ M. The better performance of the mediator-amended enzyme may be explained by two factors attributable to the radical species generated from SA by laccase: i) higher redox potential, and ii) reduction of steric hindrance. Compounds with high electrochemical potential can be oxidized by radical mediators (e.g., phenoxyl produced from SA) through the operation of H-abstraction mechanism. The laccase-mediator system can achieve better removal of compounds with high electrochemical potential as the oxidized mediator species have redox potentials higher than that of laccase only (Nguyen et al.,

2014). In this study, tests with TrOCs and SA (without laccase) yielded no TrOC degradation (data not shown). Therefore, the observed improvement in degradation was due to the enhancement of enzymatic degradation by SA, not due to direct degradation by SA.

Overall, the batch test data reveals two options for a continuous flow reactor to achieve high removal of the TrOCs, particularly DCF: i) to operate at acidic pH with frequent replenishment of enzyme (due to aggravated enzyme deactivation under this pH), or ii) to operate at neutral pH with mediator dosing. The latter option was chosen in this study from the point of view of enzymatic stability. For an in depth comparison of these options, further work with a diverse set of TrOCs is recommended. However, that is beyond the scope of this study.

[FIGURE 2]

3.1.3 Impact of mediator dose on performance

The effect of mediator concentration could be clearly observed at shorter incubation periods (Figure 2). For example, for an incubation period of 2 h, DCF was removed by 90% at an SA concentration of 1000 μM . In comparison, the removal of DCF for the same period ranged between 30 and 70% for SA concentrations between 5 and 500 μM . Similarly, Lloret et al. (2010) observed complete degradation of DCF by laccase within 1 h in presence of 1000 μM SA, while the removal ranged from 40 to 80% for the lower SA concentrations (i.e. 100 - 500 μM). The effect of mediator concentration has also been reported for other combinations of resistant pollutants and mediators. A notable example is the enhanced degradation of iso-butylparaben and n-butylparaben due to the dosing of a mediator (1-hydroxybenzotriazole, HBT) in the range of 200-2000 μM (Mizuno et al., 2009).

In contrast to better DCF removal, higher SA concentrations (i.e. 500 and 1000 μM) resulted in a remarkable decrease in laccase activity (Figure 2b). For example, within 4 h of incubation, enzymatic activity of the test media reduced by 60% for an SA dose of 500 μM . Mediator-induced inactivation of enzyme has been previously reported (Khelifi-Slama et al., 2012; Kurniawati and Nicell, 2007). Loss of enzymatic activity has been attributed to the degradation of essential amino acid residues or the glycosyl moieties at the surface of the enzyme (Khelifi-Slama et al., 2012). However, the range of mediator concentrations beyond which a fast and significant laccase inactivation occurs tends to vary depending on the source of laccase and the

type of the mediator. For example, in the study by Khelifi-Slama et al. (2012), the inactivation of laccase from *Trametes trogii* was proportional to the mediator (HBT) concentrations in the range from 100 - 10,000 μM . The authors explained that free radicals generated from HBT inactivated the laccase. On the other hand, Kurniawati and Nicell (2007) observed that up to a mediator concentration of 500 μM , there was only a slight inactivation ($< 5\%$) of laccase (from a white-rot fungus - *Trametes versicolor*). However, when the mediator concentration was increased to 10,000 μM , the inactivation was remarkable (90%). In another study, in the presence of 50 μM SA, laccase from *Trametes versicolor* was inactivated within 30 min (Mendoza et al., 2011). However, reducing the SA concentration to 25 μM resulted in a similar inactivation profile as without the mediator. In general the use of mediators can enhance TrOC transformation; however, in certain instances their practical application may be limited since they lead to significant inactivation of laccase.

Overall, in this study, it has been demonstrated that laccase offers greater stability at neutral pH (Figure 1) and at an SA concentration of up to 100 μM (Figure 2). Because there was virtually no difference in removal of TrOCs between SA concentrations of 5 and 100 μM , a SA concentration of 5 μM was selected for the operation of the EMR.

3.2 Enzymatic activity within EMR

3.2.1 Enzyme retention by the UF membrane

The EMR operating pH (7) and mediator dosage (5 μM) were selected based on the batch test data regarding TrOC removal and the stability of laccase activity. However, in the context of continuous operation, enzyme retention and enzymatic stability within an EMR is particularly important. López et al. (2004) confirmed the retention of manganese peroxidase (MnP) within the reactor by a polyethersulfone UF membrane (MWCO of 10 kDa). Laccase from *Cyathus bulleri* (molecular weight of 53 kDa) was retained by a polyacrylonitrile UF membrane (MWCO of 20 kDa) (Chhabra et al., 2009). Considering the molecular weight of the enzyme (56 kDa) and the MWCO of the membrane (6 kDa) used in this study, laccase was expected to be retained. But enzyme retention and activity can be influenced by factors such as membrane material, wastewater matrix and other operational parameters (Rios et al., 2004). Thus, the suitability of the selected membrane was tested for the retention of laccase under the operating conditions used

in this study (Section 2.4). The enzymatic activity in the permeate and in the reactor-supernatant was monitored during continuous filtration over a period of 24 h. No enzymatic activity in the permeate was observed, while that in the reactor supernatant remained stable (Supplementary Data Figure S3), confirming that the membrane effectively retained the enzyme.

3.2.2 Maintenance of enzymatic activity during EMR operation

The maintenance of enzymatic activity is an important parameter for long term stable operation of an EMR. As mentioned in Section 3.2.1, the enzyme was completely retained within EMR. However, to assess the probable change of enzymatic activity during continuous operation of the EMR, the enzymatic activity was measured periodically. As presented in Figure 3, the enzymatic activity gradually dropped to undetectable levels within 72 h of continuous operation. This was accompanied by a significant decrease in the removal of BPA and DCF (Figure 3). Denaturation of an enzyme can occur for various reasons including physical, chemical and biological inhibitors, and the effect of shear stress (Rios et al., 2004). Depending on the shear rate and flow geometry, deformation of the enzyme molecules during filtration could cause enzyme inactivation. There is only one study on continuous TrOC (i.e. estrone and estradiol) degradation by an EMR (Lloret et al., 2012). The authors of that study observed no enzyme denaturation within the short observation period of 8 h. Nevertheless, inactivation of enzyme has been reported in a few studies investigating the removal of other resistant groups of compounds. For example, Mendoza et al. (2011) observed complete inactivation of laccase within 180 h during repeated batch decolorization of Remazol Blue RR.

[FIGURE 3]

In this study, the UF membrane completely retained laccase within the EMR, but the enzymatic activity and TrOC removal decreased gradually (Figure 3). Therefore, periodic replenishment of laccase was deemed to be required to keep the enzymatic activity and TrOC removal stable. The enzymatic activity was maintained (Supplementary Data Figure S4) by the addition of 200 μ L of the commercial laccase solution (see Section 2.2) per L of the reactor volume every 12 h (equivalent to a laccase dose of 23 mg/L.d). Apart from enzyme reinjection, various additives, e.g., ethylenediaminetetraacetic acid and polyethylene glycol, which are believed to possess a

protecting role for proteins, especially under oxidative stress, may be added to an EMR (Mendoza et al., 2011). However, this aspect was beyond the scope of the current study.

3.3 *TrOC removal by EMR*

3.3.1 *Continuous removal of TrOC by EMR*

In line with the batch test data, BPA was significantly degraded ($85 \pm 7 \%$, $n=28$) during the continuous operation of the EMR (Figure 4). No reports of studies on the degradation of BPA by a continuous flow EMR could be found. However, Lloret et al. (2012) reported over 95% removal of estrone and estradiol, both $-OH$ containing TrOC, over 8 h of operation of an EMR.

In comparison to the high removal of BPA, a removal of $60 \pm 6 \%$ ($n=28$) was observed for DCF. The lower removal of DCF can also be explained by its molecular structure. DCF is a compound well-known to be resistant to bacterial degradation (Tadkaew et al., 2011). It contains an aromatic ring with two chlorine atoms and that ring is joined to another aromatic ring via N-H group. Notably chloro groups are electron withdrawing functional groups and can render the compound resistant to biological degradation. Low removal of chlorinated compounds has been widely reported in the literature. For example, laccase from a strain of *Trametes versicolor* was reported to inefficiently remove clofibric acid (containing one chlorine atom) (Tran et al., 2010).

[FIGURE 4]

A longer HRT could lead to a decrease in TrOC loading and facilitate a longer contact time between the TrOCs and the enzyme, and better TrOC removal. However, in this study the DCF removal performance improved by only 5% as the HRT was increased from 8 to 12 h (data not shown). Continuous dosing of the mediator was hence deemed imperative to attain a significant improvement in DCF removal.

3.3.2 *Effect of mediator dosing of EMR*

To date, the use of mediators to improve TrOC removal has been reported mostly for batch studies. There is only one study where repeated addition (every 8 h) of laccase and a mediator (HBT) was explored over a period of 48 h (Hata et al., 2010). Following this strategy, the authors

achieved a carbamazepine removal of 60%, which compared favourably with a removal of 20% without HBT addition. However, the effect of mediator dosing to a continuous flow reactor on TrOC degradation has not been reported to date.

Given the 90% removal of BPA even without SA addition, it was not a surprise that a high removal (i.e., 95%) of BPA continued following the start of SA dosing in this study (Table 2). Of particular interest was the significant improvement in DCF removal (from 60 - 80%) during SA dosing. As noted earlier, enhanced removal of TrOCs due to mediator addition has been demonstrated in studies involving batch tests. However, there have so far been no reports on removal of TrOCs under continuous addition of mediator(s) to the EMR. To date only a few studies have investigated mediator-enhanced enzymatic dye degradation by EMRs operated in batch or continuous mode. For example, the degradation of triarylmethane dyes by an EMR was improved by 60% when a mediator (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)) was added along with laccase (Chhabra et al., 2009). The current study reports for the first time enhanced TrOC degradation by continuous addition of a mediator to an EMR.

[TABLE 2]

It is noteworthy that apart from mediator dosing, application of a consortium of enzymes with complementary degradation capacity may enhance TrOC degradation. For example, laccase can catalyze the degradation of a wide range of phenolic substrates using oxygen as an electron acceptor, while LiP is capable of catalyzing non-phenolic substrates in the presence of H_2O_2 . Therefore, the combination of laccase and LiP may efficiently treat wastewater containing a mixture of phenolic and non-phenolic compounds. For instance, compared to application of a single fungal culture, the co-culture of two white-rot fungi *Pleurotus ostreatus* IBL-02 and *Coriolus versicolor* IBL-02, which produced all the three major ligninolytic enzymes (i.e., laccase, LiP and MnP), resulted in 10% improvement in decolorization of dye effluent (Asgher et al., 2012). However, there are no studies on combination of different enzyme for the removal of TrOCs. Further work in this line is worthwhile, however, is beyond the scope of this study.

It is further acknowledged that in this study TrOCs were added to Milli-Q water which was fed to the EMR instead of a real wastewater. The efficiency of enzymatic TrOC removal may be

different in case of real wastewater. The presence of bulk organic compounds other than TrOCs and various physico-chemical and biological inhibitors may affect TrOC degradation by enzyme. For example, the presence of ions including sulfite, sulfide, cyanide, chloride, iron (III) and copper (II) in the media resulted in a reduction of triclosan conversion by laccase (Kim and Nicell, 2006a). However, there is also evidence from batch tests that the laccase mediated degradation of natural and synthetic hormones is not significantly affected by municipal wastewater matrix (Auriol et al., 2007). Nevertheless, further work assessing performance of an EMR with real wastewater is recommended.

3.3.3 Effluent toxicity

Certain biodegradation products have been identified in recent studies investigating removal of either BPA or DCF separately. For example, Marco-Urrea et al. (2010) identified three compounds, namely, 4'-OH-diclofenac, 5-OH-diclofenac and 4-(2,6-dichlorophenylamino)-1,3-benzenedimethanol during degradation of DCF by the whole-cell of the laccase-secreting white-rot fungus *Trametes versicolor*. Different pathways of BPA degradation have also been reported. Hirano et al. (2000) reported four metabolites, namely hexestrol, phenol, 4-isopropenylphenol and 4-isopropylphenol by *Pleurotus ostreatus*, while Uchida et al. (2001) observed two metabolites, namely, bisphenol A dimer and 4-isopropylphenol for *Trametes villosa*. In this study, however, the extent of enzymatic degradation of the TrOCs i.e., the type of the metabolites was not monitored because the synthetic wastewater contained a mixture of two TrOCs. The removal efficiency was calculated as relative concentration of the parent compounds (BPA and DCF) in the feed and permeate. However, the toxicity of the treated media was measured, which provides a measure of the overall toxicity of all compounds, including by-products.

Enzymatic TrOC transformation often leads to detoxification, but can also cause increase in effluent toxicity, despite removal of the target compound, due to toxic by-product formation (Marco-Urrea et al., 2009). A ToxScreen3 assay (Section 2.5.2) revealed no significant toxicity in the feed (4.9 ± 0.1 rTU; n=2) indicating that BPA and DCF were not particularly toxic to the indicator bacteria (*P. leiognathi*) used in this study. There was no appreciable increase in toxicity in the EMR permeate (7.0 ± 1.8 rTU; n=2), indicating that treatment did not produce toxic by-products. Elevated toxicity of the treated media has previously been reported after addition of

mediators (Kim and Nicell, 2006b). Addition of the mediator to the EMR in the current study considerably increased the toxicity of the permeate (16 ± 2.9 rTU; $n=2$). It should be noted that toxicity to the indicator bacteria (*P. leiognathi*) does not necessarily imply that the effluent is toxic to other organisms. For example, treated media that was toxic to the bacteria *Vibrio fischeri* was not toxic to other aquatic organisms tested (Cruz-Morató et al., 2013). Furthermore, the TrOC loading used in this study was significantly higher than would be encountered in municipal wastewater. Nevertheless, further studies are required to screen mediators which enhance enzymatic degradation without raising treated media toxicity. This study reports data regarding the technical feasibility of mediator-enhanced continuous degradation of TrOCs by laccase using an EMR, and forms a solid basis for further studies as outlined above.

3.3.4 Role of the membrane in TrOC removal

Notably, the removal of DCF by the EMR was significantly higher than in the batch tests (Figure 4). For example, in batch tests, for an incubation period of 8 h, the removal of DCF was only 30%, while a removal of 60% was achieved during continuous operation of the EMR at an HRT of 8 h (without mediator addition). The concentration of BPA and DCF in both reactor supernatant and membrane permeate was measured periodically to investigate the probable contribution of the membrane and/or a cake layer on it to the TrOC removal. The permeate over supernatant concentration ratio was 0.5 ± 0.2 and 0.6 ± 0.1 ($n = 46$) for BPA and DCF, respectively (Figure 5). The extent of physical adsorption of TrOCs on the membrane surface was determined by operating the reactor in the absence of enzyme. Results showed that TrOC removal by adsorption on membrane was negligible ($< 5\%$). Moreover, the detection of enzymatic activity in the membrane cleaning solution confirmed that an enzyme gel layer was formed on the membrane surface. The enzymatic activity in 1 L of the cleaning solution was 60 $\mu\text{M}/\text{min}$, equivalent to an accumulation of at least $0.24 \text{ g laccase}/\text{m}^2$ membrane surface. Because stable TrOC removal was achieved throughout the operation period, and there was no accumulation of TrOCs within the EMR (as evidenced by a stable concentration in the reactor supernatant), it can be concluded that TrOC adsorbed on the enzyme gel layer was subsequently degraded.

[FIGURE 5]

Membrane fouling is an important aspect that requires due consideration when operating an EMR. The formation of an enzyme gel layer on the membrane can lead to gradual fouling. For example, a 10% drop in membrane flux was observed after 15 d of operation of an EMR in a study by Chhabra et al. (2009). However, under the operating conditions of this study, TMP did not exceed 20 kPa during any trial, and therefore, no membrane cleaning was required except at the start of a new trial.

4. Conclusion

Continuous removal of two trace organic contaminants (TrOCs), namely, bisphenol-A (BPA) and diclofenac (DCF) by an enzymatic membrane reactor (EMR) is reported. Laccase was completely retained by the UF membrane; however, denaturation of laccase necessitated its periodic reinjection, although with a small dose. Stable removal of BPA (85%) and DCF (60%) was achieved during continuous operation of the EMR. BPA and DCF removal could be further improved to > 95% and >80%, respectively, by dosing a redox-mediator to the EMR. TrOC degradation by both the suspended enzyme and that forming a gel layer on the membrane surface was confirmed.

5. Acknowledgement

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LIST OF TABLES

Table 1: TrOC degradation by laccase as a function of incubation pH and duration mean \pm standard deviation from triplicate experiments)

pH	BPA removal (%)				DCF removal (%)			
	Incubation Time (h)							
	2	4	8	22	2	4	8	22
3	43 ± 5.0	56 ± 3.5	77 ± 0.5	89 ± 3.0	36 ± 1.6	57 ± 3.5	69 ± 2.3	91 ± 4.6
4.5	51 ± 0.5	61 ± 3.5	82 ± 3.2	94 ± 1.5	44 ± 7.2	50 ± 1.0	82 ± 0.5	98 ± 2.0
6	79 ± 0.5	88 ± 9.4	92 ± 1.8	95 ± 0.5	42 ± 3.5	51 ± 2.8	65 ± 1.7	71 ± 4.5
7	87 ± 4.0	96 ± 1.5	100 ± 0	100 ± 0	29 ± 8.4	33 ± 2.5	40 ± 2.8	59 ± 7.8
9	69 ± 4.5	74.0 ± 1.4	84 ± 3.2	83 ± 0.7	11 ± 3.4	30 ± 5.9	48 ± 2.8	61 ± 1.7

Table 2: Enhanced TrOC removal by continuous dosing of a redox-mediator (syringaldehyde, 5 μ M) to the EMR (mean \pm standard deviation).

Experiment	Removal (%)	
	BPA	DCF
Without mediator	85 \pm 7(n = 28)	60 \pm 6 (n = 28)
With mediator	98 \pm 2 (n = 18)	80 \pm 7 (n = 18)

LIST OF FIGURES

Figure 1: Removal of BPA and DCF (a), and the change in enzymatic activity (b) during treatment by laccase at different pH. Control samples contained TrOC in Milli-Q water in absence of laccase. Error bars represent the standard deviation of three samples.

Figure 2: Concentration of BPA and DCF (a), and the change in enzymatic activity (b) during treatment by laccase in the presence of a redox-mediator (syringaldehyde) at different concentrations (5, 100, 500, and 1000 μM). Control samples contained TrOC and mediator in Milli-Q water in absence of laccase. Error bars represent the standard deviation of three samples.

Figure 3: Enzymatic activity decrease during continuous operation of the EMR following addition of a single dose of laccase, and concomitant reduction in removal of BPA and DCF.

Figure 4: Continuous removal of BPA and DCF by the EMR at the loading rates of 570 ± 70 $\mu\text{g/L.d.}$ and 480 ± 40 $\mu\text{g/L.d.}$ ($n=46$) for BPA and DCF, respectively, and maintenance of stable enzymatic activity by the addition of 200 μL of the commercial laccase solution per litre of the reactor volume every 12 h (equivalent to a laccase dose of 23 mg/L.d.).

Figure 5: Variation of ratio of TrOC concentration in reactor supernatant and permeate confirming the retention of TrOC on the enzyme gel layer onto membrane.

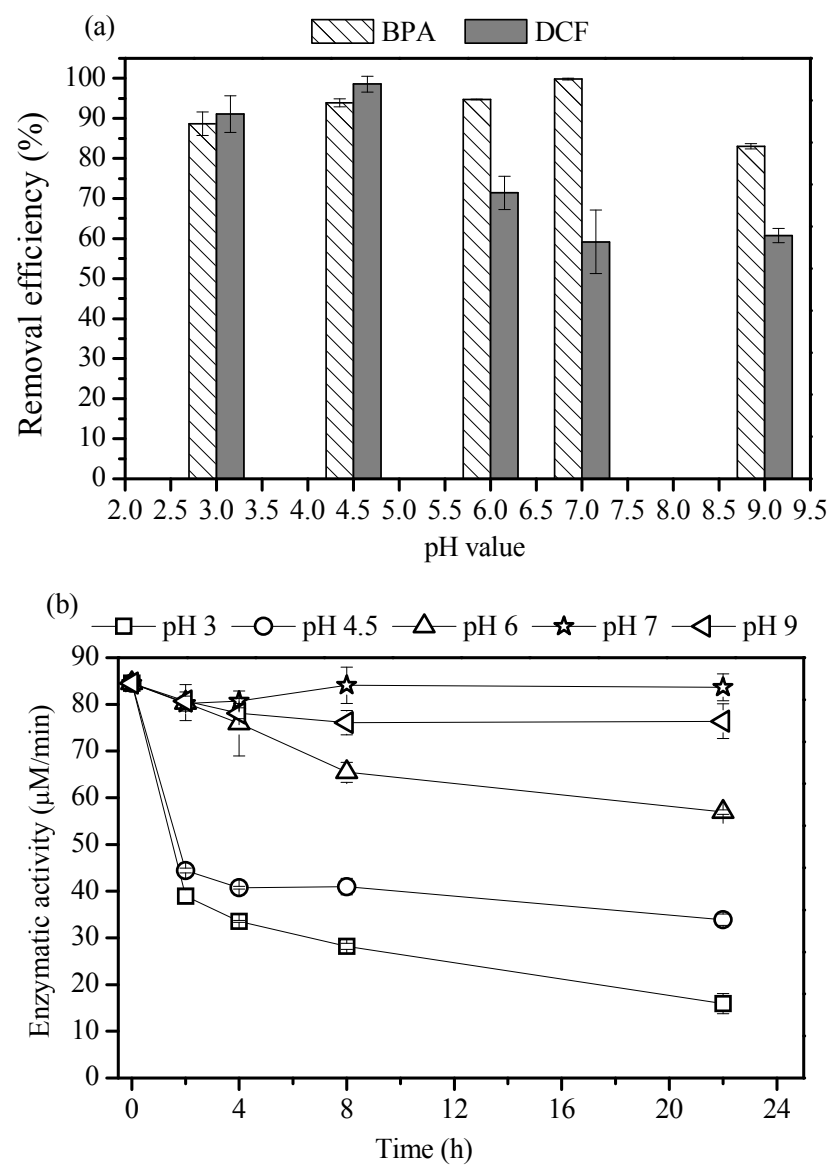


Figure 1

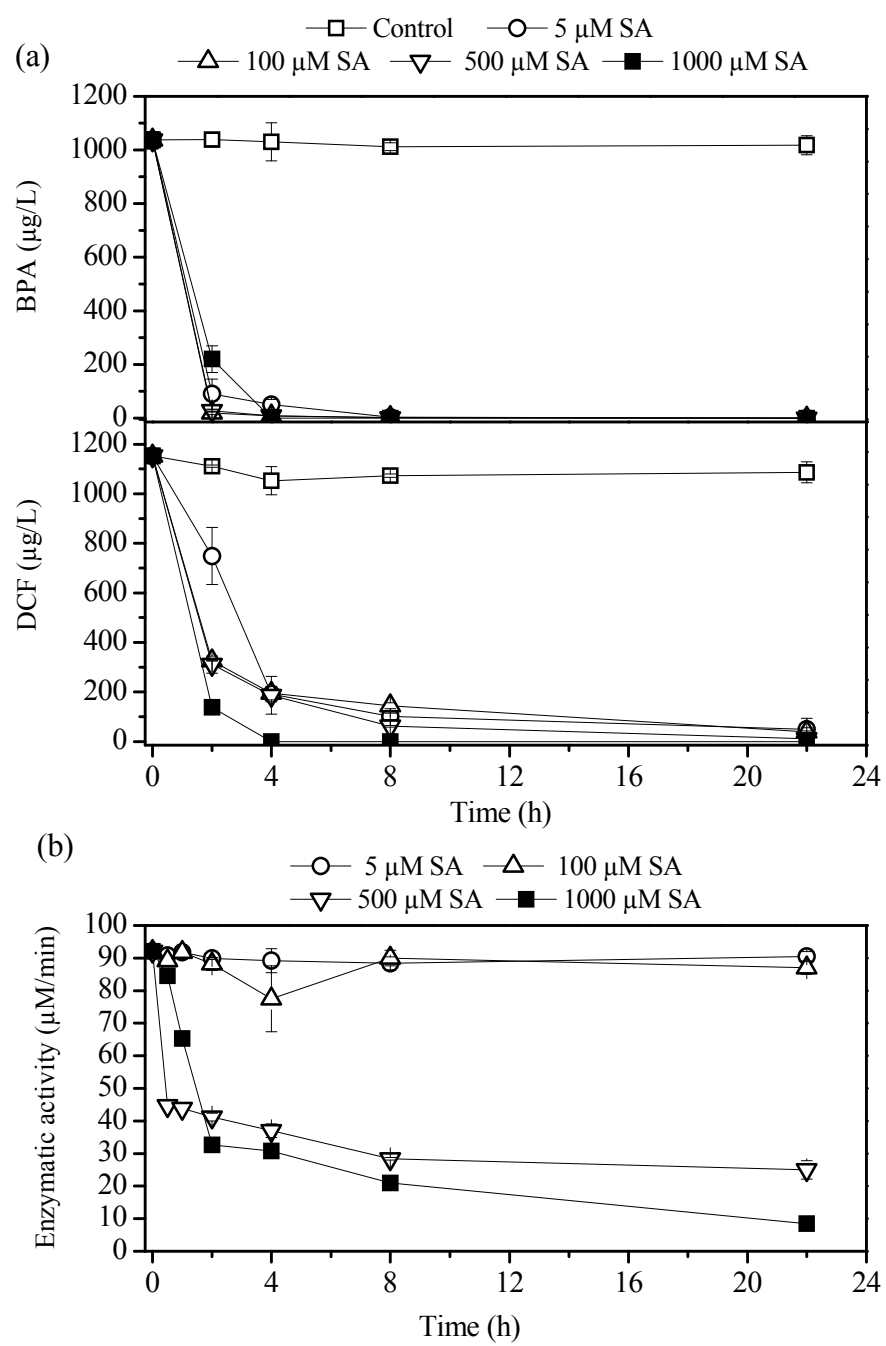


Figure 2

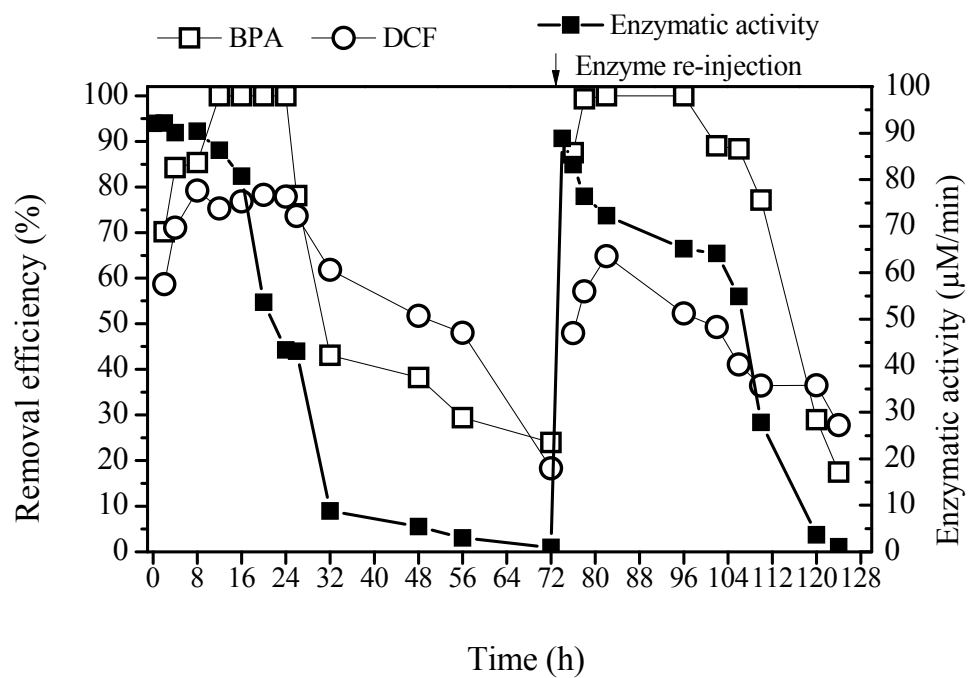


Figure 3

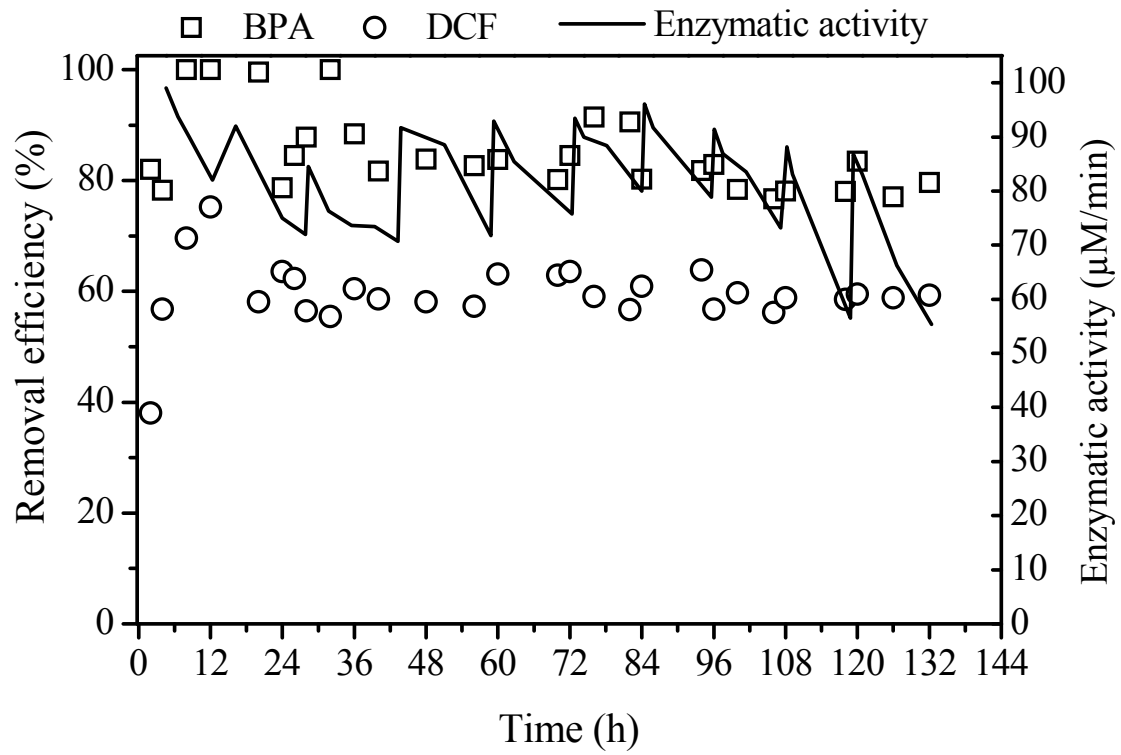


Figure 4

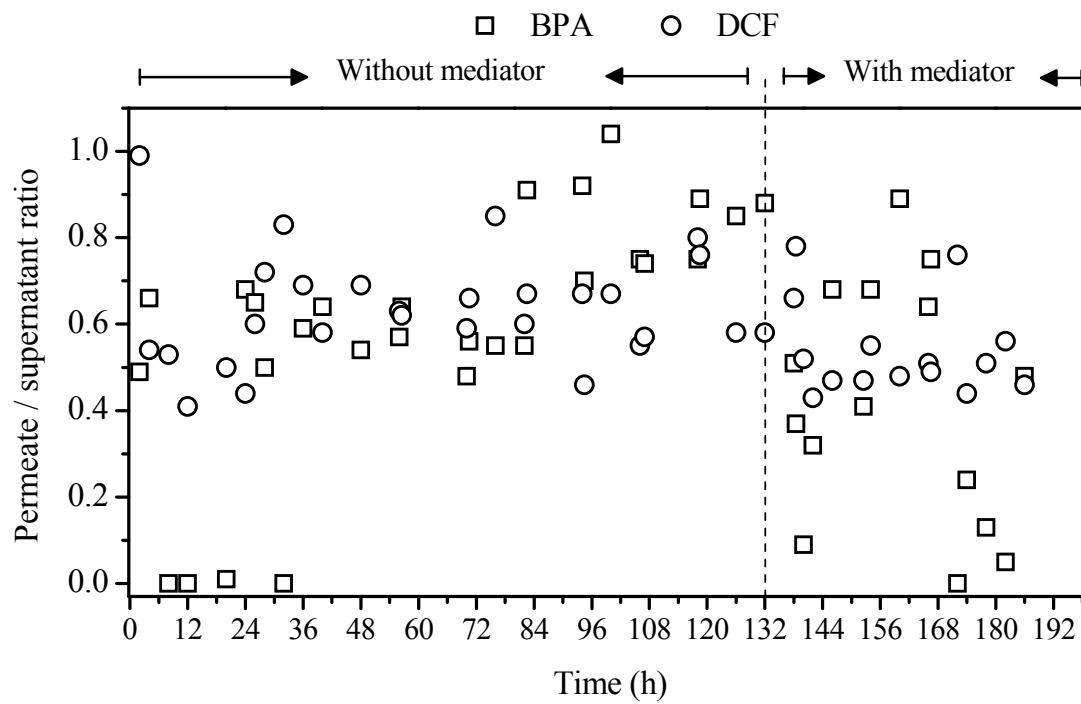


Figure 5

Continuous biotransformation of bisphenol A and diclofenac by laccase in an enzymatic membrane reactor

Supplementary Data

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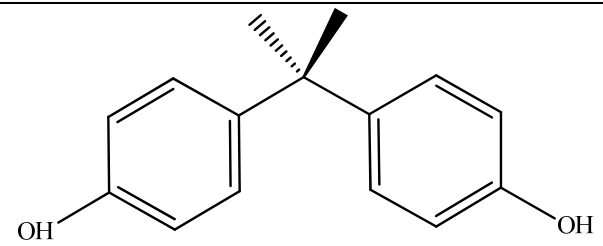
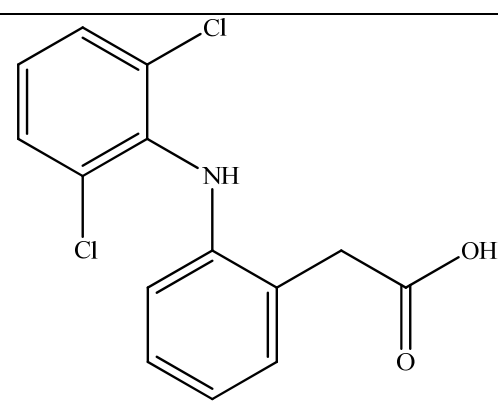
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Table S1: Physicochemical properties of bisphenol A and diclofenac.

Compounds (CAS number)	Molecular weight (g/mol)	Log K _{OW} [*]	Log <i>D</i> at pH 7 [*]	Dissociation constant (pKa) [*]	Vapour pressure (mg Hg) at 25°C [*]	Chemical structure
Bisphenol A (C ₁₅ H ₁₆ O ₂) (80-05-7)	228.29	3.64 ± 0.23	3.64	10.29 ± 0.10	5.34 × 10 ⁻⁷	
Diclofenac (C ₁₄ H ₁₁ Cl ₂ NO ₂) (15307-86-5)	296.15	4.55 ± 0.57	1.77	4.18 ± 0.10 -2.26 ± 0.50	1.59 × 10 ⁻⁷	

^{*} Source: SciFinder database: <https://origin-scifinder.cas.org>

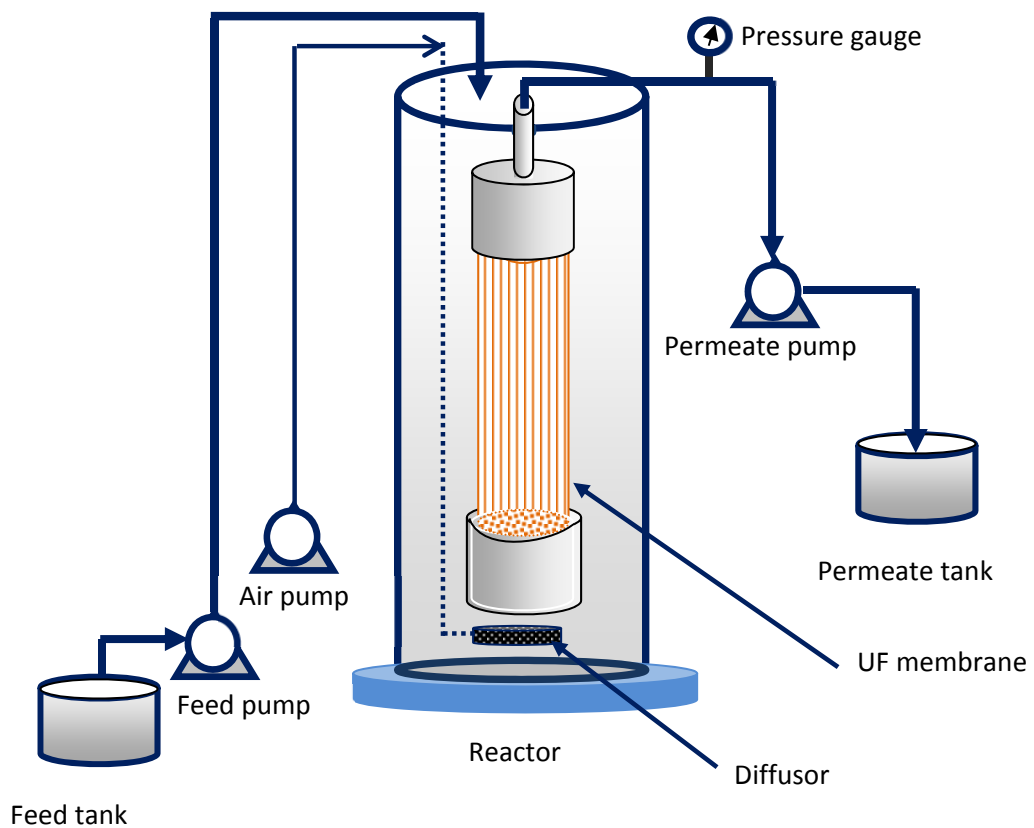
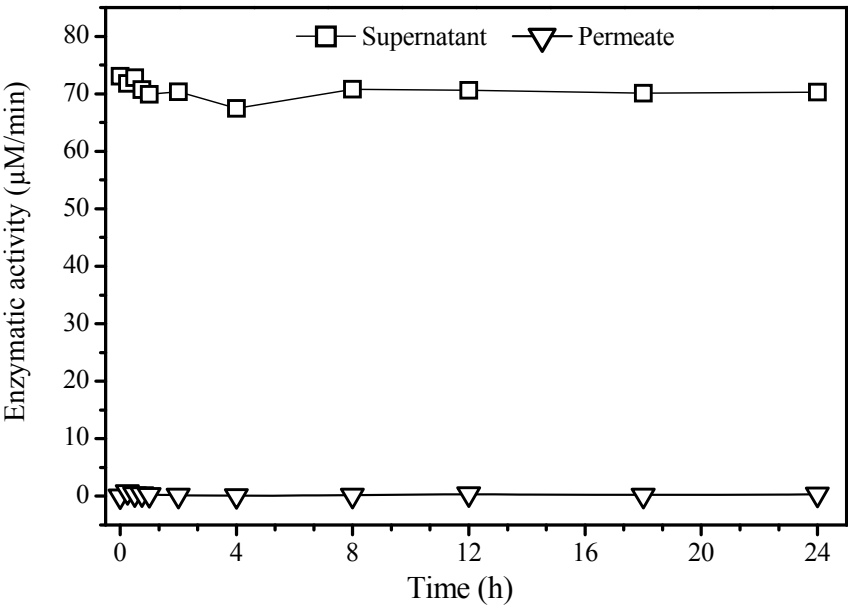


Figure S2: Schematic diagram of the enzymatic membrane reactor

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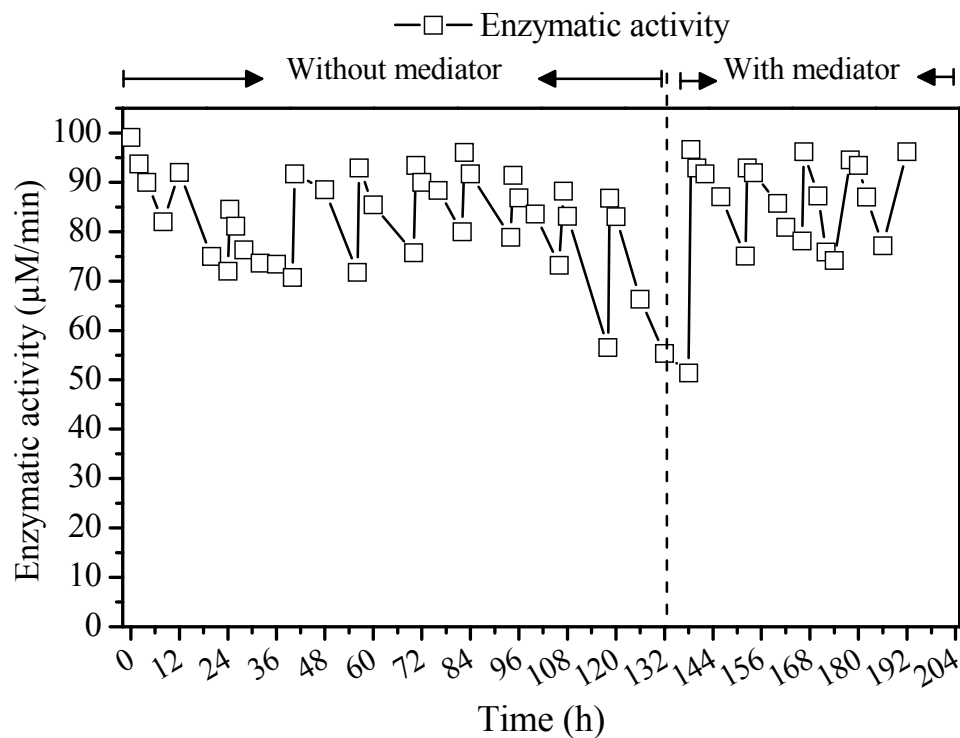
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19 Figure S3: Confirmation of complete retention of the enzyme by the membrane

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23 Figure S4: Denaturation of the enzyme within the reactor and maintenance of stable enzymatic
 24 activity over long-term operation by the addition of 200 μL of the commercial laccase solution
 25 per L of the reactor volume every 12 h (equivalent to a laccase dose of 23 mg/L.d).

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