

A novel high retention enzymatic bioreactor system for the removal of pharmaceuticals and personal care products

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Abstract: In previous studies we developed an enzymatic bioreactor coupled with an ultrafiltration (UF) membrane, which prevented enzyme washout, thereby allowing continuous enzymatic degradation of pharmaceuticals. Moreover, some resistant compounds such as naproxen and salicylic acid were retained by enzyme gel layer formed on membrane surface, subsequently resulting in their enhanced biodegradation. Based on this observation it was postulated that integration of high retention membranes with an enzymatic bioreactor can facilitate biodegradation of recalcitrant compounds by retaining both enzyme and PPCPs. This study explores a novel membrane distillation-enzymatic bioreactor (MD-EMBR) system for the removal of PPCPs including four pharmaceuticals namely, diclofenac, naproxen, salicylic acid and ibuprofen as well as two ingredients of personal care products namely, oxybenzone and salicylic acid using purified laccase from genetically modified *A. oryzae*. The results confirmed almost complete retention (>95%) of the PPCPs by the MD membrane. Of particular interest was the fact that the complete retention of the PPCPs improved the enzymatic degradation (10-30%) of compounds that have been reported to be poorly removed in UF-EMBR. Addition of 1-hydroxybenzotriazole improved the oxidation reduction potential of the solution in the enzymatic bioreactor by 16%, thereby enhancing the degradation of diclofenac, naproxen, salicylic acid and ibuprofen by 5-22%. Toxicity analysis showed that the final effluent of MD-EMBR system was nontoxic.

Keywords: Membrane distillation-enzymatic bioreactor (MD-EMBR); Pharmaceuticals; Laccase; Redox-mediators; Effluent toxicity

1. Introduction

Laccase (EC 1.10.3.2) has been increasingly studied for the degradation of pollutants that are resistant to bacterial degradation such as dyes, aromatic hydrocarbons and trace organic contaminants (Chang *et al.*, 2016; Hai *et al.*, 2012). Among trace organic contaminants (TrOCs), pharmaceuticals and personal care products (PPCPs) are of particular interest due to: (i) their widespread occurrence in municipal wastewater; and (ii) their poor/unstable removal in conventional wastewater treatment plants (Hai *et al.*, 2014; Luo *et al.*, 2014).

Laccase catalyzed oxidation of PPCPs is governed by their physicochemical properties (*e.g.* chemical structure and

availability of phenolic moiety) and operational parameters (*e.g.* temperature and pH). In general, laccase can efficiently degrade phenolic PPCPs or those having strong electron donating groups (EDGs). On the other hand, rate of degradation for PPCPs containing non-phenolic moiety or electron withdrawing groups (EWGs) is slow (Yang *et al.*, 2013). Degradation of such PPCPs can be improved with the addition of low molecular weight phenolic substrates, also termed as redox-mediators that facilitate the transfer of electron between enzyme and target compound (Asif *et al.*, 2017).

Performance of laccase for the removal of PPCPs has initially been studied in batch-fed reactors because of enzyme washout (Suda *et al.*, 2012; Tran *et al.*, 2010). This problem has been solved by coupling a membrane having an appropriate pore size with an enzymatic bioreactor, thereby allowing continuous operation without any apparent mass transfer limitations (Lloret *et al.*, 2012; Nguyen *et al.*, 2014). Although UF membrane is not expected to retain PPCPs, it partially retained some PPCPs such as diclofenac, octocrylene and amitriptyline due to the adsorption of these pollutants onto an enzyme gel layer formed following the flushing of enzyme solution, thereby improving the removal of adsorbed compounds. Moreover, it was also confirmed that compounds adsorbed on to enzyme gel layer were subsequently degraded by laccase (Nguyen *et al.*, 2015), indicating the significance of complete PPCP retention. Therefore, it was postulated that complete retention of both laccase and PPCPs by integrating a high retention membrane with an enzymatic bioreactor may facilitate their degradation. Different configurations of activated sludge based high retention membrane bioreactor (HR-MBR) such as membrane distillation MBR and nanofiltration MBR have been investigated for the removal of PPCPs recently, providing almost complete retention (95-99%) for most pollutants (Phan *et al.*, 2016; Wijekoon *et al.*, 2014b). However, complete retention did not improve PPCP degradation in bioreactor and was comparable with UF-MBR, resulting in the accumulation of PPCPs. Despite the better performance of laccase compared to activated sludge for PPCP removal, laccase catalyzed degradation of PPCPs in high retention enzymatic membrane bioreactor (HR-EMBR) is yet to be explored.

In this study, we examined the enzymatic degradation of selected pharmaceuticals and personal care products in a

membrane distillation (MD)-EMBR using a commercially available laccase from *Aspergillus oryzae*. In addition, effect of a redox-mediators namely, 1-hydroxybenzotriazole (HBT) on enzymatic degradation and effluent toxicity was also assessed.

2. Materials and methods

2.1. Laccase, redox-mediators and PPCPs

Laccase from *Aspergillus oryzae* (molecular weight: 56 kDa; purity: 10% w/w; and activity: 150,000 mM_(DMP)/min) was supplied by Novozymes Australia Pty Ltd. One N-OH type mediators (HBT) was purchased from Sigma Aldrich (Australia). 50 mM stock solution of HBT was prepared and stored at 4 °C.

For this study, PPCPs were selected based on their ubiquitous occurrence in municipal wastewater as well as in surface water (Luo *et al.*, 2014). Physicochemical properties of the selected PPCPs are given in Table 1. Stock solution (25 mg/L) of PPCP mixture was prepared in methanol and kept in dark at -18°C

2.2. Experimental setup

A lab scale MD-EMBR setup consisting of an enzymatic bioreactor (1.5 L) and a direct contact membrane distillation system (DCMD) was used in this study (Figure 1). Detailed description of DCMD system is given elsewhere (Wijekoon *et al.*, 2014a). A hydrophobic microporous polytetrafluoroethylene (PTFE) membrane (GE, Minnetonka, MN) having thickness of 175 µm, nominal pore size of 0.22 µm and porosity of 70% was used. The enzymatic bioreactor was placed in a water bath and temperature of the enzymatic bioreactor was kept at 30±0.2°C using a heating unit (Julabo, Germany). On the other hand, temperature of the distillate unit was maintained at 10±0.1°C using a cooling unit (SC100-A10, Thermo Scientific, USA) and was placed on a precision balance (Mettler Toledo Inc, USA) connected to a computer for the measurement of permeate flux. Solution from enzymatic bioreactor and ultrapure water from distillate tank were recirculated through the external DCMD system at 1 L/min.

Table 1. Physicochemical properties of selected PPCPs

PPCPs	Molecular Weight	Log D at pH=7	Water Solubility at 25°C	Vapor Pressure	pK _H at pH 7
	g/mole		mg/L	(mmHg)	
Naproxen	230.26	0.73	435,000	3.01 × 10 ⁻⁷	12.68
Diclofenac	296.15	1.77	20,000	1.59 × 10 ⁻⁷	11.51
Ibuprofen	206.28	0.94	928,000	1.39 × 10 ⁻⁴	10.39
Salicylic acid	138.12	-1.13	2240	8.2 × 10 ⁻⁵	8.18
Bisphenol A	228.29	3.64	73	5.34 × 10 ⁻⁷	8.66
Oxybenzone	228.24	3.89	2700	5.26 × 10 ⁻⁶	9.23

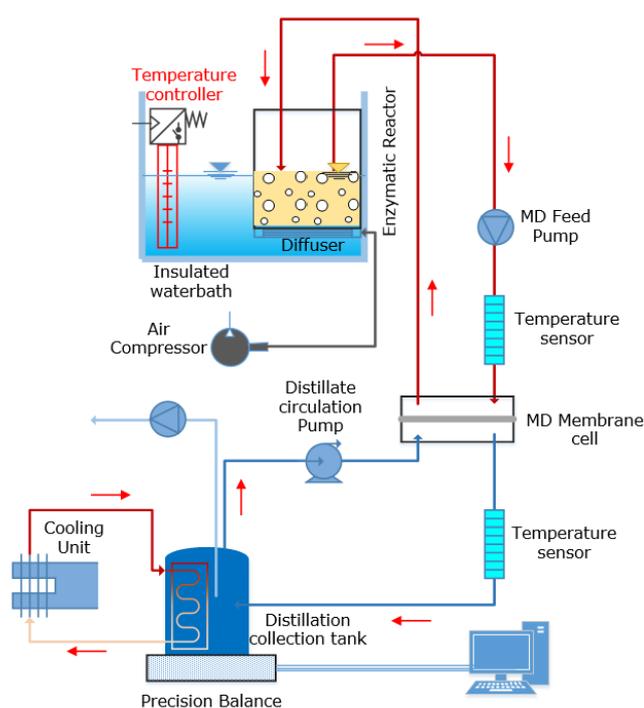


Figure 1. A lab scale MD-EMBR setup

2.3. Experimental protocol

A series of experiments were conducted to evaluate: (i) the rejection of PPCPs by MD; and (ii) the enzymatic degradation of PPCPs in batch-fed MD-EMBR using synthetic wastewater containing ultrapure water and PPCPs. Initial enzymatic activity was kept at 95-100 $\mu\text{M}_{(\text{DMP})}/\text{min}$. After confirming the complete retention of laccase by MD, PPCPs each at 20 $\mu\text{g}/\text{L}$ were added in the enzymatic bioreactor and the MD-EMBR system was operated for 12 h. The impacts of a redox-mediator *i.e.*, HBT on enzymatic degradation and laccase stability was assessed via separate runs. At the end of each experiment, 100 and 500 mL samples (in duplicate) from enzymatic bioreactor and distillate, respectively, were collected for PPCP analysis. Samples from enzymatic reactor were then diluted to 500 mL before solid phase extraction and GC/MS analysis.

2.4. Analytical methods

PPCP (500 mL) were extracted using solid phase extraction and were quantified using Shimadzu GC/MS (QP5000) system as previously described by Hai *et al.* (2011). Laccase activity was assayed by recording the change in absorbance (468 nm) due to oxidation of 2,6-dimethoxyl phenol (DMP) in the presence of sodium citrate (pH 4.5). Enzymatic activity was calculated using a molar extinction coefficient of 49.6/ $\text{mM}\cdot\text{cm}$ and expressed as $\mu\text{M}_{(\text{DMP})}/\text{min}$.

The ORP of the laccase solution before and after the addition of redox-mediator was measured using an ORP meter (WP-80D dual pH-mV meter, Thermo Fisher Scientific, Australia)..

3. Results and discussion

3.1. Removal of PPCPs in MD-EMBR

Performance of MD-EMBR for PPCP removal was evaluated based on: (i) their rejection by MD membrane; and (ii) their degradation by laccase in enzymatic bioreactor (Figure 2). Hence these two aspects are discussed separately in this section.

PPCP rejection by MD system mainly depends on their volatility, expressed as (pK_{H}) (Wijekoon *et al.*, 2014b). In a recent study by Wijekoon *et al.* (2014a), TrOCs including PPCPs were divided into two broad categories as follows: (i) incomplete rejection (54-90%) of volatile compounds ($\text{pK}_{\text{H}} < 9$); and (ii) complete rejection (>99%) non-volatile compounds ($\text{pK}_{\text{H}} > 9$). However, volatility of a compound varies with the change in operating temperature of the feed to DCMD system (Davie-Martin *et al.*, 2015). In this study, PPCP rejection by MD membrane was in the range of 95-99% (Figure 2). Since the operating temperature of feed in previous studies was kept at 40°C (Wijekoon *et al.*, 2014a; Wijekoon *et al.*, 2014b) compared to 30 °C in this study, complete rejection of PPCPs can be expected in this study.

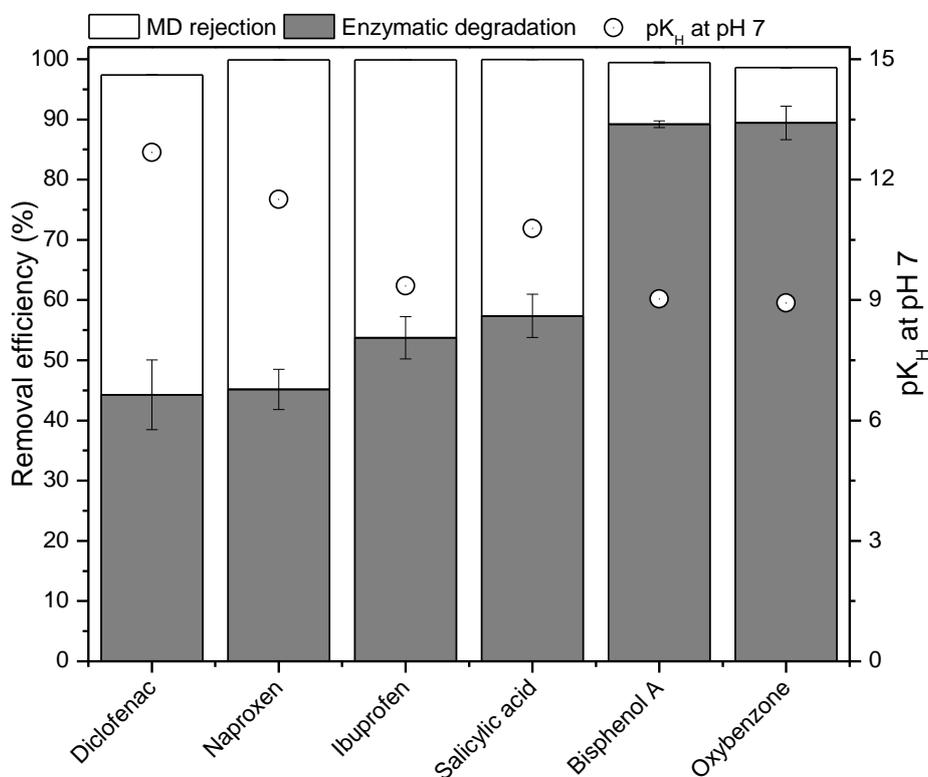


Figure 2. Removal of selected PPCPs in MD-EMBR systems categorized as MD rejection and enzymatic degradation. Temperature of enzymatic bioreactor and distillate tank was maintained at 30 ± 0.2 and 10 ± 0.1 °C, respectively. Initial enzymatic activity and PPCP concentration was 95-100 $\mu\text{M}/\text{min}$ and 20 $\mu\text{g}/\text{L}$, respectively. Error bars represents the standard deviation between duplicate samples.

The main focus of this study was to investigate the improvement in enzymatic degradation of PPCPs after their complete rejection by MD. Degradation of PPCPs containing phenolic moiety or EDGs in their chemical structure is generally high (80-95%) in laccase based treatment (Asif *et al.*, 2017). Indeed, 90% degradation of bisphenol A and oxybenzone was achieved via laccase catalyzed oxidation in the enzymatic reactor of the MD-EMBR (Figure 2). On the other hand, remaining PPCPs including diclofenac, naproxen, ibuprofen and salicylic acid were moderately removed (45-55%) by laccase in the enzymatic reactor (Figure 2). Since these compounds contain strong EWGs in their chemical structure, their laccase catalyzed oxidation is slow (Yang *et al.*, 2013). Despite the moderate degradation of these compounds in MD-EMBR system, it was still 10-30% better than UF-EMBR system as reported in previous studies (Nguyen *et al.*, 2015; Nguyen *et al.*, 2016). These results indicate that complete retention of PPCPs can facilitate their degradation. This is probably because the concentration of PPCPs in enzymatic bioreactor increases following their complete retention, hence more time and substrate is available for enzymatic oxidation (Purich, 2010). Notably, phenolic PPCPs such as bisphenol A and oxybenzone can act as redox-mediators, meaning that these compounds can act as an electron shuttle between laccase and non-phenolic compounds such as diclofenac, naproxen and salicylic acid, thereby facilitating the improvement in the removal of non-phenolic pollutants (d'Acunzo *et al.*, 2006). It is possible that the actual potential of phenolic PPCPs as redox-mediators could not be harvested in UF-EMBR because UF membrane cannot completely retain these compounds.

3.2. Impact of redox-mediators on PPCP degradation

One N-OH type redox-mediator namely, HBT (0.25 mM) was added to improve the degradation of PPCPs, particularly of those that were moderately degraded by laccase. In a laccase-mediator system, highly reactive radicals produced following the oxidation of a redox-mediator by laccase react with PPCPs, thereby improving the oxidation reduction potential (ORP) and rate of their degradation (Asif *et al.*, 2017). However, performance of redox-mediators depends on their type and concentration as well as on the pollutants of interest. In this study, a slight improvement (2-5%) in the removal of bisphenol A and oxybenzone was observed with HBT because these compounds are already well degraded (85-90%) by laccase. On the other hand, improvement in the degradation of remaining four PPCPs was compound specific (Figure 3). Addition of HBT improved the degradation of diclofenac, naproxen, ibuprofen and salicylic acid by 5, 10, 15 and 22%, respectively. This can be attributed to a 20% increase in ORP of the reaction mixture after the addition of HBT in this study.

Although redox-mediators can significantly improve PPCP removal, they can negatively affect the stability of enzymes (Ashe *et al.*, 2016; Hata *et al.*, 2010). This is because the highly reactive radicals formed due to the oxidation of redox-mediators by laccase can react with enzymes and convert them into non-productive complexes. Moreover, charged metabolites formed following PPCP degradation can also block the active sites of laccase (Purich, 2010). In this study, reduction in laccase activity was observed both with and without the addition of HBT (Figure 4). However, the extent of laccase inactivation was higher in the presence of HBT. Laccase inactivation was 16% higher in the presence of HBT, but HBT overall enhanced the removal of PPCPs.

3.3. Permeate flux

The permeate flux in MD system depends on the difference in the temperature of the feed tank and distillate tank (Alkudhiri *et al.*, 2012). In this study, temperature of enzymatic bioreactor (the feed) and distillate was maintained at 30 ± 0.2 and 10 ± 0.1 °C to avoid thermal inactivation of laccase. A stable permeate flux in the range of $3.7 - 4$ L/m².h was observed in this study.

4. Conclusion

A novel high retention enzymatic membrane bioreactor was developed by integrating a MD membrane with an enzymatic bioreactor, providing complete retention of both laccase and PPCPs. In this study, complete retention of PPCPs provided a high contact time between laccase and PPCPs, thereby facilitating their enzymatic degradation. As expected, significant degradation (up to 90%) of bisphenol A and oxybenzone was achieved because these compounds contain phenolic moiety and EDGs in their chemical structure. On the other hand, moderate degradation (40-55%) for non-phenolic PPCPs containing EWGs including diclofenac, naproxen, ibuprofen and salicylic acid was achieved in MD-EMBR. These compounds have been reported to be poorly removed (5-20%) in UF-EMBR. Addition of a redox-mediator HBT caused faster inactivation of laccase but achieved 10-22% improved degradation of non-phenolic compounds

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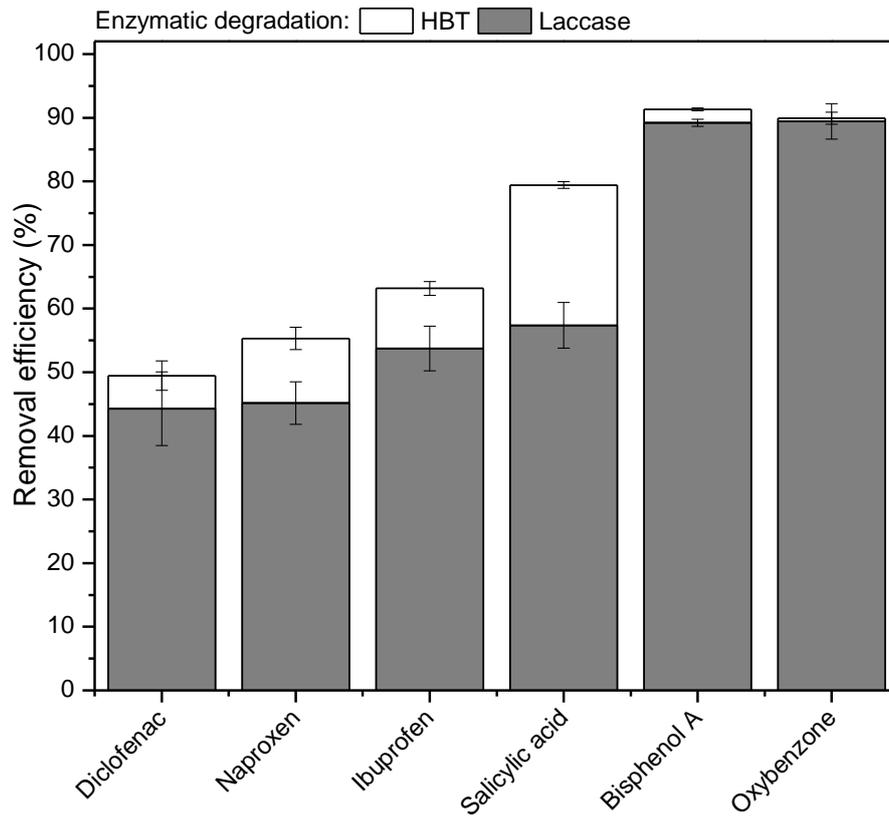


Figure 3. Improvement in the degradation of PPCPs following the addition of HBT at a concentration of 0.25 mM. Temperature of enzymatic bioreactor and distillate tank was maintained at 30 ± 0.2 and $10 \pm 0.1^\circ\text{C}$, respectively. Initial enzymatic activity and PPCP concentration was 95-100 $\mu\text{M}/\text{min}$ and 20 $\mu\text{g}/\text{L}$, respectively. Error bars represents the standard deviation between duplicate samples.

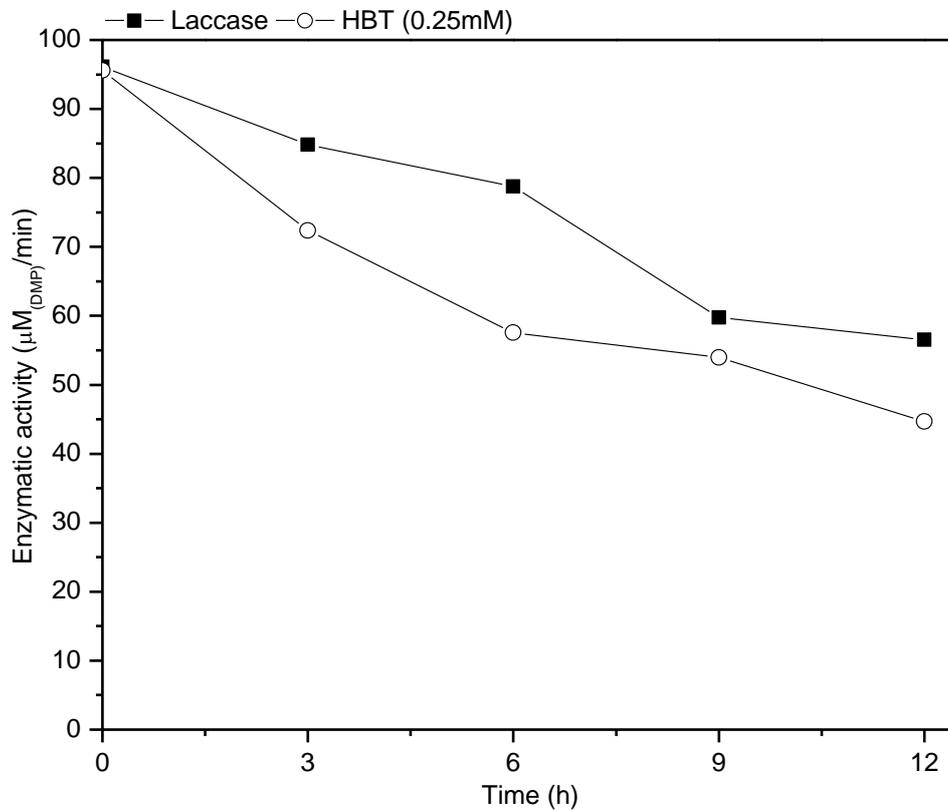


Figure 4. Inactivation of laccase with and without the addition of redox mediators (0.25 mM each). Temperature of enzymatic bioreactor and distillate tank was maintained at 30 ± 0.2 and $10 \pm 0.1^\circ\text{C}$, respectively. Initial enzymatic activity and PPCP concentration was 95-100 $\mu\text{M}/\text{min}$ and 20 $\mu\text{g}/\text{L}$, respectively.

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