Degradation of Chlortetracycline using immobilized laccase on Polyacrylonitrile-biochar composite nanofibrous membrane

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Abstract

The continuous release of antibiotic compounds through wastewater effluent into environment has raised concerns about their potential problems for different organisms. Enzymatic degradation with laccase is a green option for removal of pharmaceutical compounds from aqueous media. In this study, laccase was immobilized onto homemade Polyacrylonitrile-biochar composite nanofibrous membrane and the obtained biocatalyst was employed for removal of chlortetracycline, a widely used antibiotic, from aqueous media in continuous mode. The results showed that the immobilized laccase has improved storage, temperature and pH stability compared to free laccase. Also, it retained more than 50% of its initial activity after 7 cycles of ABTS oxidation which indicated improved enzyme reusability. Finally, while using immobilized laccase for degradation of chlortetracycline in continuous mode exhibited 58.3 %, 40.7 % and 22.6 % chlortetracycline removal efficiency at flux rates of 1, 2 and 3 mL/h.cm^2.

Keywords: Nanofibrous, Immobilization, Antibiotics, Enzyme, Degradation,

1- Introduction

In the past 70 years, antibiotics have been widely used in animal husbandry for controlling and preventing the infectious diseases. However, a considerable portion of the prescribed antibiotics is not metabolized in animal body and therefore it is released into soil, rivers, lakes and groundwater through animals' urine and feces [1, 2]. Chlortetracycline (CTC) is among the known veterinary antibiotics which is used for controlling the diseases as well as for promoting the growth rate of cattle and swine on a regular basis [3]. The presence of CTC along with other pharmaceutically active compounds (PhACs) in water bodies raised concerns among environmentalists over their potential effects on ecosystem and human health [4]. For example, it is reported that tetracycline compounds caused histological alteration in gills of fish as well as exerting a pro-oxidative activity [5]. Also, there are numerous reports on development of antibiotic resistance among bacteria against members of tetracycline family [6, 7]. Therefore, removing these compounds from water and wastewater is crucial to prevent their release into the environment and to avoid consequent ecological and health problems [8].

As conventional water treatment systems cannot efficiently remove these micropollutants from aqueous media, different physical, biological and chemical removal methods have been proposed and studied for removal of PhACs from aqueous media. However, technical, economical and environmental issues related to each approach halted the development of an acceptable strategy for removal of these compounds [9-11]. For example, production of toxic by-products in advanced oxidation processes, production of a concentrated stream in membrane separation and instability and sensitivity of biodegradation system are among the challenges that need to be addressed.

Using ligninolytic enzymes, especially laccases, that are capable of non-specifically oxidizing a broad range of organic molecules is a promising option for future strategies of wastewater treatment due to the low environmental impact of enzymatic treatment [12]. Numerous researchers reported the capability of laccase for degradation of different PhACs, such as ibuprofen, diclofenac, naproxen and sulfamethoxazole and reported degradation efficiencies up to 95% [13-15]. For example, Suda *et al.* degraded CTC with laccase and obtained 48% degradation after 4 hours of reaction [16]. In another study, Cazes *et al.* reported that laccase can degrade up to 30% of tetracycline after 24 hours [4]. In a related work, Ding *et al.* reported

>90% efficiency for degradation of CTC with laccase in presence of mediator after 3 hours of reaction [17]. However, using enzymes in their free forms has drawbacks, such as high cost of production, low stability and problems with reusability which should be addressed before scale-up [18]. Immobilization of enzyme onto a variety of solid supports and especially porous membranes, which are called enzymatic membrane reactors (EMR), is a promising approach to overcome these problems since membranes can provide high surface area for catalytic reaction [4]. The benefits of laccase immobilization on membranes, such as longer shelf life, reusability and stability against temperature and pH variations have been extensively studied in numerous research works [19, 20]. In most of the reported investigations, pure and costly laccase was employed while it is possible to use the white-rot fungi crude laccase. Furthermore, high concentrations of target compounds (>20 ppms) reported in the literature is far beyond the real concentrations in surface water and municipal wastewater. Moreover, there is scant literature on removal of micropollutants using EMRs in continuous mode. In this work, the enzymatic degradation of chlortetracycline (CTC) in continuous mode and at environmentally relevant concentration was studied. The laccase was produced by growing *Trametes versicolor* fungi and was immobilized onto an electrospun Polyacrylonitrile-biochar composite membrane. Using biochar for treatment of wastewater is of high interest due to its low cost, availability, interesting physico-chemical properties and role in value-addition to wooden residues [21]. In this work, the activated biochar was incorporated into nanofibers to create the adsorption capability in the membrane and increase the contact time between pollutants and immobilized enzyme. The properties of free and immobilized enzymes were compared and the performance of the EMR in continuous mode was investigated.

2. Materials and methods

2.1 Chemicals

CTC (purity > 97%) was purchased from Toronto Research Chemicals (TRC-Canada). 2, 2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) was purchased from Sigma-Aldrich (Oakville, Canada). Tween 80 and methanol were purchased from Fisher scientific (Ottawa, Canada). HPLC grade water was prepared in the laboratory using milli-Q/Milli-Ro system (Millipore, USA). Polyacrylonitrile (PAN), with an average weight molecular weight of 1.5×10^5 (g/mol), was obtained from Scientific Polymer Product Company (USA) and used without further purification. Biochar was donated by Pyrovac Inc. (Canada) and it was derived from pine

white wood (80%) purchased from Belle-Ripe in Princeville and the rest was spruce and fir (20%). This biochar was produced at 525±1 °C under atmospheric pressure for 2 minutes and used as obtained from the reactor outlet. Sodium hydroxide and hydrogen chloride with 98% purity and N,N'-Dimethyl-Formamide (DMF) and Dimethyl-Sulfoxide (DMSO) with 99.5% purity were supplied by Fisher Scientific (USA). HPLC grade water was prepared in the laboratory using milli-Q/Milli-RO system (Millipore, USA).

2.2 Preparation of inoculum

Fungus, *Trametes versicolor* (ATCC 20869) was grown aerobically in liquid medium by inoculating the flasks containing potato dextrose broth (PDB) using lyophilized powder and then incubating the flasks in orbital shaker at 30±1 °C and 150 rpm for 7 days. Later, 100 µl of PDB medium was inoculated in potato dextrose agar (PDA) plates at 30±1 °C for 9 days and the plates were stored at 4±1 °C, prior to use.

2.3 Solid-state fermentation

Apple pomace (Vergers Paul Jodoin Inc., Quebec, Canada) was used as solid substrate for the production of laccase by *T. versicolor*. About 40 grams of solid substrate (70% (w/w) of moisture and pH 4.5), along with 0.5% v/w Tween 80 in 500 ml flask was thoroughly mixed and autoclaved at 121±1 °C for 30 min. Later, each flask was inoculated with the biomass content of one petri plate and incubated in a static incubator at 30±1 °C for 15 days.

2.4 Enzyme extraction and assay

One gram of fermented sample was mixed with 20 ml of 50 mM sodium phosphate buffer (pH 4.5), mixed at 35±1 °C for 1 h and then centrifuged at 7,000 × g for 30 min. The relative laccase activity of collected supernatant was analyzed spectrophotometrically at pH=4.5 and 45±1 °C by monitoring the oxidation of ABTS at 420 nm (ε_{420} = 36,000 M^{-1} cm^{-1}) . Each unit of laccase was defined as the amount of required laccase to oxidize one micromole of ABTS in one minute under assay condition. For evaluation of laccase activity of immobilized enzyme, 20 mg of the sample was thrown in a 4 ml sodium phosphate buffer (pH 4.5) containing 0.5 mM ABTS and after 10 min of agitation at 45 °C, the absorbance at 420 nm was recorded. The measuring procedure was performed in triplicate and the averages were reported.

2.5 pH, temperature and storage stability

For evaluation of pH stability, 50 μL of free laccase and 20 mg of immobilized laccase onto PAN-biochar nanofibrous membrane were added to separate tubes containing 3 mL of buffers with different pH values (1.5 to 9.5) and kept for 24 h at 25 °C and 200 rpm. Then, the laccase activities of immobilized and free samples were measured (Section 2.4). For thermal stability, samples were incubated at different temperatures (20–70 °C) for 1 h at constant pH of 4.5 and then activities were measured at same temperature. The storage stability of the immobilized laccase was evaluated by keeping the samples at 4 °C and 25 °C for one month and measuring their laccase activity during one month period. Same procedure was performed for free laccase for comparison. The measuring procedure was performed in triplicates and the averages along with their standard errors are presented in figures. The ANOVA, obtained from Excel software, showed p-value<0.01 for each graph which confirmed the changes in the laccase activity are directly related to changes in studied parameters i.e. temperature, time, number of cycles and pH.

2-6- Activation of biochar

About 10 g of biochar was added to 100 ml of water containing 20 g of NaOH. The mixture was stirred with a magnetic stirrer at room temperature and 150 rpm and for 2 h and dried at 80±1 °C for 24 h. The sample was heated in a horizontal furnace under nitrogen flow of 200 ml/min. The temperature of the furnace was increased to 800±1 °C at the rate of 10 °C/min, and held for 2 h before cooling. The sample was later washed with water and neutralized with 0.1 M HCl. Finally, the sample was again washed with distilled water to remove sodium salt and dried at 60±1 °C for 24 h. The properties of activated biochar were discussed elsewhere [22].

2-7- Preparation of PAN-biochar membrane

About 2 g of PAN was dissolved in DMF/DMSO solvent mixture (9:1 v/v) at the concentration of 10 Wt% and stirred on a magnetic stirrer until a clear solution was obtained. Activated biochar at the ratios of 1.5 % (w/w) of the polymer was added to the solution and the mixture was stirred for another 48 h. PAN-biochar nanofibrous membrane was fabricated through electrospinning process under ambient conditions (T=25 °C, RH=35%) and with a rotary drum collector (length= 25 cm, diameter= 10 cm). The electric field strength, flow rate and collector rotational speed was adjusted to 1.1 KV/cm, 1.4 ml/h and 400 rpm, respectively. Also, the distance of center of collecting drum to the needle tip was 18 cm and the needle gauge was 22. The electrospinning continued for 10 h and the deposited membrane was washed with methanol for 120 min for

removal of residual solvents. Later, nanofibrous membrane sample was washed with distilled water and dried for 10 h at 50±1 °C.

2-8- Characterization of fabricated NFMs

The surface morphology of the fabricated membranes was examined using an EVO-50 (Zeiss, Germany) scanning electron microscope (SEM) at acceleration voltage of 10 kV. For this analysis, a small piece of the nanofibrous membrane was coated with a thin layer of gold using an SPI Module sputter coater.

2-9- Immobilization of laccase onto PAN-biochar membrane

Immobilization of laccase onto PAN membranes was described elsewhere in details. In brief, samples were treated with 0.5 mol/L hydroxylamine hydrochloride aqueous solution at pH 6 and 70±1 °C for 2 h. After completion of this reaction, the treated samples were rinsed with distilled water and dried at 40±1 °C. In this case, amidoxime linkage is formed on the surface of nanofibers (Figure 1). Later, the samples were immersed in 3 g/L laccase solution (acetate buffer with pH 4.5) at 4±1 °C for 12 h. Finally, the samples were washed several times with same buffer, until no laccase was detected in the drained buffer.

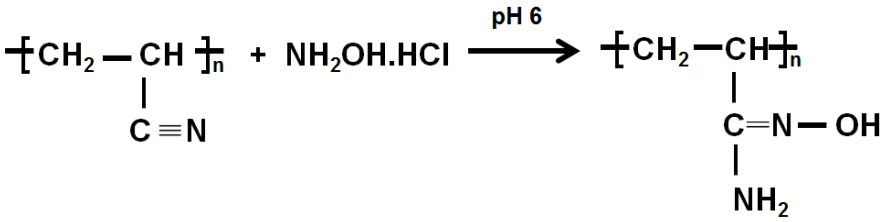

Figure 1: Formation of amidoxime linkage

2-10- Biodegradation in continuous mode

The capability of immobilized laccase on PAN membrane for degradation of micropollutants in continuous mode was studied using a 15*15 cm^2 stainless steel (SS-316) membrane test module connected to a precise syringe pump. A solution with CTC concentration of 200 ppb in milli-Q water was pumped at three different fluxes (1, 2 and 3 $ml/cm^2.h$) into the test setup in dead-end configuration. Samples for measuring CTC concentration were taken at 1 liter interval for 30 liters of total passed volume. CTC concentrations were estimated by using Laser Diode Thermal Desorption (LDTD) (Phytronix technologies, Canada) coupled with a LCQ Duo ion trap tandem

mass spectrometer (Thermo Finnigan, USA). The daughter ions identified for CTC in LDTD were 464 and 444 Da.

3- Results and discussion

3-1- Immobilization

Determination of laccase activity revealed that the immobilization of laccase onto PAN-biochar electrospun membrane using amidoxime linkage resulted in the immobilization loading of around 10.1 unit/g dry membrane under experimental conditions. Also, it was observed that for unmodified membranes, almost no laccase was immobilized. Binding onto the activated surfaces of membrane is driven by the hydrogen bonding of amine structures in the proteins toward amine and hydroxyl structures in amidoxime-modified PAN-biochar membrane. Figure 2 demonstrates the SEM pictures of PAN nanofibers with and without laccase immobilization. The surface of nanofibers before laccase immobilization is quite smooth but after immobilization, they showed a rough texture which may be due to formation of amidoxime bonds as well as attachment of enzyme macromolecules. Figure 2 also showed that activated biochar particles are entrapped into the nanofibers and increase the specific surface area of membrane to more than 12 m^2/g. These adsorbent particles can adsorb the target compounds from influent and consequently prepare enough time for degradation with laccase [21].

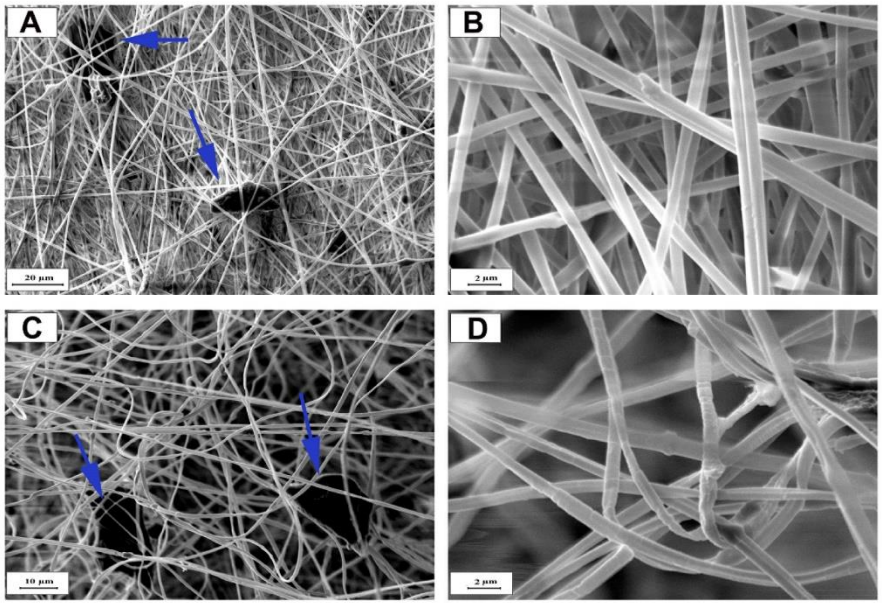

Figure 2: SEM micrographs of PAN nanofibers at different magnifications A & B: before laccase immobilization and C & D after laccase immobilization

3-1- Storage stability of immobilized laccase

Stability of the enzyme as reaction biocatalyst is very important for various biotechnological processes. However, the denaturation of enzyme and depletion of its activity is a natural phenomenon that happens in a period of time. Fortunately, immobilization of enzyme onto supports could considerably mitigate the degree of enzyme activity reduction. Generally, the immobilization restricts the freedom of macromolecules and leads to increased stability towards deactivation [23]. Figure 3 shows the residual activity of free and immobilized enzymes stored at 4±1 °C and 25±1 °C The immobilized enzymes retained more than 71% and 31% of their initial activities after one month, but the free laccase samples preserved only 37% and 2% of their initial activities. It indicated that the immobilization of laccase on PAN-biochar nanofibrous membrane enhanced the biocatalyst storage stability compared to free laccase. This enhancement is due to the fact that enzyme immobilization restricts conformational changes, which in turn prevent denaturation and increase the stability [20]. Similar results for residual activities of immobilized enzymes through amidoxime linkage after storage for 20 days at 4±1 °C were reported by Wang *et al.* (50%), Feng *et al.* (52%) and Zhang *et al.* (60%) [19, 20, 24]. In a related study, Xu *et al.* reported high storage stability of the immobilized laccase onto PAN nanofibrous membrane as it retained 92% of the initial activity after 18 days of storage at 4±1 °C, whereas the free laccase showed only 20% of initial activity [25]. Further, they investigated the storage stability of immobilized laccase on chitosan nanofibers and observed 60% residual activity after 10 days of storage at room temperature while free laccase lost most of its activity under same conditions [26]. Also, Jiang *et al.* immobilized laccase onto chitosan microspheres and observed 70% of activity retention after one month storage at 4±1 °C, while free enzyme resulted in 30% of its initial activity [27].

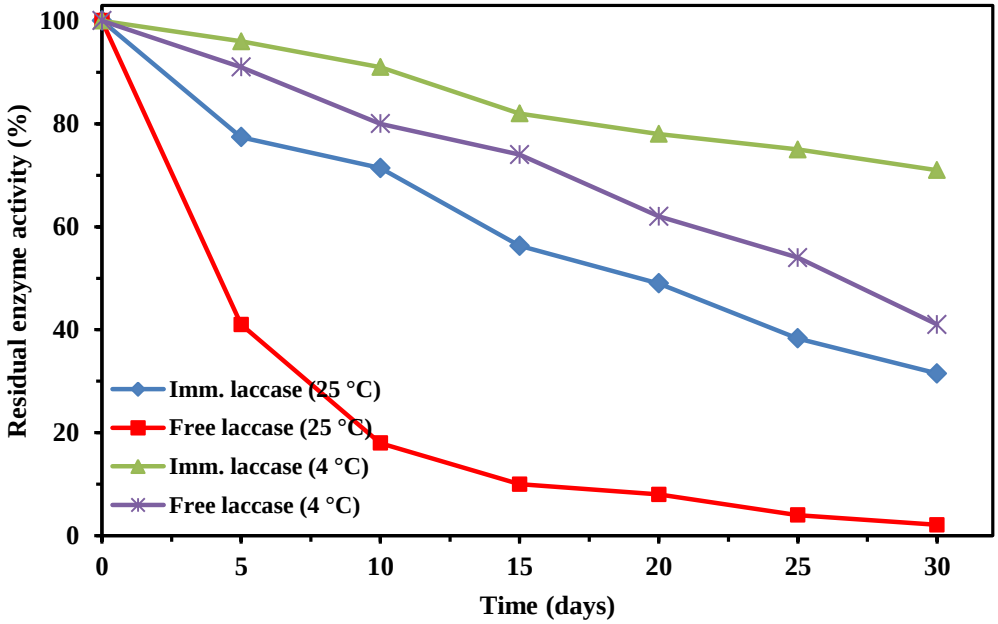

Figure 3: Storage stability of the free and immobilized laccase stored at 4±1 °C and 25±1 °C

3-2- Operational stability of immobilized laccase

Reusing laccase in free form is a big issue since it is soluble in aqueous reaction media and therefore its discharge along with effluent or product flow increases the operational cost. Unlike free laccase, immobilized laccase can be easily separated from the reaction media and reused, which considerably decreases the cost for practical application. However, still decreasing enzyme activity as a result of repeated usage is expected due to possibility of denaturation during the process. Therefore, the knowledge of operational stability of immobilized laccase is essential to evaluate its industrial exploitation. This stability parameter is estimated by reusing the same immobilized laccase sample for 7 successive cycles of ABTS oxidation and the retained activity in each run was determined. Each run had duration of 30 min and the activity of the immobilized laccase was measured in each cycle and the results are shown in Figure 4. There is a sharp reduction of activity (32.7%) from first to third run and then the activity was reduced slightly until run #7 (16.2%). Decrease in the laccase activity is expected due to denaturation or desorption of enzymes during repeated usage. Denaturation may happen due to formation of radicals during the reaction of laccase with ABTS which can block the active sites on the enzyme [28]. Zhang *et al*. used the same procedure for immobilization of laccase onto PAN nanofibrous membrane and observed around 60% activity loss after 7 cycle of ABTS oxidation [24]. Feng *et al*. modified this method by using glutaraldehyde as a crosslinking agent between amidoxime

and laccase and therefore lost only 30% of laccase activity after 7 cycles of ABTS oxidation [29]. Likewise, Xu *et al.* immobilized laccase onto PAN nanofibers through amidination process and reported that the immobilized laccase lost 30 % of the initial activity after 7 cycles [25].

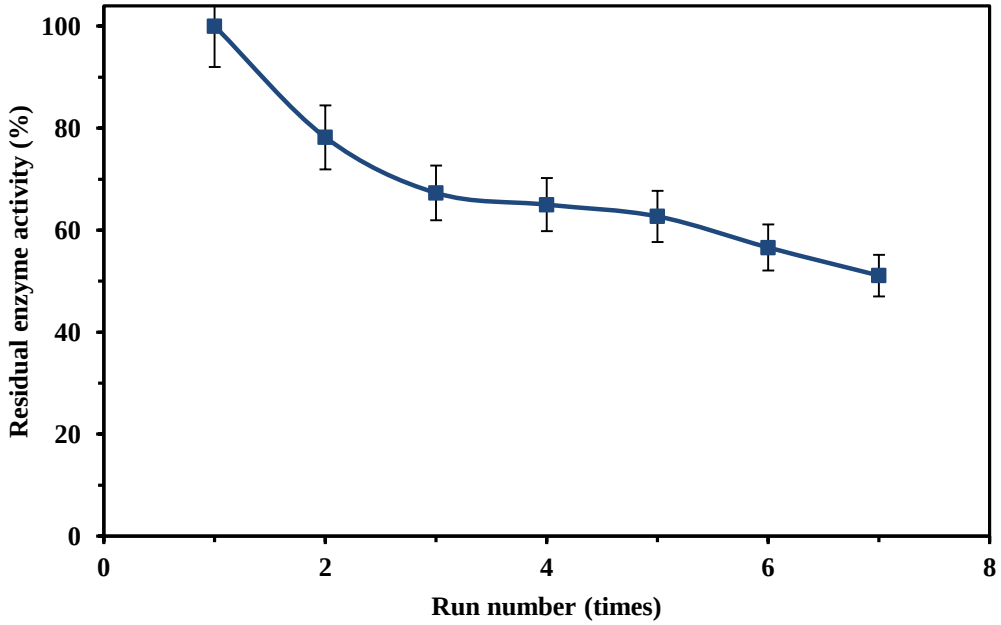

Figure 4: Operational stability of immobilized laccase

3-3- pH stability of immobilized laccase

The solution pH determines the ionization state of amino acids in enzymes which affect their 3-D structure, activity and denaturation [30, 31]. At a specific pH, the best combination between enzyme and the substrate can occur which results in a highly efficient catalytic reaction. In this work, the stability of immobilized and free laccases was evaluated in the solution pH range of 1.5 to 9.5 and the results are shown in Figure 5. Accordingly, the optimum pH value to obtain maximum activity of laccase was shifted from around 4.5 for free laccase to around 4 for immobilized laccase. The partitioning concentrations of H^+ and OH^- in microenvironment of the bulk solution and the immobilized enzyme is usually unequal due to electrostatic interactions with the matrix [27]. These interactions led to the displacement in the pH activity profile and optimum pH [32]. In this work, it seemed that biochar and amidoxime groups affected the partitioning of H^+ and OH^- and shifted the optimum pH. In a study by Wang *et al.*, it is reported that the optimum pH for maximal activity of laccase shifted from 3.5 for free laccase to 4 for immobilized laccase onto PAN membrane with metal chelation. They attributed this shift to the

influence of electrostatic interactions exerted by the carrier on the microenvironment [19]. In contrast, Xu *et al.* immobilized laccase onto PAN nanofibers through amidination process and observed no changes in the optimum pH for laccase activity. They attributed this behavior to neutral amidine bonds [25]. Similar results were reported by Catapane *et al.* who employed diazotization process for linking enzyme to PAN beads as they observed no changes in the optimum pH for maximum activity of laccase and related it to the zero surface electric charge of PAN beads [33].

Furthermore, the immobilized laccase onto PAN-biochar membrane in this work showed lower sensitivity to pH variation. This enhancement is due to the multi-point attachments between proteins and nanofibers which in turn rigidify the enzyme and protect it from deactivation [31]. Also, the effect of electrostatic charge of biochar in resisting against pH variation in enzyme microenvironment can be considered. Other researchers observed same behavior for immobilized laccase [24, 27]. For example Xu *et al.* found that immobilized laccase onto chitosan/poly(vinyl alcohol) nanofibrous membrane was less sensitive to the pH than free laccase, especially in a basic environment. It was related to the effect of the charge of the carrier since the surface of nanofiber had a large amount of free hydroxyl groups that resulted into an acidic microenvironment for immobilized laccase [26]. Also Nicolucci *et al.* found that the pH range where the enzyme activity is more than 90% of the maximum activity pH 4.0-6.2 while for the free form it is 4.6-5.4 which showed less sensitivity of immobilized laccase to the pH variations [34].

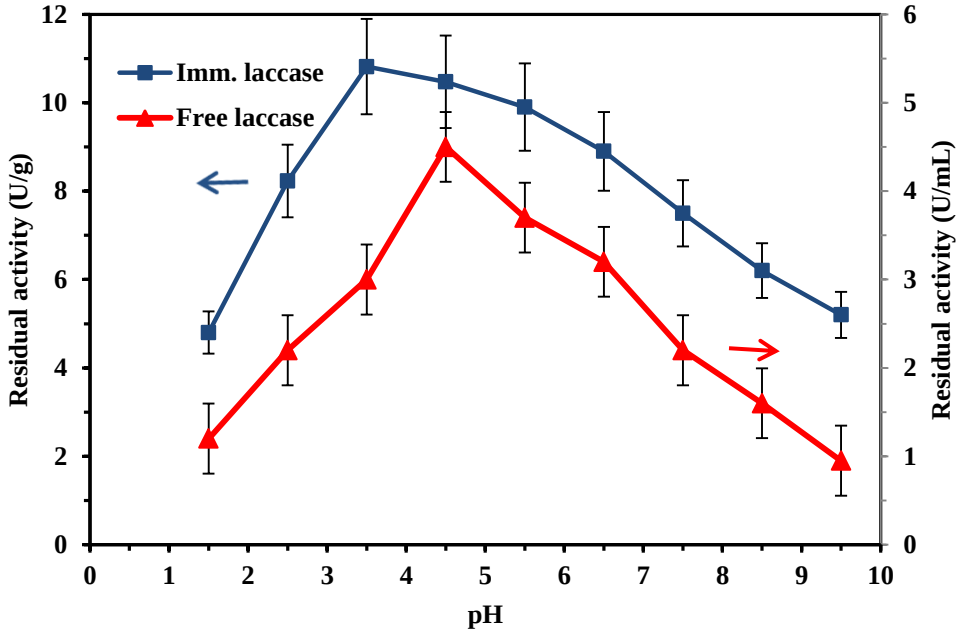

Figure 5: Effect of pH on activity of free and immobilized laccases

3-4- Thermal stability of immobilized laccase

Increasing the temperature enhanced the mobility of enzyme macromolecules which significantly affect its activity. In one hand, increasing the temperature leads to better supply of activation energy of reactions but in the other hand enhancing he mobility of macromolecule branches can increase the possibility of denaturation. Therefore an optimum temperature is expected for obtaining maximum catalytic activity. According to Figure 6, free laccase showed its maximum activity at 30 °C-40 °C while for immobilized laccase it is shifted to 40 °C-50 °C. Also the immobilized enzyme showed a slight improvement in thermal stability which is due to the bonds between enzyme and membrane [18]. The multipoint interactions between membrane and laccase can reduce the extent of conformational change at higher temperature and protected it from denaturation and shifted the optimum temperature to higher values [20]. However, the reported effects of temperature on stability and shifting optimum temperature are not in accordance with each other. For example, Jiang *et al.* reported higher sensitivity of immobilized laccase onto magnetic chitosan microspheres compared to free laccase while other researchers reported at least a slight improvement in thermal stability of immobilized laccase [19, 27, 29].

Also, Wang *et al.* and Xu *et al.* immobilized laccase on PAN and chitosan nanofibers and observed no changes in the optimum temperature [19, 25, 26] while other researchers, such as

Nicolucci *et al.* and Zhang *et al.* reported a 10±1 °C shift in optimum temperature of free laccase and immobilized laccase on PAN support [24, 33, 34]. Therefore, the behavior of immobilized laccase at different temperatures should be carefully investigated for practical applications.

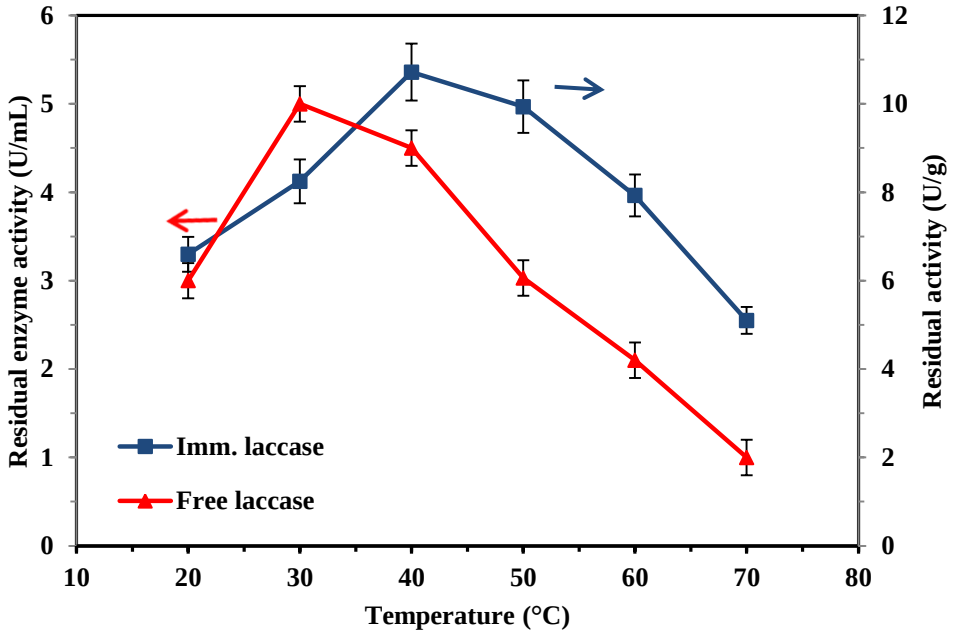

Figure 6: Effect of temperature on residual activity of free and immobilized laccases

3-5- CTC Biodegradation in continuous mode

The hydrophilicity of CTC favors its mobility in the environment and the multi-ring structure of CTC complicates its degradation [3, 21]. Laccase is a single-electron oxidoreductase known for non-specific oxidation of organic molecules. The four copper ions in laccase play important role in generation of free radicals [15]. In this research work, the immobilized laccase onto PAN-biochar nanofibrous membrane was used for degradation of CTC in continuous mode and the results are depicted in Figure 7. The concentration of CTC in feed stream was set to 200 ppb since the reported concentrations in literature ranged from 1.2 ppb in municipal wastewater to several ppm in wastewater effluent from pharmaceutical industries [35, 36]. According to Figure 7, at the flux rate of 1 mL/h.cm^2, the concentration of CTC decreased to the average of 83.25 ppb which is equivalent to 58.3 % removal. By increasing the flux rate to 2 mL/h.cm^2 and 3 mL/h.cm^2, the removal efficiency was reduced to 40.7% and 22.6%, respectively. Changing the flux rate directly affected the contact time among CTC molecules, biochar particles and enzyme biomolecules. Therefore, it is expected that by increasing the flux rate, the collision frequency

among them is decreased and as a result, the removal efficiency is decreased. Xu *et al.* took advantage of immobilized laccase onto PAN nanofibers for removal of 2,4,6- trichlorophenol from aqueous media in a batch reactor and obtained more than 40% and 90% removal efficiency in 1 and 4 h contact time, respectively [25]. Also, they used immobilized laccase onto chitosan/poly(vinyl alcohol) nanofibers for degradation of 2,4-dichlorophenol and observed 87.6% removal after 6 h of contact time [26]. In a related work, Nicolucci *et al.* used immobilized laccase on PAN beads for removal of bisphenol A, bisphenol B, bisphenol F and tetrachlorobisphenol A in a batch reactor and obtained more than 90% of removal efficiency for these compounds after 90 min of treatment [34]. They also used immobilized laccase on PAN beads for removal of octylphenol and nonylphenol and obtained more than 60% and 80% of removal efficiency for octylphenol and nonylphenol, respectively after 90 min [33]. In this work, considering the membrane thickness to be around 200 µm, the contact time (or residence time) corresponding to flux rates of 1, 2 and 3 mL/h.cm^2 were 1.2, 2.4 and 3.6 min, respectively are far lower than those reported in literature. The better performance of this system can be attributed to the presence of adsorptive particles i.e. activated biochar which adsorbed CTC and provided enough time for biodegradation with laccase. In real field applications, the reduction in residence time can result in a downsized reactor and less energy consumption for mixing. Therefore, a significant reduction in capital and operational costs may be expected [37, 38].

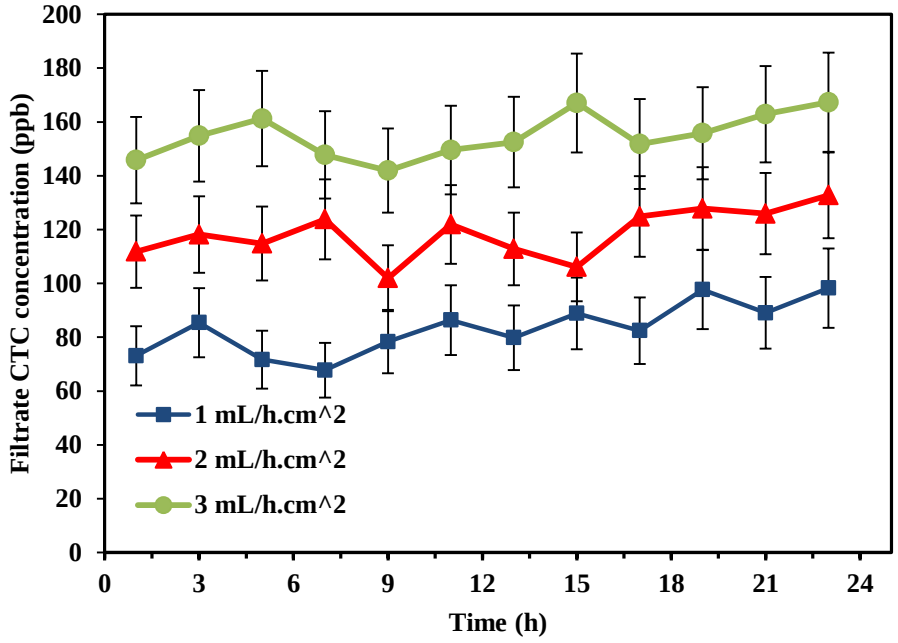

Figure 7: Degradation of chlortetracycline in continuous mode

Conclusion

An adsorptive membrane was fabricated by entrapment of activated biochar into PAN nanofiber through electrospinning process and laccase was immobilized onto this membrane through formation of amidoxime linkage. The obtained biocatalyst showed the enzyme loading of 10.1 Unit/g and it was used in a continuous mode for degradation of a widely used veterinary antibiotic, chlortetracycline. Compared to free laccase, pH, temperature and storage stability of immobilized laccase was improved. Also, it retained more than 50% of its initial activity after 7 cycles of ABTS oxidation which indicated improved reusability. Finally, using immobilized laccase for degradation of chlortetracycline in continuous mode exhibited 58.3%, 40.7% and 22.6% removal efficiency at 1, 2 and 3 mL/h.cm^2 demonstrating its potential application in wastewater treatment.

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References

1. Weng, S.-S., S.-M. Liu, and H.-T. Lai, *Application parameters of laccase–mediator systems for treatment of sulfonamide antibiotics.* Bioresource Technology, 2013. **141**: p. 152-159.
2. Hoese, A., et al., *Chlortetracycline and tylosin runoff from soils treated with antimicrobial containing manure.* Journal of Environment Science and Health, Part B, 2009. **44**(4): p. 371-378.
3. Puicharla, R., et al., *A persistent antibiotic partitioning and co-relation with metals in wastewater treatment plant—Chlortetracycline.* Journal of Environmental Chemical Engineering, 2014. **2**(3): p. 1596-1603.
4. de Cazes, M., et al., *Design and optimization of an enzymatic membrane reactor for tetracycline degradation.* Catalysis Today, 2014. **236, Part A**: p. 146-152.
5. Nunes, B., et al., *Acute Effects of Tetracycline Exposure in the Freshwater Fish Gambusia holbrooki: Antioxidant Effects, Neurotoxicity and Histological Alterations.* Archives of Environmental Contamination and Toxicology, 2015. **68**(2): p. 371-381.
6. Li, D., et al., *Antibiotic Resistance Characteristics of Environmental Bacteria from an Oxytetracycline Production Wastewater Treatment Plant and the Receiving River.* Applied and Environmental Microbiology, 2010. **76**(11): p. 3444-3451.

7. Tao, R., et al., *Detection of antibiotic resistance and tetracycline resistance genes in Enterobacteriaceae isolated from the Pearl rivers in South China.* Environmental Pollution, 2010. **158**(6): p. 2101-2109.
8. Davis, J., et al., *Antibiotic transport via runoff and soil loss.* J Environ Qual, 2006. **35**(6): p. 2250-2260.
9. Chang, P.H., et al., *Adsorption of tetracycline on 2:1 layered non-swelling clay mineral illite.* Applied Clay Science, 2012. **67-68**: p. 158-163.
10. Gómez-Pacheco, C.V., et al., *Tetracycline removal from waters by integrated technologies based on ozonation and biodegradation.* Chemical Engineering Journal, 2011. **178**(0): p. 115-121.
11. Taheran, M., et al., *Membrane processes for removal of pharmaceutically active compounds (PhACs) from water and wastewaters.* Science of The Total Environment, 2016. **547**: p. 60-77.
12. Tran, N.H., T. URASE, and O. KUSAKABE, *Biodegradation characteristics of pharmaceutical substances by whole fungal culture Trametes versicolor and its laccase.* Journal of Water and Environment Technology, 2010. **8**(2): p. 125-140.
13. Marco-Urrea, E., et al., *Degradation of the drug sodium diclofenac by Trametes versicolor pellets and identification of some intermediates by NMR.* Journal of Hazardous Materials, 2010. **176**(1–3): p. 836-842.
14. Marco-Urrea, E., et al., *Biodegradation of the analgesic naproxen by Trametes versicolor and identification of intermediates using HPLC-DAD-MS and NMR.* Bioresource Technology, 2010. **101**(7): p. 2159-2166.
15. Lloret, L., et al., *Laccase-catalyzed degradation of anti-inflammatories and estrogens.* Biochemical Engineering Journal, 2010. **51**(3): p. 124-131.
16. Suda, T., et al., *Treatment of tetracycline antibiotics by laccase in the presence of 1-hydroxybenzotriazole.* Bioresource Technology, 2012. **103**(1): p. 498-501.
17. Ding, H., et al., *Simultaneous removal and degradation characteristics of sulfonamide, tetracycline, and quinolone antibiotics by laccase-mediated oxidation coupled with soil adsorption.* Journal of Hazardous Materials, 2016. **307**: p. 350-358.
18. Naghdi, M., et al., *Immobilized laccase on oxygen functionalized nanobiochars through mineral acids treatment for removal of carbamazepine.* Science of The Total Environment.
19. Wang, Q., et al., *Laccase immobilization by chelated metal ion coordination chemistry.* Polymers, 2014. **6**(9): p. 2357-2370.
20. Feng, Q., et al., *Preparation of amidoxime polyacrylonitrile nanofibrous membranes and their applications in enzymatic membrane reactor.* Journal of Engineered Fibers and Fabrics, 2014. **9**(2): p. 146-152.
21. Taheran, M., et al., *Development of adsorptive membranes by confinement of activated biochar into electrospun nanofibers.* Beilstein Journal of Nanotechnology, 2016. **7**: p. 1556-1563.
22. Taheran, M., et al., *Adsorption study of environmentally relevant concentrations of chlortetracycline on pinewood biochar.* Science of The Total Environment, 2016. **571**: p. 772-777.
23. Leonowicz, A., J.M. Sarkar, and J.-M. Bollag, *Improvement in stability of an immobilized fungal laccase.* Applied microbiology and biotechnology, 1988. **29**(2): p. 129-135.
24. Zhang, P., et al., *Preparation of amidoxime-modified polyacrylonitrile nanofibers immobilized with laccase for dye degradation.* Fibers and Polymers, 2014. **15**(1): p. 30-34.
25. Xu, R., et al., *Laccase-polyacrylonitrile nanofibrous membrane: highly immobilized, stable, reusable, and efficacious for 2, 4, 6-Trichlorophenol removal.* ACS applied materials & interfaces, 2013. **5**(23): p. 12554-12560.
26. Xu, R., et al., *Laccase immobilization on chitosan/poly (vinyl alcohol) composite nanofibrous membranes for 2, 4-dichlorophenol removal.* Chemical engineering journal, 2013. **222**: p. 321-329.

27. Jiang, D.-S., et al., *Immobilization of Pycnoporus sanguineus laccase on magnetic chitosan microspheres.* Biochemical Engineering Journal, 2005. **25**(1): p. 15-23.
28. Purich, D.L., *Enzyme kinetics: catalysis and control: a reference of theory and best-practice methods*. 2010: Elsevier.
29. Feng, Q., et al., *Study on the Preparation of the AOPAN/MMT Composite Nanofibers and Their Application for Laccase Immobilization.* Journal of Engineered Fabrics & Fibers (JEFF), 2016. **11**(3).
30. Jordaan, J., et al., *Improved chemical and physical stability of laccase after spherezyme immobilisation.* Enzyme and Microbial Technology, 2009. **45**(6–7): p. 432-435.
31. Nguyen, L.N., et al., *Continuous adsorption and biotransformation of micropollutants by granular activated carbon-bound laccase in a packed-bed enzyme reactor.* Bioresource Technology, 2016. **210**: p. 108-116.
32. Wan, L.-S., B.-B. Ke, and Z.-K. Xu, *Electrospun nanofibrous membranes filled with carbon nanotubes for redox enzyme immobilization.* Enzyme and Microbial Technology, 2008. **42**(4): p. 332-339.
33. Catapane, M., et al., *Enzymatic removal of estrogenic activity of nonylphenol and octylphenol aqueous solutions by immobilized laccase from Trametes versicolor.* Journal of hazardous materials, 2013. **248**: p. 337-346.
34. Nicolucci, C., et al., *Biodegradation of bisphenols with immobilized laccase or tyrosinase on polyacrylonitrile beads.* Biodegradation, 2011. **22**(3): p. 673-683.
35. Kim, S., et al., *Removal of Antibiotics in Wastewater: Effect of Hydraulic and Solid Retention Times on the Fate of Tetracycline in the Activated Sludge Process.* Environmental Science & Technology, 2005. **39**(15): p. 5816-5823.
36. Hou, J., et al., *The occurrence and fate of tetracyclines in two pharmaceutical wastewater treatment plants of Northern China.* Environmental Science and Pollution Research, 2016. **23**(2): p. 1722-1731.
37. Wingren, A., M. Galbe, and G. Zacchi, *Techno-Economic Evaluation of Producing Ethanol from Softwood: Comparison of SSF and SHF and Identification of Bottlenecks.* Biotechnology Progress, 2003. **19**(4): p. 1109-1117.
38. Åkerberg, C. and G. Zacchi, *An economic evaluation of the fermentative production of lactic acid from wheat flour.* Bioresource Technology, 2000. **75**(2): p. 119-126.