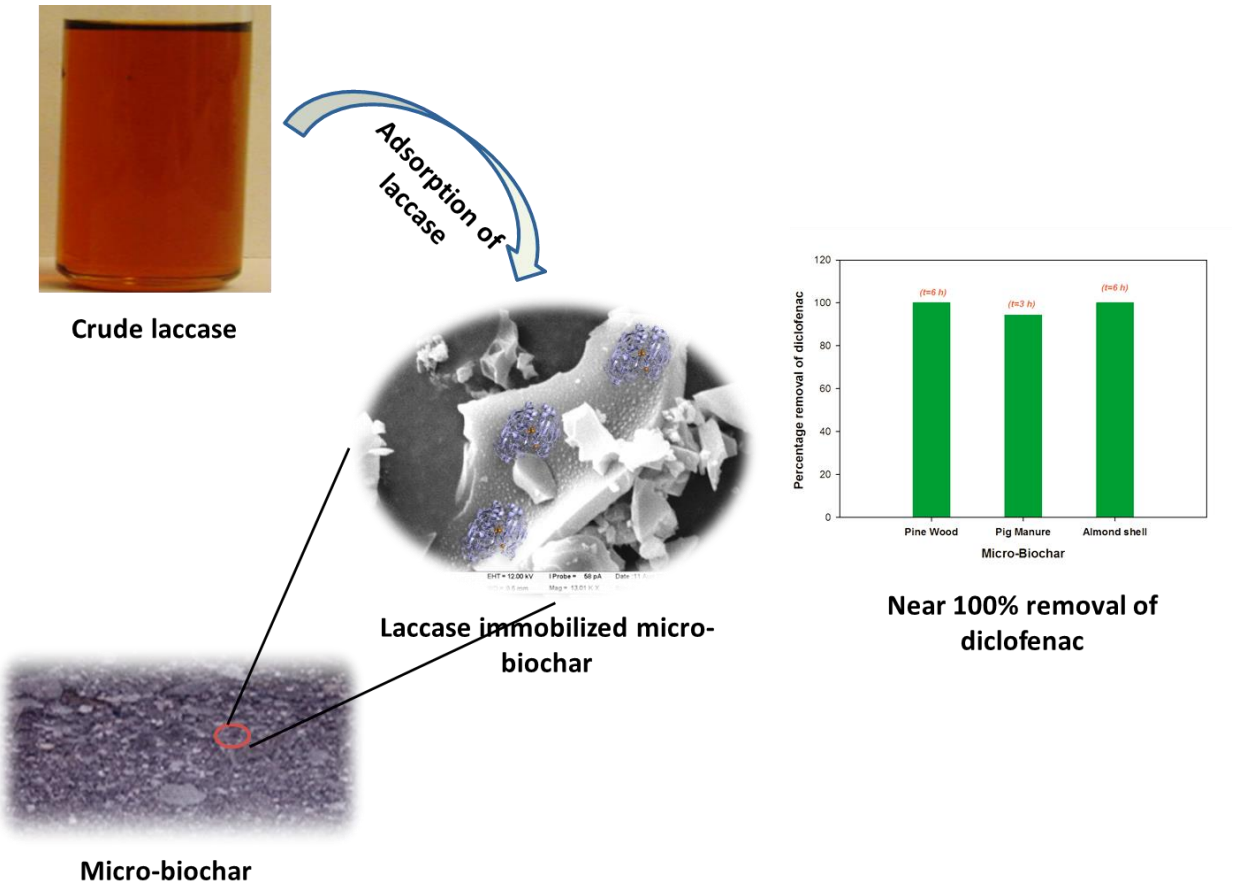
Graphical Abstract



Adsorptive immobilization of agro-industrially produced crude laccase on various micro-biochars and degradation of diclofenac

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Abstract

Although enzymes are gifted with unique and unprecedented catalytic activity and selectivity over a wide range of pollutants, still their stability related issues often hinder their application in real environmental conditions. In this study, agro-industrially produced crude laccase was concentrated using ultrafiltration. Crude laccase was immobilized on pine wood (BC-PW), pig manure (BC-PM) and almond shell (BC-AS) biochar microparticles. Immobilization of laccase was investigated at various laccase activities on micro-biochars and the release (desorption) of the enzyme has been studied. It was observed that for all the biochars, as the initial concentration of laccase increased in the crude solution, the binding capacity and as result immobilization efficiency also increased. BC-PM was found to be the most effective (31.4±3.1 Ug^{-1}) at 10 U

mL^{-1} of enzyme activity followed by BC-AS (24.3±4.8 Ug^{-1}) and BC-PW (14.58±3.3 Ug^{-1}). In addition, the biochars were functionalized with citric acid for possible surface modifications and the effect of biochars for the adsorption of enzymes has been investigated. Isotherm studies of enzyme loading onto biochar established homogeneous monolayer adsorption as the major mechanism. The desorption of laccase from all biochars followed pseudo-second-order model. Immobilized laccase exhibited superior storage ability/ shelf-life which were three times higher than free laccase. Finally, the immobilized laccase was used for the degradation of micropollutant, DCF and near 100% removal was obtained within 5 hours at an environmentally relevant concentration (500 μg L^{-1}).

Keywords: laccase; biochar; biotransformation, adsorptive immobilization; isotherms; desorption

1. Introduction

Gifted with unique unprecedented catalytic activity and selectivity over a wide range of pollutants, enzymes offer tremendous potential for environmental remediation. In particular, ligninolytic enzymes are well known for degradation of recalcitrant pollutants(Tien and Kirk, 1983), (Majeau et al., 2010; Strong and Claus, 2011). Along with manganese peroxidase and lignin peroxidase, laccases are known to be the prominent enzymes in this category due to its wide substrate range, simple requirements for catalysis, apparent stability and lack of inhibition in comparison with other enzymes (Majeau et al., 2010). However, low thermal and pH stability (narrow pH range in activity), loss of catalytic activity after one cycle and huge consumption loss while being used in water environment have remained as major obstacles in large-scale environmental applications(Jesionowski et al., 2014). Thus, past studies investigated the

47 improvement of enzyme stability and their properties for environmental applications, and
48 immobilization of enzymes on solid supports was suggested as an effective method (Cowan and
49 Fernandez-Lafuente, 2011; Mateo et al., 2007).

50 Immobilization of enzymes on solid supports is primarily based on two approaches: physical
51 binding and covalent binding between the enzyme and the supports. Physical binding (includes
52 van der Waals forces, ionic interactions, and hydrogen bonding) through adsorption is often
53 considered as the most economically viable method (Jesionowski et al., 2014). Enzyme-support
54 affinity is the most important factor in adsorptive immobilization along with a larger surface
55 area. The presence of specific active functional groups on the surface of the carrier along with a
56 large surface area of the carrier can enhance the adsorptive immobilization of enzymes on solid
57 supports. Recently, carbonaceous material, such as biochar is getting wide attention due to its
58 great potential in various domains including agricultural applications and environmental
59 remediation(Glaser et al., 2009). The possibility of production from waste materials, moderate
60 surface area and abundant presence of surface functional groups make biochar an excellent
61 adsorbent for various contaminants (Fang et al., 2014). Also, due to these dependable properties,
62 biochars can be used as immobilization support for laccases.

63 Diclofenac (DCF), being an emerging micropollutant poses toxic concerns towards several
64 aquatic as well as terrestrial organisms (Lonappan et al., 2016a; Vieno and Sillanpää, 2014).
65 Therefore, DCF must be removed from the environmental compartments, for a sustainable and
66 healthy environment and ecosystem. Previous studies have demonstrated the efficiency of
67 laccase for the degradation of DCF (Lonappan et al., 2017; Marco-Urrea et al., 2010).
68 Nevertheless, these studies were mostly focused on the production and application of free laccase
69 for the degradation of DCF and thus, incurred disadvantages of application of free enzyme

limiting their applicability at large scale and in long-term. Therefore, the possibility of immobilizing crude non-purified laccase produced from agro-industrial materials (most economically convenient production method as well as through valorization of waste materials) on micro-biochar (efficient, least expensive and valorization of waste materials) derived from different feedstocks was investigated in this study. This approach has the dual advantage of continuous adsorption as well as degradation of the contaminants (DCF in this study). Moreover, this study employed the simple adsorption technique for immobilization which can be considered as green as it avoids usage of any chemicals. Immobilization of laccase at various concentrations on various biochars and the release of the enzyme have been studied. In addition, the biochars were functionalized with organic acids for possible surface modifications, and their effect on adsorption of enzymes has been investigated. Finally, the laccase-immobilized biochars were applied for the degradation of DCF in batch mode under various experimental conditions and the results are presented. The immobilization of non-purified laccase on organically functionalized biochars (and non-functionalized) along with its potential application for the degradation of model micropollutant presents the novelty elements of this study. To the best of our knowledge, no previous studies used biochars from various origins (feedstock and method of production) for the immobilization of crude laccase. In this study, the relationship between biochar surface properties and adsorptive immobilization of enzymes has been studied. The present work demonstrates the potential application of biochar as an immobilization support and explains briefly how the surface features of biochar can affect the adsorptive properties/immobilization enzymes (laccase) and thus further enhacing the adsorptive immobilization.

2. Materials and Methods

2.1 Materials

Pinewood and almond shell biochar samples were obtained from Pyrovac Inc. (Quebec, Canada).Pinewood biochar (BC-PW) was derived from pine white wood (80% v/v) spruce and fir (20% v/v) and was produced at 525±1°C under atmospheric pressure by increasing the temperature of biomass at the rate of 25 °C min^{-1} in the presence of nitrogen. Almond shell biochar (BC-AS) was produced from almond shells slightly under atmospheric pressure at 520±1°C for 4h. Pig manure biochar (BC-PM)was a gift sample from Research and Development Institute for Agri-Environment (IRDA), Quebec, Canada. BC-PM was derived from the solid fraction of pig slurry and prepared at 400±1 °C for 2 h.

Juice industry residue, apple pomace was used as the substrate for the microorganism for enzyme production which was gifted by Vergers Paul JodoinInc., Quebec. White-rot fungus, *Trametes versicolor* (ATCC 20869) was used as the microorganism for the production of laccase.

Diclofenac sodium salt (98%; CAS 15307-79-6) was purchased from Fisher Scientific (Ottawa, ON, Canada) and internal standard (IS), diclofenac-d_4 was obtained from C/D/N Isotopes Inc. (Montreal, QC, Canada). All other chemicals used in this study (including organic acids used for the functionalization of biochars) were of analytical grade and they were obtained from Fisher Scientific and Sigma-Aldrich (Ontario, Canada).

2.2 Methods

2.2.1 Pre-treatment of biochar

All the biochars were ground and sieved to obtain biochar microparticles less than 75μm. This pre-treatment produced compact, almost uniformly sized (distributed) biochar with an increased surface area. Functionalization procedure of biochar microparticles is given in a previous

publication (Lonappan et al., 2018b). In short, the micro-biochars were shaken with 2M citric acid for 24 hours. Then, the samples were centrifuged at 3000 x g and washed twice using deionized water. Further, micro-biochar samples were dried at 60°C and used for the immobilization of laccase.

2.2.2 Preparation of laccase

The detailed procedure for the production of laccase has been presented elsewhere (Lonappan et al., 2017). In short, the fermentation substrates were adjusted to 75 ±1 % (w/w) moisture with sterile milli-Q water. Fermentation was carried out at 30 ± 1°C for 9 d in 500 mL Erlenmeyer flasks containing 20 g of apple pomace. Extraction of laccase was carried out using 50mM sodium phosphate buffer at pH 6.5 (10/1: *v/w*).

Crude laccase obtained by fermentation from apple pomace was concentrated by ultrafiltration using Sartorius stedim- SartoJet ultrafiltration system (Göttingen, Germany). Sartocon Slice Hydrosart Cassette- 10 kDa and 100 kDa were used as the filtration membranes. The harvested enzyme was filtered using 100 kDa membrane to remove the particles having size more than 100 kDa. Later, the filtrate was passed through 10 kDa and the retentate having particles of molecular weight less than 10 kDa have been discarded. This resulted in semi-purification of laccases.

2.2.3 Enzyme assay

Laccase activity was determined by measuring the rate of color generation due to the enzymatic oxidation of 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) to ABTS radical ($ABTS^{+}$) at 420 nm and pH 4.5. The extinction coefficient (ε) used was 36,000 M^{-1} cm^{-1}(Wolfenden and Willson, 1982). The assay solution contained 100 mM citrate-phosphate as

135 the buffer (pH 4.5), 0.5 mM ABTS and enzyme. Enzyme-ABTS mixture was incubated for 4min
136 and the average increase in absorbance per min was taken. One unit of laccase activity (U L^{-1}) is
137 defined as the production of 1 μM $ABTS^{+}$ per min under assay conditions (Bourbonnais and
138 Paice, 1992; Collins and Dobson, 1997).

139 **2.2.4 Immobilization of laccase on biochars**

140 Throughout the experiment, a constant solid: liquid ratio of 1: 5 (w/v) was maintained between
141 biochars and enzyme extract. Various enzyme concentrations were tested for immobilization as
142 the highest enzyme activity obtained after concentration of enzyme was 10 U mL^{-1}. Biochars
143 were mixed with crude enzyme using a vortex blender (Fisher Scientific 02215370 Vortex
144 mixer-deluxe 120V) at 1200 rpm. Initially, the mixing was carried out for 5 h and in later
145 experiments; the mixing was done for 12 h (overnight) to have maximum possible adsorption of
146 enzymes on biochars. Thus, the effect of contact time and adsorptive immobilization was
147 studied. Various enzyme concentrations of 0.5, 2.5, 5, 7.5, and 10 U mL^{-1} were studied by
148 keeping the biochar amount constant. Similar experiments were repeated with the functionalized
149 biochar obtained from pinewood, pig manure and almond shell. Citric acid was used for the
150 functionalization of micro-biochars. After immobilization experiments, enzyme- biochar mixture
151 was centrifuged at 6000 xg and the liquid portion was removed. The solid fraction was gently
152 washed using deionized water, later centrifuged and the liquid part was removed. Then, the
153 remaining enzyme in the liquid was analyzed for enzyme (activity) and hence effective enzyme
154 immobilized on biochar was calculated in U g^{-1}. Enzyme- immobilized biochar was dried at
155 room temperature/ or by using compressed air carefully without increasing the temperature
156 above 28±1°C. Moreover, 0.1 g of enzyme immobilized biochar (dried) was shaken for 15 min
157 with 10 mL of sodium phosphate buffer at pH 6.5 and the enzyme activity in the supernatant was

158 measured after centrifugation. This procedure was carried out to make sure the immobilization of
159 enzyme on biochar surface; however, the values were not used for calculation due to the
160 variations in release kinetics from different biochars.

161 Studies on release of enzymes (desorption) from biochars were carried out under shaking
162 conditions over time for two days (Fisher Scientific 02215370 Vortex mixer-deluxe 120V) at
163 1000 rpm). The stability of enzyme- immobilized biochar (shelf-life) was tested after 3 months.
164 In short, free and immobilized laccase were stored at 4± 1 °C for up to 60 days and their
165 activities were determined in 20 days interval

166 Since immobilization process was carried out using adsorption, the adsorption isotherms were
167 plotted to better understand the mechanisms behind protein (laccase) binding to micro-biochars.
168 Langmuir and Freundlich isotherms were used to study the adsorption of laccase onto biochars.
169 Equation (1) presents Langmuir isotherm model, whereas Equation (2) presents Freundlich
170 isotherm model.

$$C_e/q_e = 1/Q_0K_L + C_e/Q_0 \text{ ---------- (1)}$$

171 $$\log q_e = \log K_F + 1/n \log C_e \text{ ---------- (2)}$$

172 Where C_e (U L^{-1}) is the equilibrium concentration of the adsorbate, q_e (U g^{-1}) is the amount of
173 adsorbate adsorbed per unit mass of adsorbent, Q_o and K_L are Langmuir constants for adsorption
174 capacity and rate of adsorption, respectively. K_F is the adsorption capacity of the adsorbent and n
175 is the favorability factor of the adsorption.

176

2.2.5 Characterization of laccase immobilized biochar

Particle size distribution was measured using a "Horiba particle size analyzer"(LA-950).Mean values were taken after analyzing the samples twice. Surface morphology of micro-biochar and enzyme immobilized micro-biochar was investigated using an "EVO® 50 smart scanning electron microscope" (SEM) (Zeiss, Germany). The specific surface area of micro-biochars before and after laccase immobilization was obtained from Brunauer, Emmett and Teller (BET) N_2 adsorption isotherms at 77 K (Autosorb-1, Quantachrome, USA).Analysis of changes in surface functional groups of micro-biochars before and after laccase immobilization was carried out using Fourier transform infrared (FT-IR)spectrometer (Nicolet iS50, Thermo Scientific, USA).

2.2.6 Diclofenac degradation experiments

Laccase-immobilized biochars (most suitable sample from each micro-biochar) was tested for the degradation of DCF. The degradation experiments were carried out at room temperature and at pH 6.5 and the experiments were carried out in duplicates. About 10 mL of 500 µg L^{-1} DCF was used as the initial concentration. About 0.5g of laccase immobilized biochar was used for the experiments. Experiments were carried out for 12 h with continuous sampling under shaking conditions (Fisher Scientific 02215370 Vortex mixer-deluxe 120V) at 750 rpm. Supernatant from the samples was collected and mixed with equal amount of methanol (purity >99.8%, Fisher Scientific Ottawa, ON, Canada) to stop enzyme activity. The samples were stored at 4°C and later analyzed for DCF using LDTD-MS/MS (Lonappan et al., 2016a).

3. Results and discussion

3.1 Laccase: concentration by ultrafiltration and enzyme activity

198 The possible presence of several other biomolecules can be expected in these crude extracts since
199 extraction of the enzyme was carried out from fermented apple pomace. These
200 biomolecules/unwanted substances can include phenolic substances, various organic acids,
201 sugars and minor fractions of minerals lipids and proteins (Gassara et al., 2011; Persic et al.,
202 2017; Sato et al., 2010). Various concentrations of laccase (based on enzyme activity, U mL^{-1})
203 were selected and laccase was concentrated using ultrafiltration by 10 and 100 kDa membranes
204 following previous reports on the molecular weight of fungal laccases (60-100 kDa) (Majeau et
205 al., 2010). The filtrate after passing through each membrane was analyzed for enzyme activity to
206 measure the efficacy of the ultrafiltration. Maximum of 10 U mL^{-1} of laccase was obtained at the
207 end of concentration from an initial activity of about 0.8 U mL^{-1}.

208 **3.2 Characterization of laccase-immobilized biochar**

209 Particle size distribution of biochar microparticles was well below 75 µm as expected (data not
210 shown). SEM images of raw and enzyme immobilized biochars (samples with best enzyme
211 loading) is given as Fig.1. The porous surface texture was clearly visible, however, any
212 significant differences in the surface texture were not observed after immobilization of laccase.
213 The possible reason could be the fact that enzymes are not visible through SEM owing to their
214 small size. A similar phenomenon was observed in the SEM images reported by a previous
215 publication(Qiu et al., 2008). Fig.2 presents the FTIR spectra of micro-biochars before and after
216 laccase immobilization. Clear and distinct peaks of laccase are not visible, after immobilization.
217 This could be due to the fact that the concentration of laccase was very small to observe a peak
218 while comparing with biochar. The surface areas of micro-biochars used were 14.1 m^2 g^{-1},
219 46.1m^2 g^{-1} and17 m^2 g^{-1} for BC-PW, BC-PM, and BC-AS, respectively. After laccase
220 immobilization, the area decreased to 1.89 m^2 g^{-1}, 1.46 m^2 $g^{-1}$and 1.24 m^2 g^{-1} in the respective

order. About 86.6% decrease in surface area was observed for pinewood biochar, whereas almost 97 % of the surface of pig manure biochar was covered with the molecules which were present in the crude enzyme mixture. In the case of almond shell biochar, reduction in surface area after laccase immobilization was 92.7%. This larger decrease in surface area provided the quantitative evidence for the laccase immobilization through surface adsorption. However, this cannot be attributed solely towards laccase adsorption since other bio-molecules/impurities having a molecular size between 10-100kDa were also present in the crude extract. A similar observation in the reduction of surface area after enzyme immobilization was observed in many previous studies as well (Badgujar et al., 2013; He et al., 2006; Naghdi et al., 2017; Pirozzi et al., 2009).For example, up to 89.1% decrease in surface area was observed in an immobilization study of lipase on mesoporous silica(He et al., 2006).

3.3 Adsorptive immobilization of laccase onto micro-biochars

It is already established that reduction in particle size can increase effective surface area (Lonappan et al., 2016b) and thus the micro-biochars were prepared and used. It has also been reported that the presence of carboxylic acids on the support can enhance immobilization of enzymes (Cho and Bailey, 1979). Thus, carboxylic acids functionalized biochar (through citric acid) was tested for the improved immobilization of laccase.

Fig.3 presents laccase binding on non-functionalized biochars. Various laccase concentrations, (enzyme activities),such as 0.5, 2.5, 5, 7.5, and 10 U mL^{-1} were used for the immobilization of laccase on micro-biochars. It was observed that binding increased as the initial laccase activity increased. This observation was unanimous in all the three micro-biochars. This phenomenon can be explained based on adsorption. As the initial activity of laccase increased more laccase

molecules were available in the solution. This increased the effective interactions between micro-biochar and laccase molecules and hence better adsorption leading to higher immobilization. The previous study on the immobilization of α-amylase onto bentonite/chitosan composite has reported increased adsorption of the enzyme with increased initial concentration (Baysal et al., 2014). In addition, another study on immobilization of bovine catalase onto magnetic nanoparticle reported the same trend in loading the enzyme onto the nanoparticles. However, both studies reported that, increasing the enzyme activity beyond a particular level will not enhance the adsorption capacity. This is because once the saturation level (equilibrium of adsorption) has been reached, it is impossible to increase the loading on the support. In this study, with increased initial laccase activity, loading was also enhanced. Thus, it has to be hypothesized that the saturation was never reached for any of the three biochars used for the study. As a result, it can be assumed that increasing the activity of laccase in the crude extract will further enhance the adsorption of laccase and thus will increase the immobilization. The aim of this study was to test the loading efficiency/immobilization efficiency of crude laccase on biochars. Nevertheless, increasing the enzyme activity beyond 10 U mL^{-1} through concentration (by ultrafiltration) for the enzymes produced by agro-industrial route was not easily achievable. This was due to the comparatively lower yield of laccase by this method since a maximum of 1 U mL^{-1} of laccase was produced (Lonappan et al., 2017).

Sincenon-purified laccase has been used for the immobilization, competitive adsorption can be expected. Presence of other substances, such as polyphenols, proteins and carboxylic acids (Persic et al., 2017; Sato et al., 2010) in the crude extract can reduce the adsorption of laccase because the available sites on the biochar can be occupied by these impurities. These factors depend upon the selectivity of the molecules which have to be attached on to the surface of the

biochar. This will vary according to the surface features of each biochar. Of the three biochars used in this study, BC-PM had the largest surface area (46.1m^2 g^{-1})followed by BC-AS (17.0 m^2 g^{-1}) and BC-PW (14.1 m^2 g^{-1},). This difference in surface area has been reflected in enzyme loading and thus immobilization of enzymes on the biochar surface. BC-PM was found to be most effective (31.4±3.1 Ug^{-1}) under optimal conditions followed by BC-AS (24.3±4.8 Ug^{-1}) and BC-PW (14.58±3.3 Ug^{-1}). The effectiveness of BC-PM can also be directly correlated with their surface features which show the presence of diverse functional groups that can be potential adsorption sites (Lonappan et al., 2018a; Xu et al., 2014).

Previous studies have reported that COOH groups on immobilization supports can enhance the binding of enzymes and laccase in particular on the supports(Cho and Bailey, 1979). The citric acid treated biochars were used for the immobilization laccase and the results are presented as Fig. 4. The selective grafting of COOH groups on biochar surface by citric acid treatment has been reported in a previous study (Lonappan et al., 2018b). However, unexpectedly lowered laccase binding was observed with all the three functionalized biochars.

3.4 Loading efficiency and isotherms

Table 1 presents binding efficiency (%) of the micro-biochars at various laccase concentrations. Laccase concentrations have been presented in international units (IU) and presented for total volume used (10 mL) and 1 g of micro-biochar was used(Cabana et al., 2009). Due to the larger surface area and advanced surface characteristics in comparison with other biochars, BC-PM showed higher binding efficiency that topped at 31.4%. Maximum binding efficiency obtained from BC-AS was 24.3% and with BC-PW, a maximum binding efficiency of 14.58% was achieved. An increasing trend in binding efficiency was observed for all the biochars. For BC-

PW, the binding efficiency decreased initially at 25 U and a further increase was observed. However, inconsistencies in results were observed with BC-PW which led to 12% binding efficiency at 5U and which decreased to 6.4% at 25 U and later an increment to 8.6%, 12.4% and reached a maximum at 14.58% corresponding to 50 U, 75 U and 100 U of laccase activity , respectively. Thus, the immobilization efficiency was barely increased by 2.58% even with the 20-fold increase in effective enzyme concentration. This observation can be explained by selective and competitive adsorption and hydrophobic and electrostatic interaction between the laccase and micro-biochar(Lassen and Malmsten, 1996; Nakanishi et al., 2001). Initially, to obtain 5U enzyme activity, crude extracts were diluted using milliQ water and several fold dilutions (20 folds for 5 U) were made. Thus, this procedure could possibly eliminate the potentially competing adsorbates from the crude laccase and thus effective binding was obtained for laccase. Later, as the enzyme concentration increased, the concentration of other impurities also increased as the dilution reduced. Along with surface area and enzyme concentration, the selectivity of BC-PW for adsorbates is important. Inthis case, it has to be assumed that BC-PW had a superior affinity towards other adsorbates than the laccase molecule.

For BC-PM and BC-AS, a significant increase in binding efficiency was observed with increasing laccase concentration. This was particularly evident for BC-PM. A consistent increase in binding from 14% to 31.4% was observed from an enzyme concentration (in terms of enzyme activity) of 5U to 100U. This phenomenon can be explained based on increased adsorbate(laccase) adsorbent (BC-PM) interactions and thus resulted in higher adsorption (Baysal et al., 2014). Moreover, it must be assumed that in comparison with BC-PW and BC-AS along with an increased surface area, BC-PM exhibited superior affinity towards laccase molecules. However, an increase in binding efficiency from 6% to 24.3% was observed for BC-

311 AS, albeit this phenomenon was not consistent since irregularities in results were observed at 25
312 U.

313 Table 2 shows Langmuir and Freundlich adsorption isotherm constants for micro-biochar for
314 laccase adsorption. Langmuir isotherm model was constructed based on the assumption of
315 homogeneous monolayer adsorption onto the surface with no re-adsorption of adsorbate on the
316 surface. Therefore, for BC-PW laccase adsorption was expected to be homogeneous monolayer
317 adsorption and most likely by physical forces (physisorption) such as electrostatic interactions,
318 H-bonding and Van der Waals forces (dipole-dipole, dipole-induced dipole and London
319 (instantaneous induced dipole-induced dipole) force). However, an R^2 value of 0.92 for
320 Freundlich adsorption isotherm contradicted with this assumption. Eventhough a 1/n value of
321 1.052 (isotherm plot not shown) suggested homogeneous adsorption since adsorption becomes
322 heterogeneous when the slope 1/n equals zero (Baysal et al., 2014). Since this value is greater
323 than 1 even from Freundlich adsorption isotherm constants, homogeneous monolayer adsorption
324 can be expected. Thus, Langmuir isotherm model was suitable for BC-PW. However, for BC-
325 PM and BC-AS, Freundlich adsorption isotherm model was suitable owing to near unity R^2
326 values (0.96 and 0.98, respectively). Thus, in these two biochars, stronger binding sites on the
327 surface were occupied first and the binding strength decreased with the increasing degree of site
328 occupancy and which reduced the adsorption with time. At the same time, for both these
329 biochars, favorability factor values (1/n) were well above zero and hence instead of
330 heterogeneous adsorption, homogenous adsorption occurred through both physisorption and
331 chemisorption. Of the three biochars, high rate of adsorption (K_L) was shown by BC-AS
332 followed by BC-PM and BC-PW. Thus, theoretically and as per isotherm models, adsorption
333 loading of laccase was faster in BC-AS in comparison with other two micro-biochars.

3.5 Release kinetics (de-immobilization) of immobilized laccase

Results related to desorption been given as Fig. 5. It is evident that a complete desorption can never be expected even under vigorous shaking conditions. However, this particular experiment tried to understand the stability and binding strength of immobilized laccase as well as the nature of release (release kinetics) of various immobilization supports (micro-biochars). Almost all previous studies presented static release (leaching) of enzymes from the support. However, this study presents desorption/ release of enzymes under mild shaking conditions. It is clear that a very fast release can be expected in this manner due to continuous shaking in comparison with static conditions.

An initial slow release was observed from BC-PM, in comparison with BC-PW and BC-AS even though the initial immobilized enzyme concentration was very high (BC-PM was found to be most effective with 31.4±3.1 Ug^{-1} under optimal conditions followed by BC-AS with 24.3±4.8 Ug^{-1} and BC-PW with 14.58±3.3 Ug^{-1}). This result can be read in line with the isotherm kinetics which suggested possibly higher percentage of chemisorption than physisorption with BC-PM. Presence of metals in BC-PW (Zhang et al., 2013) can enhance the chemisorption that is adsorption by chemical bonds. Comparatively, the faster release was observed from BC-PW and BC-AS which suggested loose binding of laccase on to biochars or physisorption. Thus, a higher percentage of laccase molecules were bound by weak forces, such as Van der Waals forces and electrostatic attractive forces. Moreover, it was observed that at about 10 hours, both BC-AS and BC-PW reached desorption equilibrium despite that the data obtained for BC-PW was not consistent. However, even after 48h, desorption equilibrium was not reached for BC-PM and which suggested continuous application potential of BC-PM for longer periods. Desorption data was analyzed using kinetics plots and the results are presented in Table 3.

For BC-PM, both pseudo-first-order and pseudo-second-order release kinetics were a good fit which was observed as near unity R^2 value. However, for BC-PW and BC-AS, pseudo-second order release kinetics can be applied to explain the release kinetics. As explained previously, second-order rate constant k_2 confirmed the faster release of laccase from BC-AS and BC-PW in comparison with BC-PM.

3.6 Removal of DCF using laccase immobilized biochars

Biochar obtained with the best binding of laccase was used for the degradation studies of diclofenac. Batch mode experiments were carried out with each enzyme immobilized micro-biochar samples and the results are shown in Fig.6.

The contribution of adsorptive (Lonappan et al., 2018a), as well as enzymatic removal (Lonappan et al., 2017) over time, has already been reported for the degradation DCF for each biochar as well as for laccase. In these studies, similar experimental conditions were used. With all samples, near 100% removal was observed over time. As enzymes are immobilized onto biochars, continuous adsorption and biotransformation of DCF can be expected. It can be hypothesized that, a series of continuous adsorption of DCF onto the vacant sites (sites available after immobilization of laccase) of micro biochar and simultaneous degradation of DCF through laccase can be expected. DCF is often considered as a compound resistant to laccase degradation (Nguyen et al., 2014; Yang et al., 2013). The pooroxidative efficiency of laccase and steric hindrance between laccase and the target molecule are the major reasons for inadequate degradation of certain micro-pollutants (d'Acunzo and Galli, 2003). Simultaneous and complete removal of DCF is possibly due to the enhancement of electron transfer between laccase and DCF after adsorption on the GAC surface. Furthermore, in line with previous release kinetics

experiments (section 3.5); the immobilized laccase can be used for several cycles without loss in enzyme activity.

It will be rather difficult to make differentiation between adsorption and degradation at this point. As a result, the process has been explained as removal of DCF and which includes both adsorption and biotransformation.

3.7 Storage and stability and shelf-life of immobilized laccase

Low thermal and pH stability (narrow pH range in activity) and loss of catalytic activity of enzymes after one cycle and consumption loss while using in water environment have remained as major obstacles in large-scale environmental remediation applications(Jesionowski et al., 2014).Thus, past studies investigated the improvement of enzymes stability through immobilization on solid supports (Cowan and Fernandez-Lafuente, 2011; Jesionowski et al., 2014; Mateo et al., 2007; Sheldon and van Pelt, 2013). About 80% reduction in laccase activity (residual) was observed for free-form laccase while being stored at 4 °C over 60 days. The loss in residual activity was about 38% after 20 days and 65% after 40 days. However, higher enzyme activity stability was observed with all immobilized biochar samples at 4± 1 °C over 60 days. The loss in residual activity was less than 35% for all laccase immobilized biochar samples over 60 days. After 60 days, laccase immobilized BC-PM lost 21% activity, laccase immobilized BC-PW lost 24% residual activity and laccase immobilized BC-AS lost 32% residual activity. A similar but lower storage stability was observed for laccase immobilized on chitosan/poly(vinyl alcohol) composite nanofibrous membranes(Xu et al., 2013), however, this study employed covalent binding. Another study reported upto 98% retention in residual activity of immobilized laccase while immobilized on Eupergit supports (Lloret et al., 2012).

To the best of our knowledge, for the first time, this study presented concise and first-hand information on adsorptive immobilization of crude enzyme (laccase) on various biochars by using the principles of physical chemistry (adsorption). Moreover, this study demonstrated one potential application of laccase immobilized micro-biochar for the removal of an emerging contaminant DCF. Thus, the laccase immobilized micro-biochar can be used for several other applications including but not limited to soil remediation, wastewater treatment etc. and which further signifies the importance of this study.

Conclusions

Adsorptive immobilization of crude laccase on pine wood (BC-PW), pig manure (BC-PM) and almond shell (BC-AS) micro-biochars was investigated using the principles of physical chemistry and through isotherms. With all biochars, as the initial activity of laccase increases in the crude solution, the binding capacity also increased and thus the immobilization efficiency. BC-PM was found to be most effective (31.4±3.1 U/g) at 10 U $mL^{-1}$of enzyme activity followed by BC-AS (24.3±4.8 U/g) and BC-PW (14.58±3.3 U/g).Homogeneous monolayer adsorption was key mechanism behind enzyme binding on biochar. Immobilized laccase exhibited superior storage ability/ shelf life over free laccase and which was quantified to be more than 3 times. Immobilized laccase degraded diclofenac with near 100% and obtained within 5 hours at an environmentally relevant concentration (500 ug L^{-1}).

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Figures

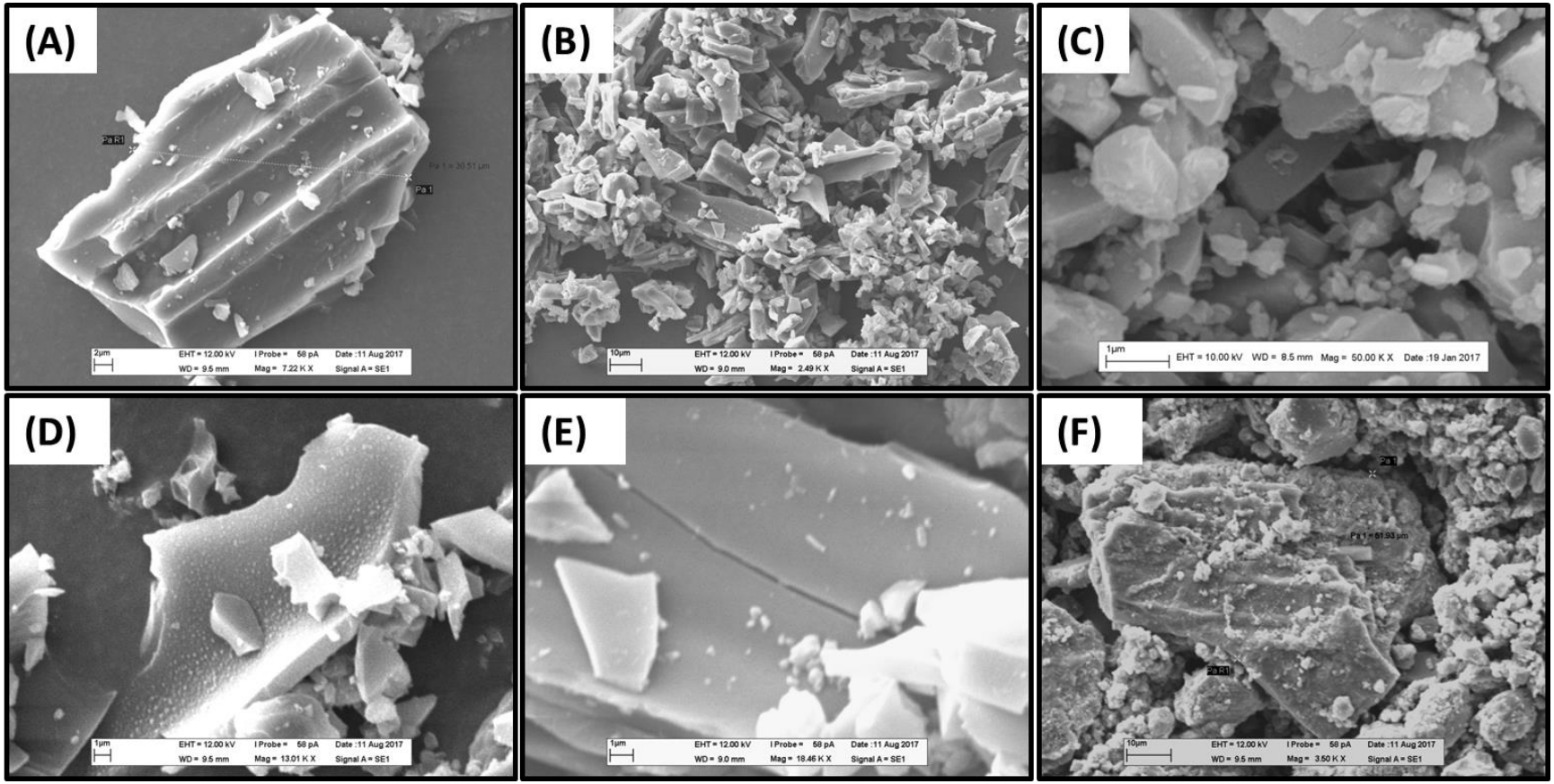

Fig. 1: SEM of micro-biochars before and after laccase immobilization (A, D: pine wood biochar before and after laccase immobilization; B, E: pig manure biochar before and after laccase immobilization; C, F: almond shell biochar before and after laccase immobilization)

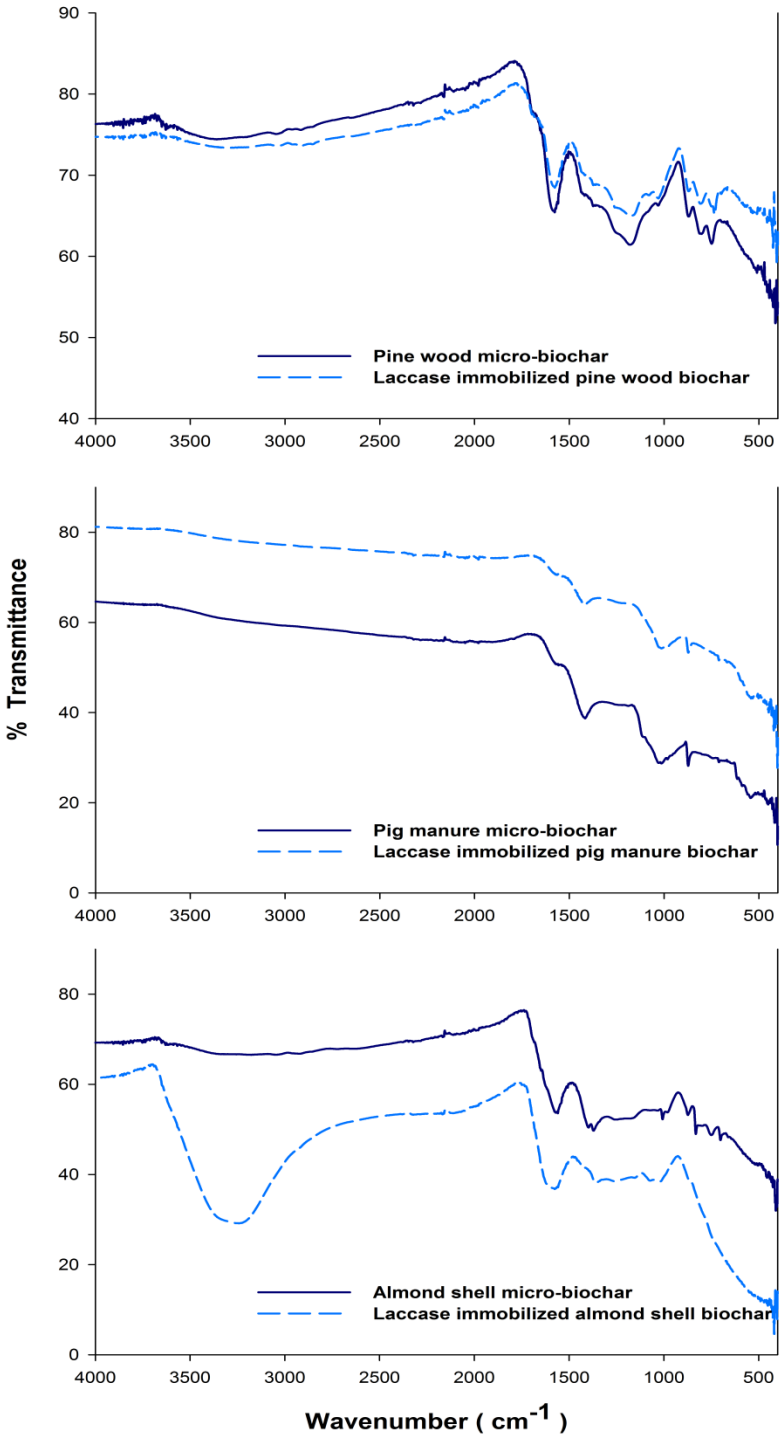

Fig. 2: FTIR spectra of micro-biochars before and after laccase immobilization

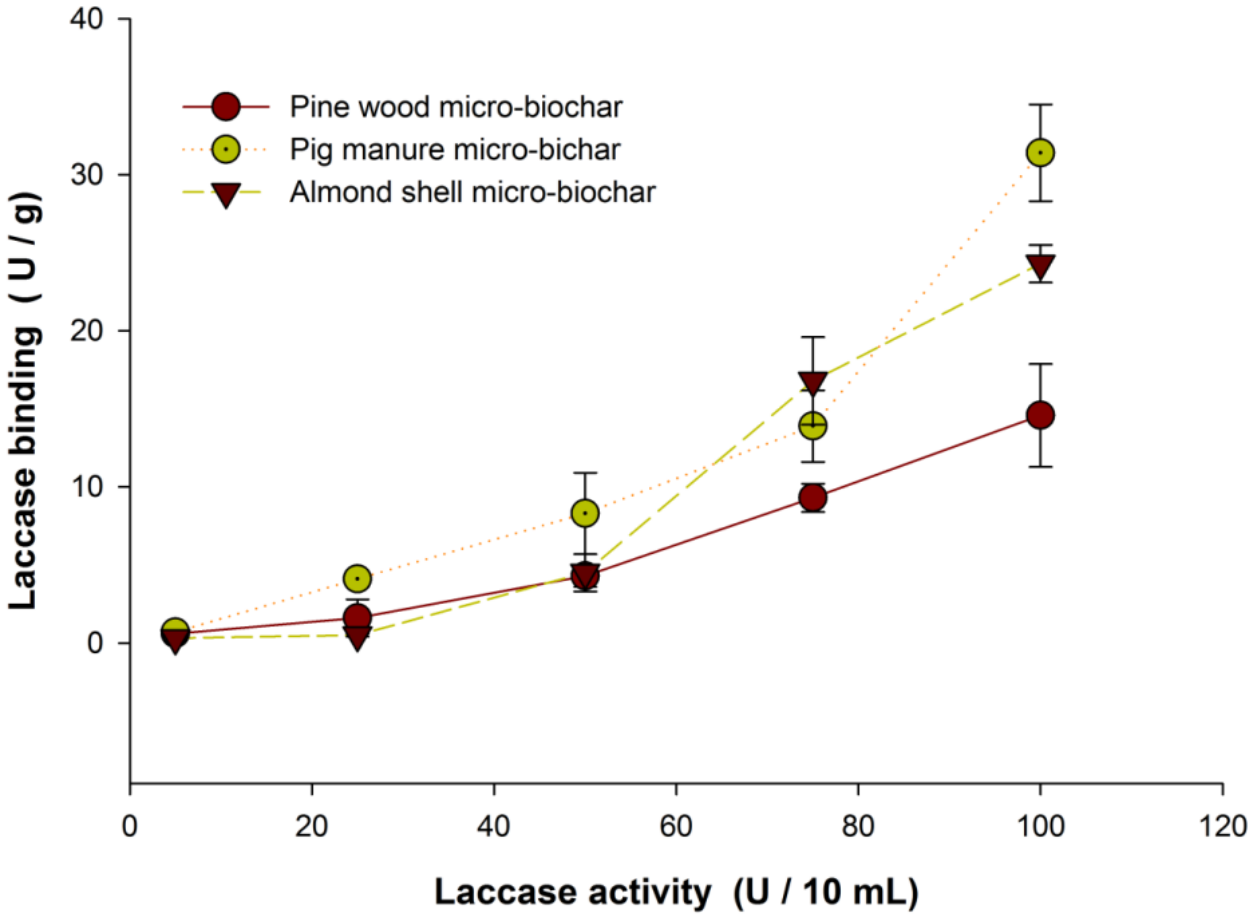

Fig. 3: Laccase loading on non-functionalized biochars at different laccase activities

**Room temperature, pH 6.5, 1200rpm*

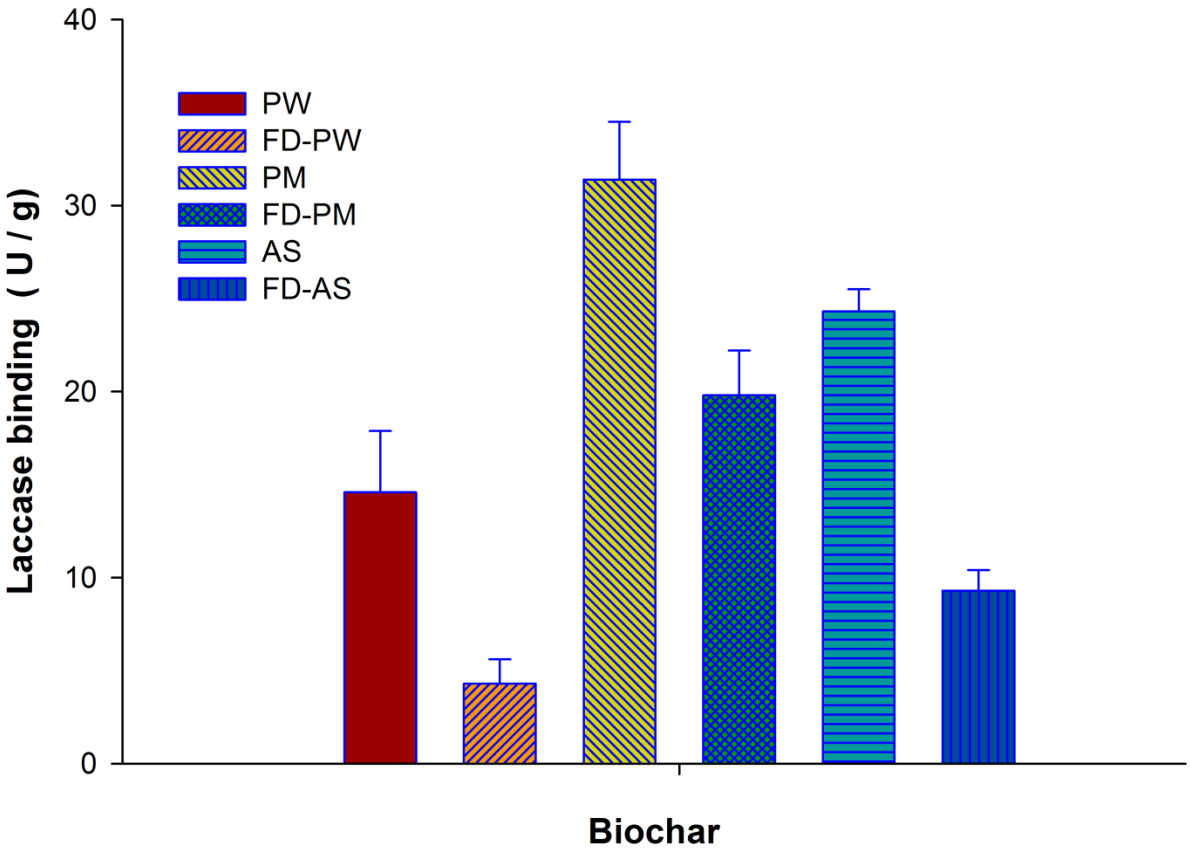

Fig. 4: Laccase loading on functionalized biochars at 10 U mL^{-1} laccase activity

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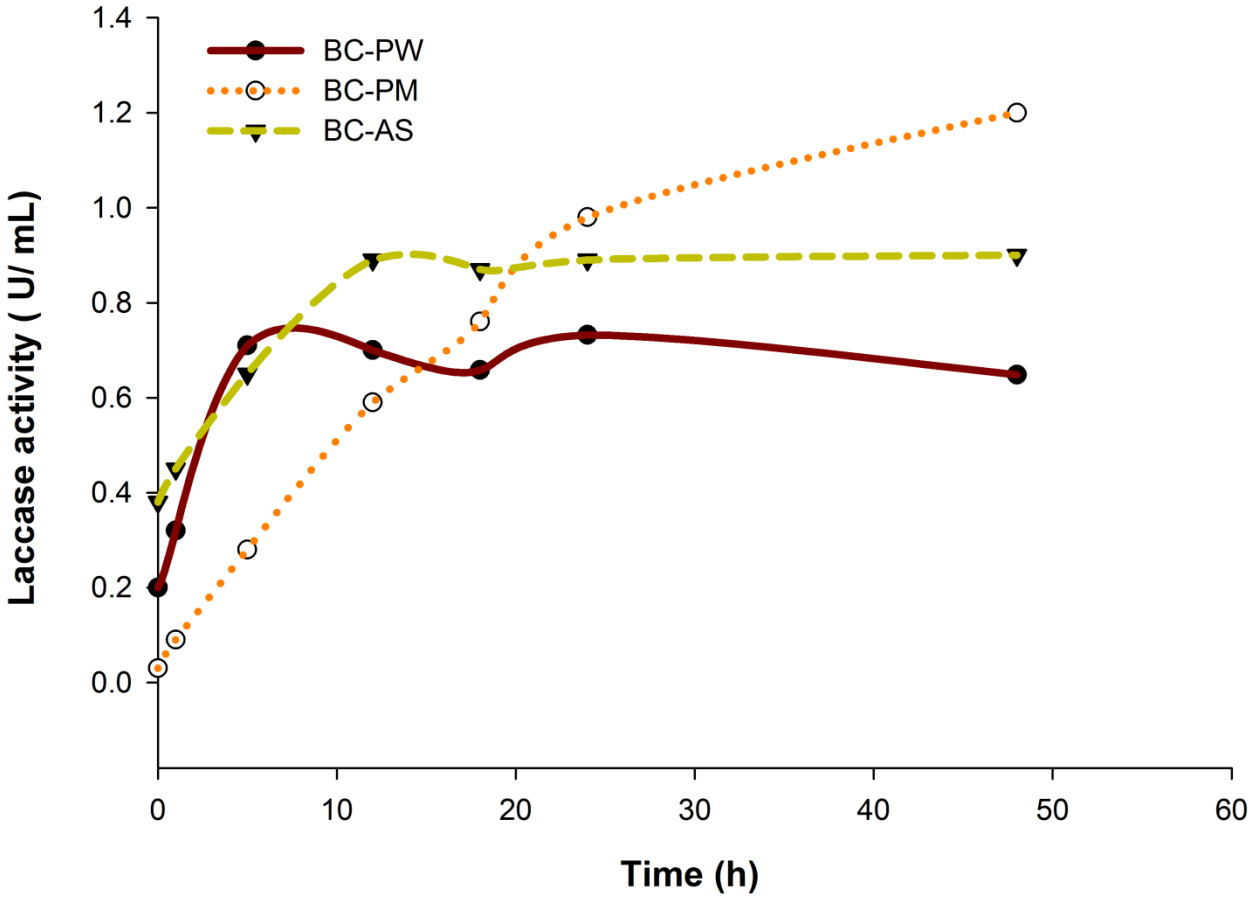

Fig. 5: Release (de-immobilization) of immobilized laccase on micro-biochars

**Room temperature, pH 6.5, 0.5 g of laccase immobilized biochar,1000rpm*

Initial laccase activities in biochar=BC-PM: 31.4±3.1Ug^{-1}, BC-AS: 24.3±4.8 Ug^{-1} , BC-PW: 14.58±3.3 Ug^{-1}

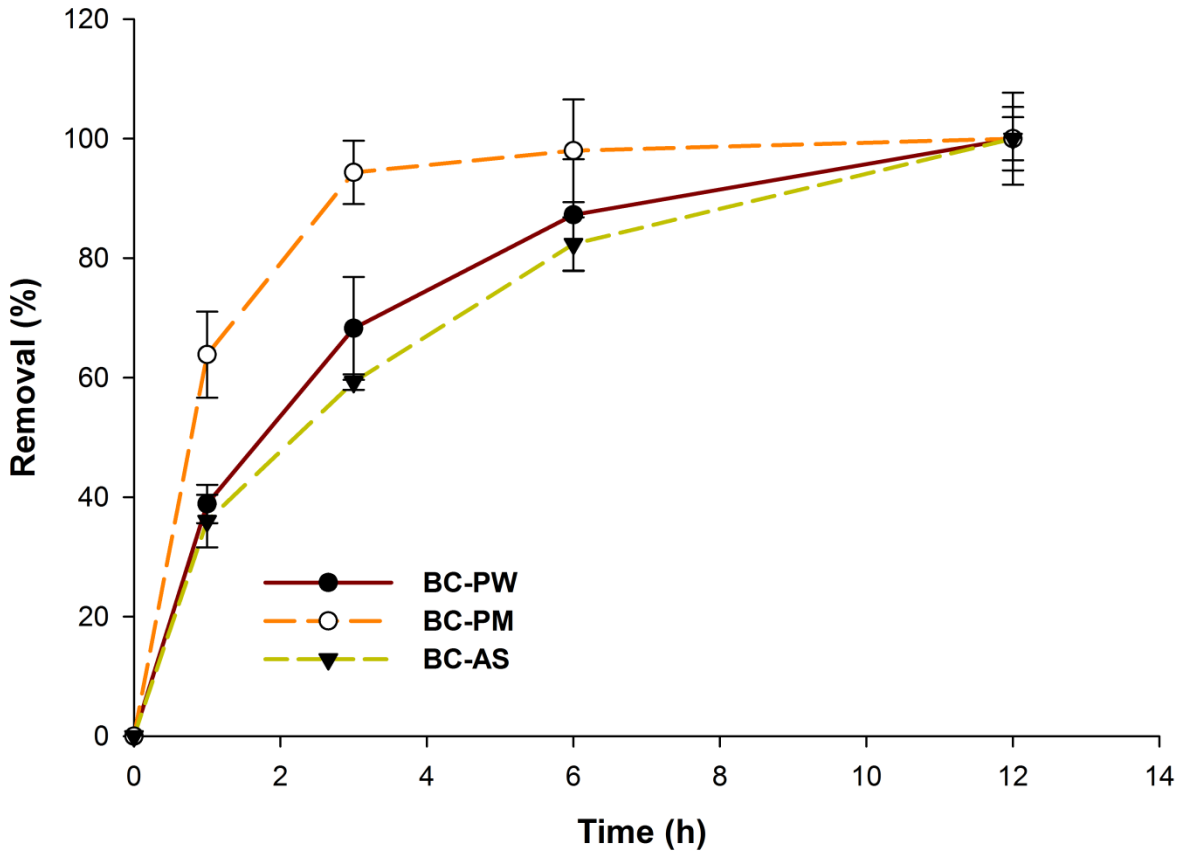

Fig. 6: Removal of DCF using enzyme immobilized micro-biochar over time

**Room temperature, pH 6.5, 500 µg L^{-1} DCF (initial), 0.5 g of laccase immobilized biochar , 750rpm*

Tables

Table 1: Immobilization efficiency of laccase at various activities on micro-biochars

Micro-biochar	Theoretical binding efficiency of laccase (%) at				
	5 U	**25 U**	**50 U**	**75 U**	**100 U**
BC-PW	12	6.4	8.6	12.4	14.58
BC-PM	14	16.4	16.6	18.53	31.4
BC-AS	6	2	9	22.4	24.3

BC-PW: pine wood biochar, BC-PM: pig manure biochar, BC-AS: almond shell biochar

Table 2: Langmuir and Freundlich adsorption isotherm constants for micro-biochar for laccase adsorption

Micro-biochar	Langmuir isotherm model			Freundlich isotherm model		
	Q_0 (U g^{-1})	K_L (LU^{-1})	R^2	K_F (U g^{-1})	n	R^2
BC-PW	6.87	8.28×10^{-5}	0.96	1288.2	0.95	0.92
BC-PM	21.4	7.26×10^{-5}	0.74	2917.4	0.80	0.96
BC-AS	2.54	1.20×10^{-4}	0.33	28543.0	0.648	0.98

BC-PW:pine wood biochar, BC-PM: pig manure biochar, BC-AS: almond shell biochar

Table 3: Desorption kinetics of laccase from biochars

Micro-biochar	Pseudo-first order model			Pseudo-second order model		
	q_e, cal. (U g^{-1})	k_1 (h^{-1})	R^2	q_e, cal. (U g^{-1})	K_2 (g $U^{-1}h^{-1}$)	R^2
BC-PW	----	-----	0.1663	16.0	1.95×10^{-4}	0.99
BC-PM	-891.2	0.00092	0.99	2500	5.42×10^{-8}	0.99
BC-AS	------	------	0.369	30.3	8.12×10^{-2}	0.99

BC-PW:pine wood biochar, BC-PM: pig manure biochar, BC-AS: almond shell biochar

q_e and q_t are desorbed (U g^{-1}) laccase from biochar at equilibrium and at time t (hour) respectively and k_1 is the rate constant of desorption (h^{-1}). k_2 (g U^{-1} h^{-1}) is the desorption rate constant of pseudo-second-order desorption.