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Pinewood Nanobiochar: a Unique Carrier for the Immobilization of Crude Laccase by Covalent Bonding

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Abstract

Nanotechnology-inspired biocatalytic systems attracted attention for many applications since nanosized supports for enzyme immobilization can improve efficiency-determining factors e.g. enhancing the surface area and loading capacity and reducing the mass transfer resistance. Among the nanomaterials, nanobiochar has unique features as a support for enzyme immobilization i.e. high surface to volume ratio, porous structure, and presence of functional groups on its surface. However, the performance of the immobilization is highly dependent on the immobilization conditions and the properties of the enzyme and the support material. In this research, crude laccase was covalently immobilized onto functionalized nanobiochar using a two-step method of diimide-activated amidation. The effect of different parameters were investigated. The optimal conditions were found to be 14 mg/mL of laccase concentration, 5 mg/mL of nanobiochar, 8.2 mM of cross-linker and 3 h of contact time. For investigating the pH, thermal, storage, and operational stability, the sample obtained from the optimized conditions was used. The results showed the higher stability of immobilized laccase against temperature and pH variation compared to free laccase. In addition, immobilized laccase maintained its catalytic performance up to seven cycles of utilization and showed more than 50% of initial activity after two months of room temperature storage.

Keywords: Nanobiochar, Laccase, Immobilization

Introduction

Application of enzymes as catalysts has gained significant attention due to their biodegradability, high selectivity and ability to operate in a wide range of pH, temperature, and salinity. Laccase (EC 1.10.3.2) is an oxidoreductase enzyme that can catalyze the oxidation of phenolic compounds with reduction of oxygen to water without requiring hydrogen peroxide as a co-substrate [1-3]. Laccase has attracted attention for different applications, from delignification of pulp to remediation of water and soil because of its capability of catalyzing the oxidation of various compounds [1, 4, 5]. It has the immense potential for industrial processes since it requires only air as a co-substrate and releases water as a by-product so that it can be classified as a green catalyst [6].

However, utilization of free enzymes in industrial processes has encountered some limitations including non-reusability, poor stability, inactivation by inhibitors and the high cost of isolation and purification [2, 4]. In contrast, immobilized enzymes exhibited advantages, such as stability against pH and thermal variations and easy separation of the enzyme from reaction medium [5]. Selection of suitable support for enzyme immobilization is very important since it affects enzyme loading, operational stability, and cost of the process [1, 4]. A varied spectrum of materials has been employed for the immobilization of enzymes as well as whole cell microorganisms and among them, carbonaceous materials showed superior textural properties and higher water stability [7, 8]. For example, charcoal has attracted much attention for enzyme immobilization due to its application in many areas, such as biotechnology, medicine, biology, and food processing [9].

Conversion of the agro-forestry residues by thermochemical and biological conversion for compost and biofuel production is a potential strategy for conserving natural resources, saving costs, and production of added-value products [10]. Pyrolysis of agro-forestry residual biomass is considered as a promising strategy for value-addition of these residues. In this treatment, residues are heated in the absence of oxygen to produce synthesis gas, bio-oil, and biochar [11]. The latter has a large surface area, enriched surface functional groups, porous structure, slow biological decay and moderate content of essential elements [12]. Biochar showed the excellent capability to remove contaminants, such as organic pollutants and heavy metals from aqueous solutions [12]. Soil amendment, nutrients retention, and bioremediation of contaminated soils

and water are among applications of biochar [13]. Recently, biochar has been employed by numerous researchers in a new application i.e. as a support for enzyme immobilization [14]. Using biochar for such a purpose can increase the product value and improve the cost to benefit ratio of the enzyme immobilization. Cea *et al.* evaluated the capability of biochar samples obtained from pyrolysis of oats husk at 300 °C (BCA 300) and 450 °C (BCA 450) and pretreated or not with 99% ethanol for immobilization of lipase. They found that the treatment with ethanol had no effect on biochar prepared at 300 °C, but the immobilized enzyme onto the treated biochar prepared at 450 °C showed 22.4 % more activity compared to untreated sample [14]. Davis and Burns covalently immobilized laccase onto activated carbon using four different derivatization methods. The highest immobilized activity was obtained using coupling of diimide to carboxyl groups in laccase. The immobilized laccase showed improved stability against pH and temperature variations [15]. Similar behavior in the enhancement of stability of the enzyme was observed in the research of Bezerra *et al.* who activated fibers obtained from green coconut husk with glyoxyl or glutaraldehyde to immobilize laccase. The thermal stability was higher with increments of 6.8-fold (with glutaraldehyde) up to 16.5-fold (with glyoxyl) compared to the free enzyme [16]. In a similar study, Cristovao *et al.* covalently immobilized commercial laccase on green coconut fiber activated with 3-glycidoxy propyl trimethoxysilane, which led to improved thermal and operational stabilities of the enzyme, but the biocatalyst showed a lower activity and affinity [4]. Modification of different agro-forestry residues for immobilization of enzyme is reported in several studies. However, there is significant knowledge gap on the effect of parameters including the reaction time, enzyme concentration, coupling reagent concentration, on the activity, stability, and recyclability of the biocatalyst.

In this work, nanobiochar obtained from residues of pinewood was modified and used to immobilize laccase through covalent bonding. To the best of our knowledge, this is the first effort for modification of pinewood-derived biochar nanoparticles for covalent immobilization of laccase. Biochar use as support and apple pomace as a substrate for enzyme production provides a waste management option for protecting the environment. The effect of important parameters on immobilization of laccase onto functionalized nanobiochar (FNBC) including FNBC dosage (mg/mL), the concentration of the coupling reagent (mM), and laccase concentration in solution (mg/mL) were investigated and optimized since the optimization is critical for application of this nanobiocatalyst in the wastewater or water treatment plants. Additionally, pH, temperature and

storage stability of free and immobilized laccase, as well as the reusability of immobilized laccase, were investigated.

2. Material and methods

2.1. Material

Pinewood biochar was supplied by Pyrovac Inc. (Quebec, Canada). This biochar was derived from pine white wood (80% w/w, size: 3 mm) and the rest 20% w/w was fir and spruce. The carbonization process was performed in the presence of nitrogen under atmospheric pressure at 525±1 °C by increasing the temperature at the rate of 25 °C/min for 20 min. 2, 2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) (ABTS), 2-(N-Morpholino) ethanesulfonic acid (MES), N-hydroxysuccinimide (NHS), N-ethyl-N'-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDAC) were purchased from Sigma-Aldrich (Oakville, Canada). Tween-80, sulfuric acid and nitric acid were purchased from Fisher Scientific (Ottawa, Canada). Apple pomace, provided by Vergers Paul Jodoin Inc. (Quebec, Canada), was used as substrate for *Trametes versicolor* (ATCC 20869) for laccase production. Ultrapure water was produced in the laboratory using Milli-Q/Milli-Ro Millipore system (Massachusetts, USA).

2.2. Nanobiochar production and functionalization

Nanobiochar with the average size of 60±20 nm and specific surface area of 47.3 m^2/g was produced using a planetary ball mill (PM100; Retsch Corporation) at ambient conditions. Briefly, 10 g of pinewood biochar was preconditioned at -80 °C for 24 h and then ball milling was carried out at 575 rpm for 100 min using stainless steel balls of 2.4 mm in diameter (800 balls with total weight of 45 g) in a 500 mL stainless steel jar. The physicochemical properties of produced nanobiochar are described elsewhere [17]. For functionalization of nanobiochar through acidic treatment, the procedure of Naghdi *et al.* was employed with some modification [18]. About 4 g of produced nanobiochar was dispersed in 500 mL of H_2SO_4/HNO_3 mixture (5 M, 3:1 V/V) and mixed at 200 rpm and room temperature for 48 h. Subsequently, the functionalized nanobiochar (FNBC) suspension was washed several times with milli-Q water to remove residual acids and to reach pH 7. The treated nanobiochar was then freeze-dried and stored at room temperature as a dry powder.

2.3 Laccase production and extraction

About 40 grams of apple pomace (pH 4.5, 78% (w/w) moisture), was mixed with Tween 80 at 0.5% v/w in several 500 mL Erlenmeyer flasks and autoclaved at 121±1 °C for 20 min. Then, the substrate was inoculated with *Trametes versicolor* strain and kept at 30±1 °C in a static incubator for 15 days. For extraction of enzyme, each gram of fermented apple pomace was mixed with 20 mL of 50 mM sodium phosphate buffer (pH 6.5). The mixture was agitated on a shaker at 150 rpm and 35±1 °C for 1 h and then the mixture was centrifuged for 30 min at 7000 ×g. The collected supernatant was dried at -55 °C, 5 Pa, for 48 h using freeze dryer (FD-1000, Eyela, Japan).

2.4 Covalent immobilization of laccase

Central composite design (CCD) and response surface methodology (RSM) were used to study the effects of FNBC concentration, enzyme concentration and EDAC concentration on the activity of immobilized laccase, which was considered as the dependent variable. RSM was employed for optimization of enzymatic activity as this method is widely used for bioprocess optimization, studying parameters interaction and building mathematical models [19]. Independent parameters and their levels are listed in Table 1. Design-Expert®-7 software (Stat-Ease Inc., Minneapolis, USA) was employed to create the experimental array composed of 20 experiments with 6 replicates in the center. The details of proposed experiments by software are listed in Table 2. Laccase was chemically attached to FNBC through diimide-activated amidation in two-steps. In the first step, different concentrations of FNBC (see Table 2) was prepared in MES buffer (50 mM, pH 6.2) and an equal volume of 400 mM NHS (prepared in MES buffer (50 mM, pH 6.2) was added to the solution and the mixture was sonicated for 30 min in an ultrasonication bath. Also, different concentrations of EDAC (see Table 2) was prepared in MES buffer (50 mM, pH 6.2) and then they were added to initiate the linking of NHS to the carboxylic groups on the FNBC and the mixture was sonicated for 2 h. Then, the FNBC mixture was centrifuged and rinsed thoroughly with MES buffer to remove excess EDC and NHS. In the second step, the activated FNBC was transferred to a solution of laccase in 10 mM phosphate buffer, pH 8.0 (see Table 2) and sonicated for 1 min to re-disperse the FNBC. The mixture was incubated at 200 rpm and at room temperature for 3 h. The immobilization time was optimized by performing a set of experiments at different contact times (1, 2, 3, 4, 5, 6, 12, 18, 24, 36, 48 and 72 h). The FNBC-laccase suspension was centrifuged and washed several times with ultrapure water to remove any unbound enzyme and freeze-dried at -55 °C, 5 Pa, for 48 h. A

control experiment was performed using an identical procedure except using EDC and NHS. The activity of immobilized laccase on FNBC was measured through the method explained in 2.7.

2.5 Data analysis

The obtained experimental data were analyzed through RSM and fitted into a second-order polynomial model. The following function (Equation 1) was employed in the analysis of response surface to correlate the independent and dependent factors.

$$Y = \beta_0 + \sum_{i=1} \beta_i X_i + \sum \beta_{ii} X_i^2 + \sum_{i=1} \sum_{j=i+1} \beta_{ij} X_i X_j \qquad (1)$$

Where: Y, β_0, X_i (or X_j). β_i, β_{ii}, and β_{ij} are the predicted responses, second-order constant, independent variables, the linear coefficient of regression, the quadratic coefficient of regression, and interaction coefficient of regression between every two independent variables, respectively.

Table 1: Independent variables used for optimization of covalent immobilization of laccase onto functionalized nanobiochar

Independent factor	**Units**	**Coded levels**				
Levels		-2	-1	0	+1	+2
FNBC	(mg/mL)	2	3	4	5	6
Laccase	(mg/mL)	2	6	10	14	18
EDAC	mM	5.7	6.7	7.5	8.2	8.9

Table 2: Variable parameters and their level in designed experiments

No	**FNBC (mg/mL)**	**Enzyme (mg/mL)**	**EDAC (mM)**	**Laccase activity (U/g)**
1	3	6	6.7	1.13
2	5	6	6.7	1.66
3	3	14	6.7	1.35
4	5	14	6.7	2.54
5	3	6	8.2	2.96
6	5	6	8.2	3.42
7	3	14	8.2	4.10
8	5	14	8.2	4.95
9	2	10	7.5	0.97
10	6	10	7.5	4.43
11	4	2	7.5	0.71
12	4	18	7.5	4.39
13	4	10	5.7	1.03
14	4	10	8.9	3.23
15 (C)	4	10	7.5	3.59

16 (C)	4	10	7.5	3.57
17 (C)	4	10	7.5	3.59
18 (C)	4	10	7.5	3.57
19 (C)	4	10	7.5	3.59
20 (C)	4	10	7.5	3.57

2.6 Stability of immobilized laccase

The effect of pH on the stability of immobilized laccase at optimum conditions (5 mg/mL of functionalized nanobiochar, 14 mg/mL of laccase and 8.2 mM of EDC) was investigated by incubating immobilized and free laccase in buffer solutions over a pH range of 3 to 10 at 200 rpm and 25 ºC. Briefly, 10 mg of immobilized laccase and 50 µL of free laccase (with an initial laccase activity of 1.2 U/mL) were added to separate tubes containing 2 mL of respective buffers. After 8 h of incubation, the residual laccase activity for free laccase was measured. For immobilized laccase, all samples were incubated for 8 h and centrifuged for 20 min at 11, 000 × g before activity measurement. The thermal stability was assessed by incubating free and immobilized laccase at different temperatures (20-70 °C) for 8 h and measuring the residual activity, in the same way, explained for pH stability. For evaluating the storage stability of free and immobilized laccase, samples were stored at room temperature for up to 30 days and their activity was measured at intervals. For evaluation of the operational stability, about 50 mg of immobilized laccase on FNBC was dispersed in 1 mL of citrate-phosphate buffer (pH 4) containing 1.5 mM ABTS and incubated at room temperature and 200 rpm for 10 min. Then, the sample was centrifuged for 10 min at 11,000 × g and the concentration of transformed ABTS in the supernatant was measured. The immobilized laccase on FNBC was washed with Milli-Q water, decanted and the procedure was repeated for 7 cycles.

2.7 Analytical methods

2.7.1 Enzyme assay

Oxidation of ABTS was used to determine the laccase activity by spectrophotometry. About 50 µL of enzyme sample was mixed with 500 µL of 1.5 mM ABTS and 2.450 mL of 50 mM citrate-phosphate buffer (pH 3.5). Oxidation of ABTS was monitored by an increase in the absorbance at 420 nm (ε_{420} = 36,000 M^{-1} cm^{-1}) [20] using a Cary 50 UV-visible spectrophotometer (Varian, Australia). One unit of laccase activity was defined as the amount of required enzyme for oxidizing one µmol of ABTS per min under the assay conditions. For immobilized laccase, 10 mg of sample was mixed with one mL of 1.5 mM ABTS and 2 mL of citrate phosphate buffer

(pH 3.5). After 10 min of incubation at 45 °C, the sample was centrifuged for 10 min at 11, 000 × g and the absorbance at 420 nm was recorded. The final activity of immobilized laccase onto FNBC was expressed in U/g nanobiochar.

2.7.2 Fourier transform infrared (FT-IR) spectroscopy

FT-IR spectra were recorded in attenuated total reflectance (ATR) mode with 4 cm^{-1} resolution in the range of 400-4000 cm^{-1} using a Nicole IS50 FT-IR Spectrometer (Thermo Scientific, USA). Briefly, the sample was placed on the diamond crystal and consistent contact between the crystal and the sample was achieved with the gripper plate. The measurement was taken 16 times for each spectrum and their average was used for plotting.

3. Results and discussions

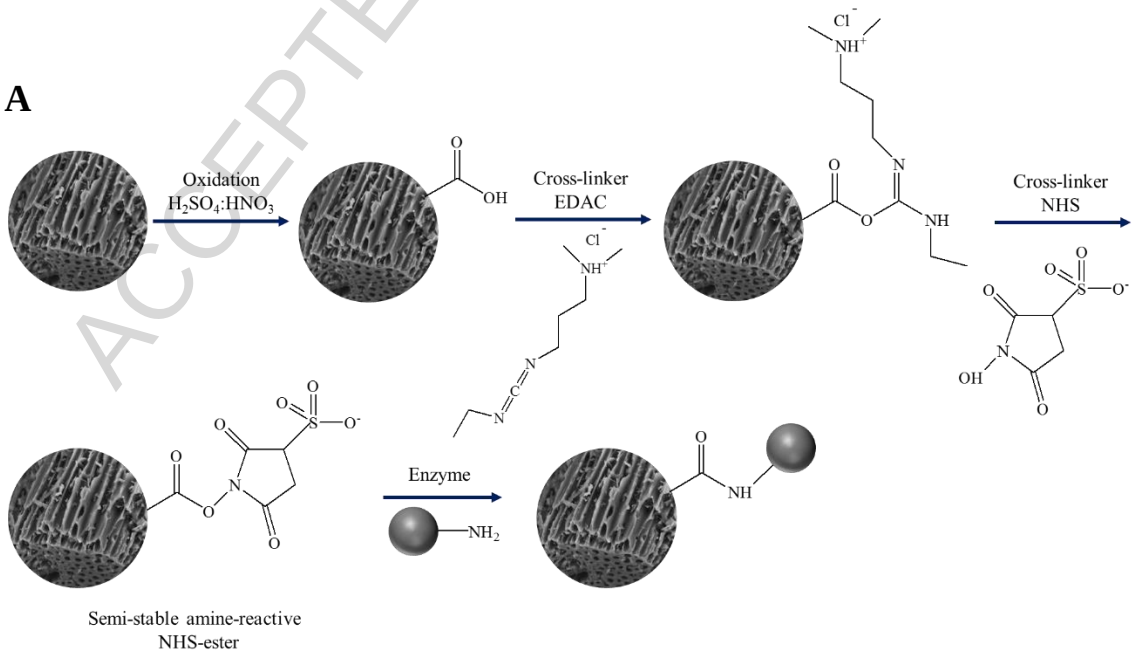
3.1. Covalent immobilization of laccase onto FNBC

Covalent and non-covalent bonding have been reported for the immobilization of various types of enzymes [21]. Non-covalent bonding retains the unique features of both supports and enzymes material, but the enzyme is lost during the repeated usage of the support-enzyme system [22]. Covalent bonding provides durable attachment between enzyme and support, but it can significantly disrupt the enzyme structure. For efficient immobilization, chemical modifications of support are required to create reactive groups, such as carboxylic groups for covalent bonding [23, 24]. The carboxylic groups on the nanobiochar surface provide anchoring points for the covalent attachment of enzyme using EDAC cross-linker. This method was employed by many researchers in recent years for functionalization of different support materials [25-27]. The results of laccase immobilization in this work indicated that the activity of covalently immobilized laccase onto FNBC using EDAC was 16 times more than the immobilized laccase onto nanobiochar without acid treatment and without using EDAC and NHS as cross-linker (4.95 U/g compared to 0.31 U/g). Also, it was 4.8 times more than immobilized laccase onto FNBC without using EDC and NHS (noncovalent interactions). Lee *et al.* reported that the activity of the immobilized horseradish peroxidase on carboxylated multi-wall carbon nanotubes was three times higher than that on un-functionalized multi-wall carbon nanotubes, indicating the critical role of carboxyl groups on carbon in the immobilization of enzyme [28]. These results indicated that laccase was immobilized on FNBC by multiple modes of binding including physical

adsorption, specific interactions between carboxyl groups on FNBC and polar or ionic groups of laccase and covalent coupling of the enzyme molecules by EDAC.

3.2 Fourier transform infrared (FT-IR) spectroscopy analysis

Figure 1a illustrate the mechanism of immobilization of laccase onto functionalized nanobiochar through diimide-activated amidation and Figure 1b presents the FTIR spectra of FNBCs, laccase, and laccase immobilized on FNBCs samples. Five main peaks were observed in the spectrum of laccase including: (i) a strong band centered at 3332 cm^{-1} attributed to OH and NH vibrations; (ii) a weak band at around 2930 cm^{-1} attributed to CH bonds; (iii) a band at 1610 cm^{-1} corresponding to CONH linkage; (iv) a band at 1240 cm^{-1} corresponding to CN stretching vibration of amines; and finally (v) a sharp band at 1037 cm^{-1} due to COC groups. The band at 3000-3500 cm^{-1} in untreated FNBCs was due to phenol groups or OH groups in the adsorbed moisture. For functionalized FNBC, the band at 1707 cm^{-1} corresponded to C=O stretching bond in carboxylic acid functional groups. After the reaction of FNBC with laccase, the bands at 3000-3500 cm^{-1} and 1610 cm^{-1} ascribed to OH vibration and CONH, respectively showed higher intensity which indicated the immobilization of enzyme molecules on the surface of FNBC [8]. Further, an additional broad peak appeared after immobilization of laccase onto FNBCs at 1586 cm^{-1} which can be mostly attributed to the Amide I in the proteins [29].



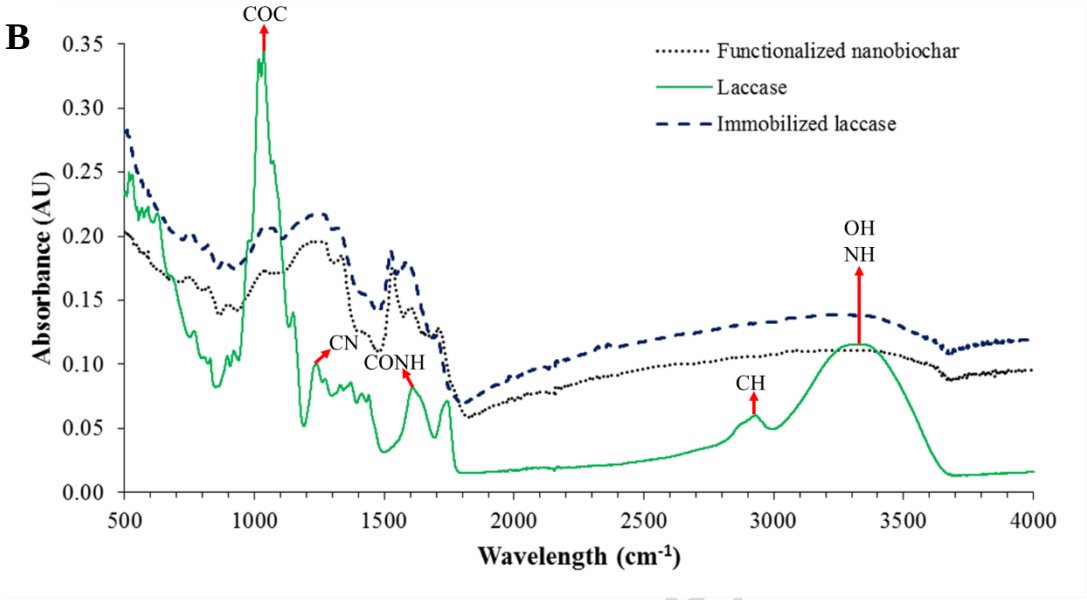


Figure 1: a) Mechanism of immobilization of laccase onto functionalized nanobiochar and; b) FTIR spectra of laccase (solid line), neat functionalized nanobiochars (short-dash line) and laccase immobilized over functionalized nanobiochars (dash line)

3.3. Optimization of covalently immobilized laccase

The experiments designed by CCD were carried out and their results have been presented in Table 2. The RSM design considered central points (0), low (-) and high (+) levels for each parameter (Table 1) and the obtained results were analyzed to determine the coefficients of the quadratic model. A mathematical expression obtained for the relationship of the activity of immobilized laccase onto FNBC with variables A, B, and C (concentration of FNBC, enzyme concentration and EDAC concentration, respectively) are given below in Equation 2 in terms of coded factors:

$$Y = + 3.58 + 0.62\ A + 0.70\ B + 0.82\ C + 0.13\ AB - 0.052\ AC + 0.19\ BC - 0.22\ A^2 - 0.26\ B^2 - 0.36\ C^2 \quad (2)$$

The results of Analysis of variances (ANOVA) for the activity of immobilized laccase showed that the probability (*P*) value and the R-squared of the quadratic model were 0.0004 and 0.9095. Therefore, the regression of the quadratic equation for immobilized laccase activity was significant and applicable for practical applications. The *P* values for quadratic enzyme and EDAC concentration and linear coefficients of all studied parameters of the model were less than

0.05, which meant they were significant. On the other hand, the *P* value for interaction coefficients was greater than 0.05 which indicated the insignificance of interactions among the parameters. The observed activity of immobilized laccase varied between 0.71 (U/g) (obtained at 4 mg/mL FNBC, 2 mg/mL laccase and 7.5 mM EDAC) and 4.95 (U/g) (obtained at 5 mg/mL FNBC, 14 mg/mL laccase and 8.2 mM EDAC). Furthermore, the control sample (untreated FNBC) showed no oxidation of ABTS (laccase substrate).

EDAC is expected to accelerate the covalent bonding of amino-groups on the enzyme molecules with carboxyl groups (COOH) on the surface of FNBC [28]. Figure 2 shows the effect of EDAC concentration in the range from 5.7 to 9 mM on the activity of immobilized laccase on FNBC. As seen, increasing the concentration of EDAC enhanced the activity of immobilized laccase. Similar behavior was observed in the work of Lee *et al.* when they immobilized horseradish peroxidase on carboxylated multi-wall carbon nanotubes using EDAC concentration of up to 10 mM [28]. Tastan *et al.* changed the EDAC/carboxylic group (mol/mol) ratios in the range of 0.05 to 0.6 and observed the maximum activity of immobilized laccase at the ratio of 0.1. At EDAC/carboxylic group ratio higher than 0.4, they observed no activity, which was attributed to a negative effect on the enzyme at high concentrations of the cross-linker [30]. In addition, Figure 2 shows that the immobilization activity of laccase increases rapidly when the initial concentration of laccase in the immobilization mixture increases up to 10 mg/mL, then increases steadily upon further increase in the laccase concentration. Hu *et al.* immobilized laccase on silica nanoparticles by physical adsorption and covalence bonding in which the nanoparticles were functionalized by concentrated HNO_3 and then activated by glutaraldehyde. They reported that covalent coupling enhanced activity than the physical adsorption [31]. Likewise, Ji *et al.* covalently immobilized *P. ostreatus* crude laccase onto the functionalized TiO_2 nanoparticles by glutaraldehyde. They reported that the apparent activity increased 8 times for purified laccase compared to crude laccase [32]. However, using cross-linkers, such as glutaraldehyde at high concentrations can result in distortion of structure, aggregation, precipitation, and loss of enzyme activity [33]. In addition, glutaraldehyde is a toxic and hazardous cross-linking agent which can induce different adverse effects on living organisms [34].

The effect of laccase concentration on the enzymatic activity of final product is shown in Figure 2. For concentrations in the range of 2-14 mg/mL, the enzymatic activity of the immobilized

laccase increased with increasing enzyme concentration and beyond this range, enzymatic activity was independent of the enzyme concentration. This suggested that up to 14 mg/mL, the enzyme molecules were covalent-bonded as a monolayer at the surface of the nanoparticles. Later, the surface of the FNBC is occupied by enzyme molecules and there is no possibility for more enzyme molecules to attach despite increasing the concentration of the enzyme [8]. Similarly, Silva *et al.* immobilized laccase on functionalized spent grains and reported that by increasing enzyme concentrations up to 5 mg/mL, the enzymatic activity increased faster, while beyond this concentration, enzymatic activity did not differ significantly [35]. In a related study, Salis *et al.* showed that enzymatic activity increased linearly with the enzyme concentration, but higher loadings resulted in decreased laccase activity. They attributed this behavior to the limitations of substrates diffusion inside the support pores before reaching the active site of the enzyme [36]. However, Cristovao *et al.* observed two slopes in the activity trend of laccase immobilized on coconut fiber when they changed the enzyme concentration from 8 to 67 mg/mL and then to 260 mg/mL. They concluded that the enzyme adsorption was not restricted to a monolayer on the support, and adsorption of secondary layers was possible [37]. Also, Tastan *et al.* observed a gradual increase in the activity of the immobilized enzyme with increasing initial enzyme concentration [30].

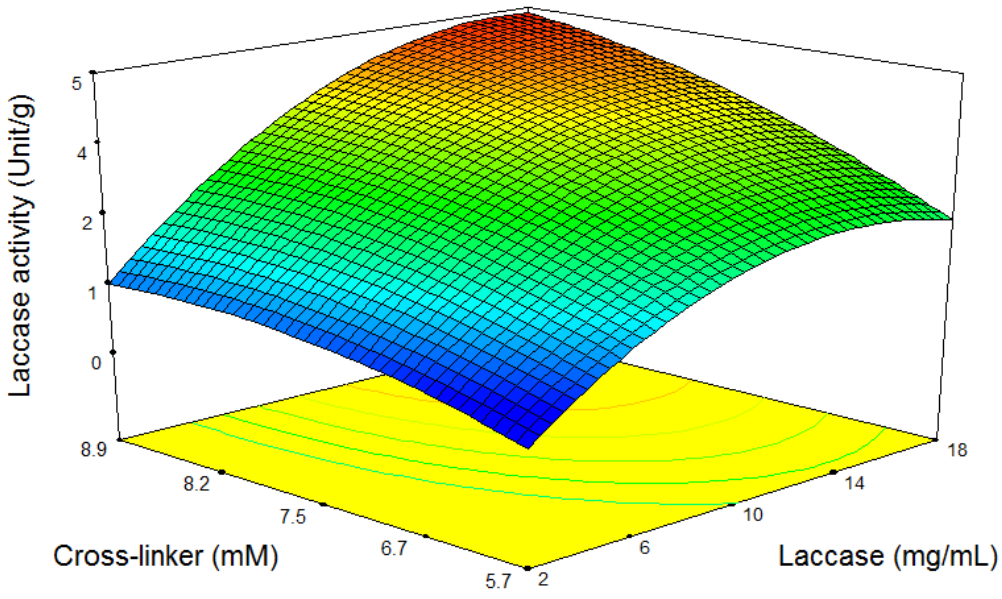


Figure 2: Effects of cross-linker concentration and laccase concentration on the immobilization activity of laccase on functionalized nanobiochar

The effect of incubation time was investigated after setting the other parameters at their optimum levels. Figure 3 illustrates the effects of incubation time on the enzymatic activity of biocatalyst, which reaches to a maximum value at 3 h and then decreases slightly for longer incubation times. The results indicated that the adsorption of laccase over FNBC was faster and 3 h was enough to attain the maximum enzyme immobilization. At longer incubation time, two or more layers of the enzyme were possibly formed over the carrier that reduces the number of free enzymes [35]. Silva *et al.* reported 3.5 h of incubation time as the optimum value for immobilization of laccase on spent grains [35]. Similarly, Cristovao *et al.* observed that the activity increased until 3.5 h, remained constant until 5-6 h and decreased after 6 h. They attributed this behavior to desorption of some enzyme or adsorption of the enzyme as the second monolayer, which ceased the availability of the enzymes [37]. Thus, the longer contact time between the support and enzyme had no advantages nevertheless more molecules were immobilized on a support. In literature, different incubation times were reported as the optimal value for different supports, such as 48 h for Eupergit® C [38]; 24 h for chitosan [39]; 24 h for activated carbon [38]; 5 h for silica [40]; 3.5 h for spent grain [1]; 1.6 h for mesoporous silica [36]; and 30 min for MWCNTs [8]. The difference among supports can be explained by the fact that the adsorption of a macromolecule onto a porous support involved complex steps including diffusion from solution to the surface of the support, diffusion inside the pores and attachment of protein. Therefore, the rate of adsorption depended on every single step, which, in turn, depended on the nature and structure of the support and the enzyme [36, 41].

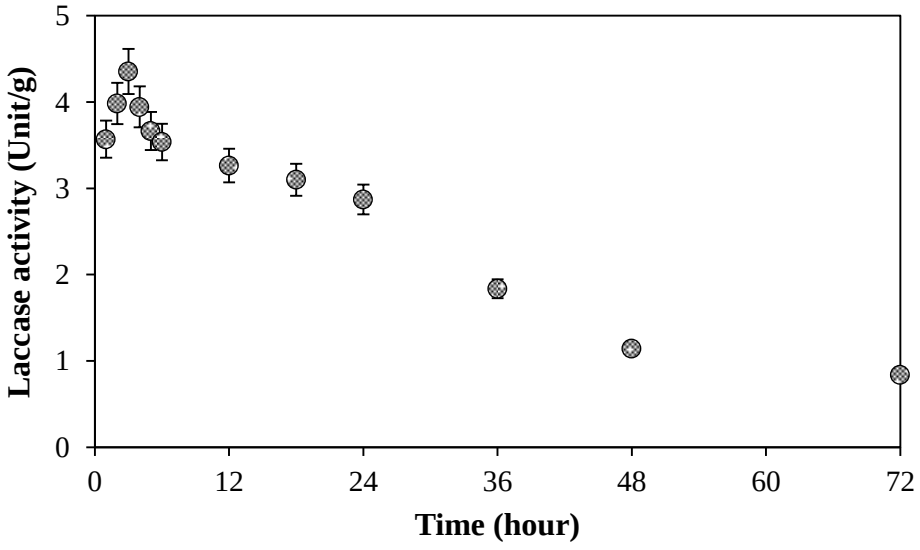


Figure 3: Effect of incubation period on the immobilization activity laccase on functionalized nanobiochar at 4 °C

The above results indicated the higher potential of FNBC as support for immobilization of laccase so that at the optimum conditions, i.e., FNBC of 5 mg/mL, initial laccase concentration of 14 mg/mL, and EDAC concentration of 8.2 and a contact time of 180 min, 5 Unit/g of activity toward ABTS oxidation was achieved. These optimized conditions were maintained for further studies on the thermal, pH, operational and storage stability of free and immobilized laccase.

3.4. Characteristics of the activity of immobilized laccase

Immobilization of enzyme has propounded effects on the enzyme's activity and performance. In this research, the effect of immobilization on the pH dependency of activity of free and covalently immobilized laccase on FNBC was investigated in the pH range of 3.0 to 10.0 (Figure 4) and the results were compared with the previously reported literature (Table 3). Accordingly, the free and covalently immobilized laccase exhibited their maximal activities at pH 4.0 and pH 3.0, respectively. Other researchers reported the same level of optimum pH shift for immobilized laccase on poly (4 vinyl pyridine) [42] and magnetic bimodal mesoporous carbon [43]. This behavior was attributed to the influence of support microenvironment on electrostatic interaction. Therefore, the activity of the immobilized enzyme is significantly impacted by the characteristics of the support and the link between the enzyme and the support. In a related research, Misra *et al.* observed same profile for free and immobilized enzyme but the immobilized laccase higher sensitivity to pH variation [29]. Similarly, Tastan *et al.* immobilized laccase on PTFE membranes through entrapment into gelatin and covalent immobilization. Their results showed optimum pH values of 5, 4 and 6 for free laccase, immobilized laccase through entrapment and immobilized laccase through covalent bonding [30]. Also, the immobilized laccase showed a broader profile for pH-activity than the free laccase as well as higher activity at pH 8-10, indicating that immobilization retained the enzyme activity in a broader pH range [44]. Similarly, Wang *et al.* showed that the immobilized laccase demonstrated higher pH stability than free enzyme, especially in the pH range of 3-7 [45]. Also, Jolivalt *et al.* reported higher stability of immobilized laccase onto PVDF membrane, though the pH activity profiles for the immobilized and the free enzymes were similar [46].

Table 3: Properties of immobilized laccase

Property	Immobilized laccase (This study)	Data from Literature	
		Value	Reference
pH stability	3-5	3-7	[30, 45, 46]
Temperature stability	30-50 °C	30-60 °C	[5, 27]
Storage stability	30 days	25-40 days	[27, 45, 47]
Reusability	7 cycles	5-10 cycles	[1, 33, 37]

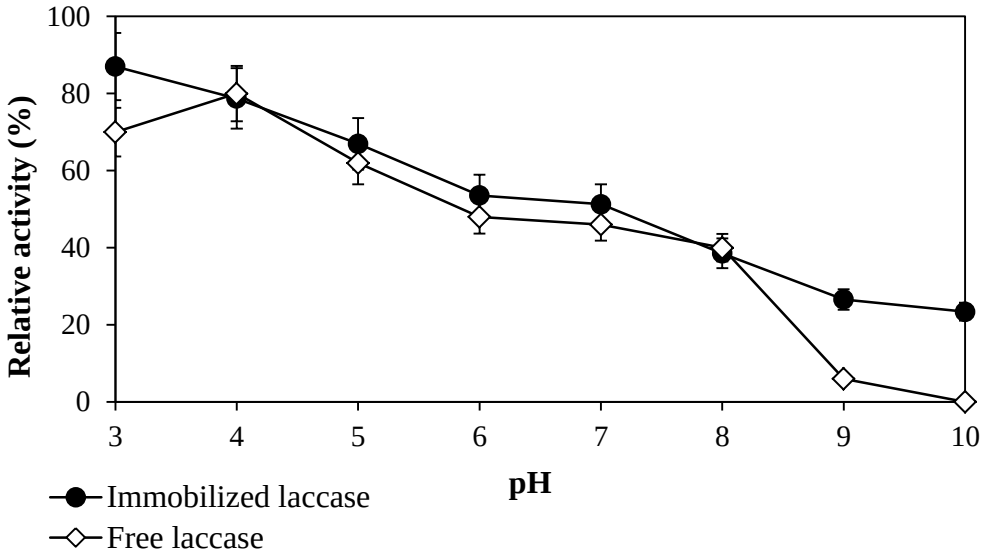


Figure 4: The effect of pH on the activity of free laccase and immobilized laccase

Determining the optimum temperature to achieve the maximum activity of the enzyme is very important since it can determine the maximum efficiency of a biocatalytic system. Immobilization of enzyme alter the activity profile of an enzyme within its working temperature range and may shift the optimum temperature [29] and affect the stability against high temperatures by limiting the conformational changes of the enzyme [4]. To assess the thermal stability of free and covalently immobilized laccase onto FNBC experiments, the samples were incubated at different temperature values ranging from 20 to 70 °C for 8 h. The measured activity of samples after incubation is illustrated in Figure 5. Accordingly, the enzymatic activity of both free and immobilized enzyme was significantly dependent on temperature and both exhibited their maximal activity at 30 °C. However, the immobilized laccase showed a broader temperature profile compared to the free laccase. The attachment of laccase to FNBC results in its thermostabilization, as reflected by both elevated activity at low temperature and decreased

deactivation extent at high temperatures. After 8 h of incubation at 30 °C, the free laccase showed around 30% deactivation while the immobilized ones lost less than 15% of its initial activity. Also, as shown in Figure 5, at a temperature range of 40-50 °C, immobilized laccase on FNBC showed higher stability compared to free laccase. Increasing the thermal stability resulted in retaining the enzymatic activity at high temperatures, so that the residual activity of immobilized laccase was ~ 6-fold higher than that for the free laccase at 60 °C. Similarly, Asuri *et al.* observed an increase in the thermal stability of soybean peroxidase immobilized on MWNT at high temperatures compared to free enzyme so that the maximal initial reaction rate for the immobilized enzyme at 90 °C was 2.5-fold higher than that for the free enzyme at 75 °C [27]. Also, the immobilized laccase onto coconut fiber was reported to be 6.86-fold more stable than the free enzyme at 60 °C [5]. Increasing the thermal stabilization can be attributed to the multi-point attachment of the enzyme macromolecule to the support and/or decreased protein-protein interactions [31, 48, 49].

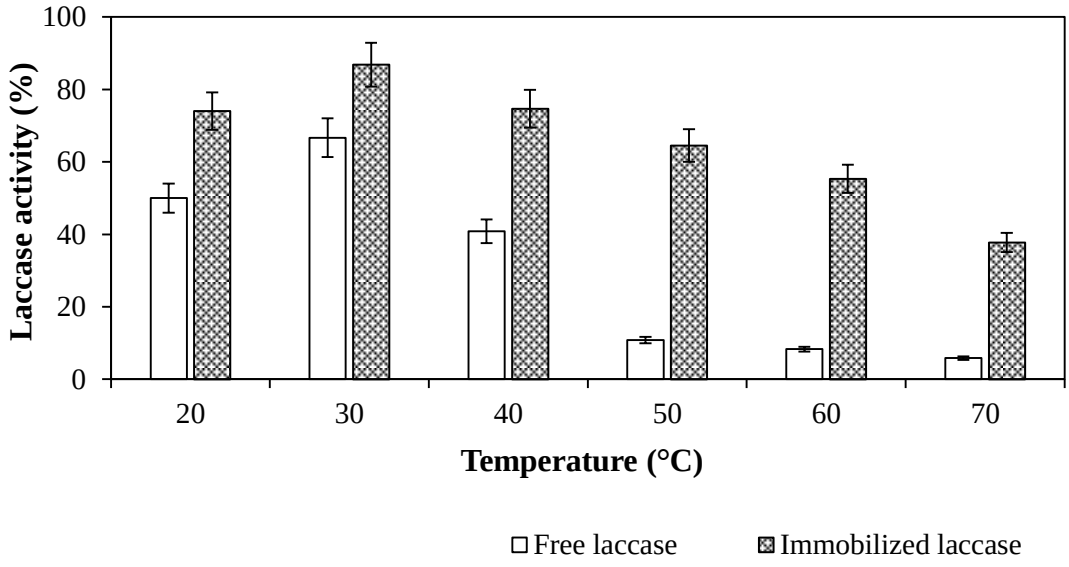


Figure 5: Influence of temperature on the activity of free and immobilized laccase after 8 h of incubation at a desired temperature

The storage stability of the immobilized laccase on FNBC was evaluated by incubating the samples at room temperature and the results were compared with the results of similar research previously reported in the literature (Table 3). According to Figure 6, after 5 and 30 days, the immobilized laccase on FNBC lost around 33% and 50% of its initial activity after 30 days, whereas free laccase lost 58% and 100% activity during the same period. Asuri *et al.* reported

that immobilized soybean peroxidase on MWNT retained 70% of its initial activity after 30 days of incubation at room temperature, while the native enzyme retained only ~ 30% of its activity [27]. Compared to other reports, the immobilized enzyme on nanosized biochar showed higher stability than immobilized enzyme on different supports. For instance, 60% of activity loss was observed after 25 days for immobilized laccase on TiO_2-montmorillonite complexes [45], 60% activity loss after 34 days for immobilized laccase on multi-walled carbon nanotubes at 4 °C [8] and 40% activity loss for laccase immobilized on poly(vinyl alcohol) cryogel after 2 days [50]. In addition, Misra *et al*. immobilized laccase on epoxy functionalized polyethersulfone and observed 12% loss in the initial activity of free laccase after 20 days, while immobilized laccase retained almost all its activity during the same period [29]. Also, Pezzella *et al*. immobilized laccase on perlite and observed 98% and 81% of activity loss for free and immobilized laccase after 27 days storage at room temperature [47].

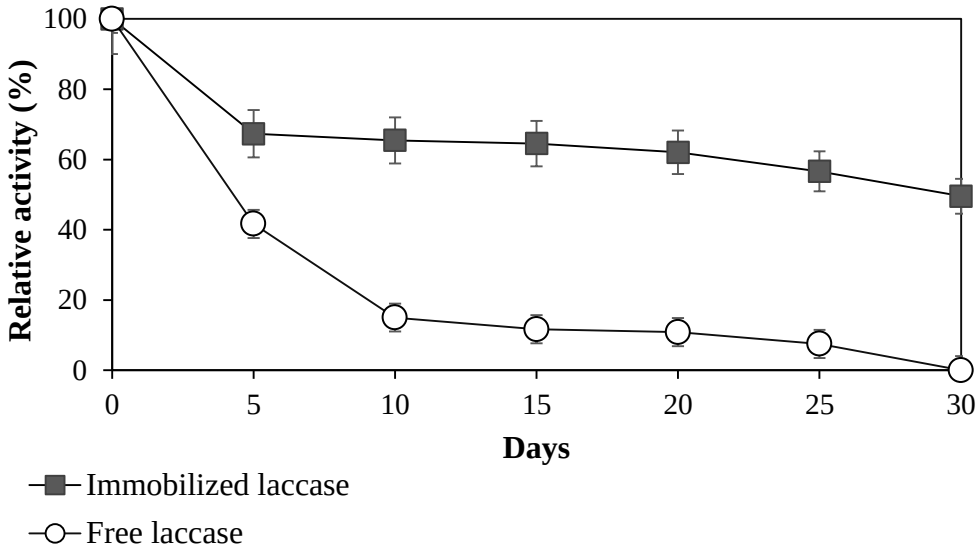


Figure 6: Retention of enzymatic activity at room temperature for free laccase and immobilized laccase on functionalized nanobiochar

3.5. Reusability of immobilized laccase on FNBC

The reusability of the biocatalyst in a batch or continuous system is an important factor in assessing the value of immobilization. Stabilization of laccase due to attachment to FNBC enabled the facile reuse of the immobilized laccase. To assess this property, the immobilized laccase was repeatedly incubated with ABTS and the catalytic activity was measured, and the results are illustrated in Figure 7. The FNBC-laccase conjugates retained around 30% and 5% of

its initial activity after four and seven cycles. In related studies, laccase immobilized on different supports showed similar activities, for example: 30% residual activity after 7 cycles for Amberlite IR-120 [33], 10-30% after 7 cycles for activated carbon [15]; 87% after 10 cycles for digested spent grain [1]; and 55% for green coconut fiber [37]. However, several researchers reported higher reusability for immobilized laccase. For example, the immobilized laccase on carbon-based magnetic mesoporous composites was reported to retain above 70% and 50% of its initial activity after 5 and 10 cycles of ABTS oxidation [43]. One reason for higher reusability was using ABTS with lower concentrations and for shorter reaction time. Tavares *et al.* observed that the immobilization of laccase onto MWCNTs increased the enzymatic activity up to cycle 4. They related the decrease in the activity after the fourth cycle of leaching from the support [8].

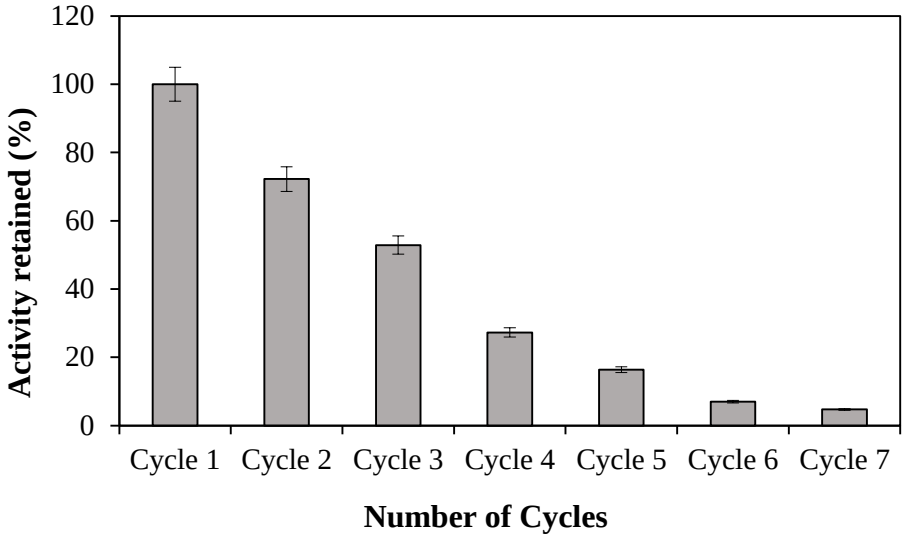


Figure 7: Reusability of functionalized nanobiochar-immobilized laccase during seven cycles of incubation

Conclusion

Enhancing the conformational stability of an enzyme through immobilization is one of the most important steps for implementing the enzymatic technology. In this research, laccase was covalently attached to functionalized nanobiochar via diimide-activated amidation under mild conditions. The two-step process was carried out at room temperature in buffer solutions in a short time (3 h), and the activity of the immobilized enzyme reached a maximum at 5 Unit/g. The characterization results showed that the proteins were intimately associated with the nanobiochar. The optimum conditions for covalently immobilized laccase were determined to be

FNBC of 5 mg/mL, initial laccase concentration of 14 mg/mL, and EDAC concentration of 8.2 and a contact time of 180 min. The thermal and pH stabilities of the immobilized laccase were improved as compared to the free laccase. Also, the immobilized laccase showed good reusability so that it retained 70% of initial activity after 4 consecutive cycles.

Acknowledgments

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Highlights:

- Crude laccase was covalently immobilized onto functionalized nanobiochar
- Conditions were optimized for future application in the wastewater treatment plants
- The maximum activity of immobilized crude laccase on nanobiochar was 5 U/g
- Immobilized laccase on nanobiochar showed higher stability compared to free laccase