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Laccase-Oriented Immobilization Using Concanavalin A as an Approach for Efficient Glycoproteins Immobilization and Its Application to the Removal of Aqueous Phenolics

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Abstract: An expanding number of human activities are contributing to the rising levels of aromatic compounds, which pose a major threat to the ecosystem. However, readily available microbial enzymes might be used to remediate contaminated wastewater in an economical and environmentally benign manner. In this study, an efficient method of laccase-oriented immobilization on modified Immobead 150P was proposed. The oriented immobilization technique using aminated laccase exceeds in both protein loading onto the carrier (4.26 mg/g) and immobilization yield (93.57%) due to the availability of more active sites. The oriented aminated laccase preserves 100% and 95% of its original activity after six and ten cycles of operation, respectively. The thermal stability performance of the oriented enzyme was the best among both free and random immobilized forms, since it was able to conserve 79% and 44% of its initial activity after 6 h at 50 °C and 60 °C, respectively. The ideal pH of oriented immobilized laccase was altered from 3.0 to 4.0, and it was more stable than both free and random immobilized laccases at pH 7.0. Finally, the integration of the adsorption capacity of Immobead 150P and the biodegradation ability of laccase promises the efficient removal of aqueous phenolics. Oriented immobilized laccase may provide a significant new approach for wastewater treatment, according to these findings.

Keywords: laccase; oriented immobilization; concanavalin A; Immobead 150P; phenolics removal

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1. Introduction

One of the most urgent environmental challenges in recent years has been the widespread detection of bioactive substances in aquatic environments [1]. These bioactive molecules are extremely heterogeneous and comprise various chemicals used in pharmaceutics, personal care products, synthetic hormones, etc. [2]. These compounds are one of the most significant families of emerging contaminants because they pose a particular hazard to aquatic ecosystems due to their capacity to bioaccumulate to large quantities [3]. Many of these bioactive chemicals slip through treatment plants unaltered and eventually wind up in surface waters because wastewater treatment plants are poorly equipped to treating them due to their unexpected specificity and biological activity [4]. Recently, an inexpensive and ecologically friendly treatment method utilizing the enzymes of white rot fungi has been proposed. It has been demonstrated that ligninolytic enzymes, such as laccases, lignin peroxidases, and manganese peroxidases, are very effective at degrading these emergent pollutants [1,5].

Laccases (EC 1.10.3.2) are ligninolytic enzymes that belong to the multicopper oxidases and specifically benzenediol:oxygen oxidoreductase. They are catalyzing the one-electron oxidation of diverse substrates joined with molecular oxygen (four-electron) reduction to

water [6–8]. Laccases have enormous potential applications in the treatment and bioremediation of contaminated soil and water, such as the degradation of pesticides [9], detoxification of polluted water [10], decolorization of dyes [11], biosensors [12], and other biotechnological procedures. In contrast, the high cost of production and low stability of most laccases have hampered their development [13,14].

The immobilization process is the most straightforward and efficient way to conquer these boundaries via enhancing the properties of enzymes such as thermal stability increase and extreme conditions struggle [15]. A variety of carriers have been described for successful laccases immobilization, such as magnetic nanoparticles [16], multiwalled carbon nanotubes [11], chitosan, and nylon membrane [17]. Among different carriers, epoxy carriers are exceedingly appropriate supports for the immobilization of enzymes due to their capacity of reusability and surface adaptation with different active groups. Traditional laccases immobilization on epoxy supports is based on physical adsorption, cross-linking, or covalent coupling (one-point or multipoint attachment). Among these techniques, covalent binding is typically more stable, and the enzyme leakage from carriers upon use in this method is usually avoided. Conversely, the activity of immobilized laccase may become stuck as the active center's amino acid residues are occupied in the covalent coupling with Immobead 150P functional groups, as an example of epoxy supports, due to the random coupling between supports and proteins [14]. Immobead 150, which is made of methacrylate polymers with particles ranging in size from 0.15 to 0.30 mm and epoxy linkers capable of interacting with amino groups, is one of the recently commercially available carriers. Epoxy carriers, such as Immobead 150, may react to a variety of nucleophilic groups, such as hydroxyl, thiol, and amino moieties, which are present on different proteins, producing well-built connections with no chemical modification to the protein [18].

Traditional techniques of immobilization comprise entrapment, adsorption, cross-linking, and covalent attachment on top of diverse supports counting graphite, gold, silica, and various nanocomposites such as nanoparticles, carbon nanotubes, and nanowires [19–21]. Such techniques are not easy to control and generally result in random enzyme immobilization. Preferably, enzyme coupling has to be accomplished at a precise preset enzyme position on the carrier's surface to assist in affording an appropriate orientation for ideal enzyme catalytic activity while obviating conformational alterations. Oriented and controlled enzyme immobilization has been accomplished via generating bioaffinity linking between an activated suitable carrier and a definite cluster of the enzyme molecule. Models incorporating bioaffinity interlinkages were illustrated among an affinity residue such as biotin, cysteine, histidine, or carbohydrate moiety existing or genetically designed at a specified site in the sequence of protein sugars and streptavidin, metal chelates, and lectins of stimulated support surfaces [22]. Mannose has been used as a sugar residue in enzymes as naturally occurring or as a tag, and it has been used to generate an affinity ligament with concanavalin A (Con-A) [23–25]. Lectins are non-immune proteins able to reversibly join, particularly to carbohydrates or carbohydrate-carrying glycoconjugates [26]. Lectins are proteins present in microorganisms, animals, and plants and are able to bind carbohydrate moieties. Con-A is one of the main fully described and extensively utilized lectins. It is initially isolated from the seeds of *Canavalia ensiformis* (Jack-bean), and it distinctively connects to the internal and nonreducing terminal α-D-glucosyl and α-D-mannosyl groups present in diverse glycolipids, glycoproteins, and sugars. The biochemical compatibility between immobilization supports and affinity immobilization ligands plays a critical role in immobilization success [27].

Con-A has a lot of fascinating features. It agglutinates several cell kinds [28], demonstrates mitogenic activity in lymphocytes [29], and restrains the development of tumors [30]. Its responses to glycoproteins and soluble polysaccharides apparently look like antigen–antibody interactions. All of these interactions originate from its capability to join carbohydrates through definite distinct configurations [31,32]. The molecular weight of the Con-A subunit is 27 kDa, and it has a binding position for divalent transition metal ions,

usually Mn^{2+}. As soon as this position is engaged, a location that joins Ca^{2+} is created; only totally metalized protein is competent to bind carbohydrates [33]. Within the acidic range of pH values (3.5–5.6), Con-A is a dimeric molecule (Mw 54 kDa), whereas at elevated pH values, Con-A tetramers and extra polymeric sorts are shaped [28,34]. Since Con-A is a metalloprotein, the existence of both Ca^{2+} and Mn^{2+} is necessary to preserve its coupling properties. Every Con-A subunit employs one ion of both manganese and calcium, which could be eliminated in an acidic environment, revoking its capability to carbohydrate-bind. Con-A at a neutral pH range is a tetramer having a glycosyl binding position on every subunit. The binding locations of Con-A are disseminated between the two opposed protein regions [35]. Con-A has a lot of applications in the fields of immobilization, biosensing, and glycoprotein separation [36,37] on the basis of the interaction between the glycosyl moiety of glycoproteins and Con-A. However, the use of Immobead 150P to be a base for Con-A immobilization first and then, consequently, the contact between Con-A and the glycosyl moiety of laccase has not so far been stated. Biotechnologically, Con-A has been applied as an affinity ligand for the immobilization and purification of glycoconjugates and glycoproteins [38,39].

Affinity attachment of glycoenzymes to immobilized lectins is vastly utilized in different preparative goals [40,41]. The laccase-oriented immobilization on glyoxyl Immobead 150P through the contact between the laccase glycosyl moiety and Con-A has not been investigated. On the basis of Con-A-specific affinity to glycosyl moieties, we utilized in the current study an innovative approach for the oriented immobilization of laccase as a glycoprotein on the surface of Immobead 150P utilizing the unique properties of Con-A as a bioaffinity ligand.

2. Materials and Methods

2.1. Chemicals

Laccase from *Myceliophthora thermophila* was provided by Novozymes (51003). Concanavalin A (Con-A) from *Canavalia ensiformis* (Jack bean) Type IV (C2010) lyophilized powder, Immobead 150P, ethylenediamine (EDA), N-(3-dimethylaminopropyl)-N′-ethyl carbodiimide hydrochloride (EDC), 1-hydroxybenzotriazole, m-cresol, 4-nitrophenol, and pyrimethanil were provided by Sigma-Aldrich (St. Louis, MO, USA). GE Healthcare provided the DEAE Sepharose 6B carrier. 2,2′-Azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) diammonium salt (ABTS) as a substrate for laccase was purchased from Sigma-Aldrich. For protein estimation, a Bio-Rad Protein Reagent was utilized.

2.2. Laccase Clarification and Amination

Myceliophthora thermophila laccase was clarified and aminated according to the method described by Othman et al. [11]. In this method, a DEAE Sepharose 6B carrier was applied to clarify laccase via an adsorption technique. The clarified laccase was then aminated using EDA and EDC solutions at pH 4.75. Finally, the resultant aminated enzyme was dialyzed for 24 h via a 14 kDa cutoff dialysis cellulose membrane (Sigma-Aldrich).

2.3. Enzyme Activity Estimation

The activity of free laccase was estimated using a JASCO UV–Vis spectrophotometer (V-630, Tokyo, Japan) applying ABTS as a substrate. ABTS was dissolved in a citrate buffer (100 mM, pH 4.5) to obtain a concentration of 0.58 mM. The absorbance change at 436 nm (ε = 29,300 $M^{-1}cm^{-1}$) was monitored for 1.0 min. One unit (U) of laccase was defined as the enzyme quantity required to oxidize 1.0 µmol of ABTS per minute. In the case of immobilized laccase, enzyme samples were shaken at 50 rpm for 4 min in an ABTS solution (3.0 mL, 0.58 mM) dissolved in a citrate buffer (100 mM, pH 4.5) at 30 °C with monitoring of absorbance at 436 nm every minute, and then the obtained data were linearly regressed. Protein concentration was determined via the Bradford method [42] at 595 nm and using BSA as a standard protein, employing a Bio-Rad protein assay reagent. The immobilized protein was quantified as the difference in concentration between the initial protein added

to the immobilization reaction and the protein quantity retrieved from the immobilization solution after the formation of the carrier–enzyme complex.

2.4. Immobead 150P Modification

Immobead 150P was modified according to Matte et al. [43] method with some modifications, to hydrolyze its epoxy groups to aldehyde units. Twenty milliliters of sulfuric acid (0.5 M) was utilized to hydrolyze 2.0 g of Immobead 150P for 15 h. Then the carrier was washed with distilled water. To complete the oxidation process, 2.0 g of the carrier suspended in 20 mL of distilled water was stirred for 2 h in a sodium periodate solution (0.1 M), rinsed with distilled water, and finally dehydrated at 60 °C for 2 h.

2.5. Random and Oriented Laccase Immobilization

The oriented immobilization of laccase on the Immobead 150 surface was performed via the interaction between Con-A attached to the Immobead 150P surface and laccase glycosyl moieties. Random immobilization between Immobead 150P and laccase via covalent binding was used as a control. For random immobilization (covalent binding), 250 mg of Immobead 150P was dispersed in 5 mL of a phosphate buffer (100 mM, pH 7.0 containing 0.1 M KCl, 0.1 mM $CaCl_2$, and 0.1 mM $MnCl_2$). The purified or aminated laccase solutions were added to the immobilization mixture and then were shaken at 200 rpm and 30 °C for 24 h.

For oriented immobilization (affinity adsorption), the same amount of Immobead 150P was added into a preactivated Con-A solution (0.8 mg mL^{-1}; Con-A was preactivated in a 0.1 M phosphate buffer at pH 7.0 containing 0.1 M KCl, 0.1 mM $CaCl_2$, and 0.1 mM $MnCl_2$ for 15 h) and shaken at 200 rpm at 30 °C for 3 h [44]. After that, the Immobead 150P–Con-A complex was washed with an excess of distilled water to remove the unbound Con-A. The laccase solution of purified or aminated laccase was added to Immobead 150P–Con-A complexes, then they were shaken at 200 rpm and 30 °C for 24 h. The immobilized laccase was removed via filtration and washed with an excess of distilled water and finally with a phosphate buffer (100 mM, pH 7.0) to eliminate the unbound laccase [14,45]. The efficiency of immobilization processes was evaluated through monitoring the decrease in both protein concentration and laccase activity in the immobilization reaction mixture [43]. Protein loading was calculated as the difference in protein concentration before (P_i) and after (P_f) immobilization reaction, divided by the weight (w) of the carrier (Equation (1)). Immobilization yield (Equation (2)) was defined as the difference between the primary enzyme activity (A_i) and the enzyme units remaining in the immobilization solution after the formation of the carrier–enzyme complex (A_f) divided by the initial enzyme activity (A_i).

$$\text{Protein loading} = \frac{P_i - P_f}{W} \quad (1)$$

$$\text{Immobilization yield} = \frac{A_i - A_f}{A_i} \times 100 \quad (2)$$

2.6. Operational Stability of Random and Oriented Immobilized Laccase

The operational stability of the random and oriented immobilized purified and aminated laccase was monitored through the repeated reaction of laccase preparations with ABTS as a substrate under the standard reaction conditions (Section 2.3) at 50 rpm through ten operational cycles (each round is 4.0 min). During every round, samples of enzymes were taken each minute for activity assessment and then returned to the reaction mixture. The rounds of the laccase reaction were stopped by the removal of all reaction media except immobilized laccase through filtration. Consequently, the immobilized enzyme was gently washed by distilled water, and then it was returned to a fresh reaction medium (new buffered ABTS) to start a new reactivity cycle.

2.7. Temperature and pH Profiles

The effect of reaction temperature on laccase activity was estimated through monitoring free and (random and oriented) immobilized laccase activity at a wide range of temperatures from 40 to 90 °C. At the specified range of temperatures, several identical standard reaction mixtures (Section 2.3) were incubated and assessed, and the relative enzyme activity was then plotted. The pH profile of laccase activity was determined by investigating the impact of reaction pH values on *M. thermophila* free and immobilized laccase activity. pH values ranging from 2.0 to 6.0 were examined using a Robinson buffer. Several identical standard reaction mixtures (Section 2.3 with the exception of a particular pH value) were incubated and evaluated at the prescribed range of pH values, and the relative enzyme activity was then reported. ABTS was employed as a substrate in both temperature and pH optimum value estimations.

2.8. Thermal and pH Stability

The thermal stability behavior of *M. thermophila* free and (random and oriented) immobilized laccase was studied. Laccase preparations were preincubated at 50, 60, and 70 °C without substrate (ABTS) for sequence time intervals in a 50 mM phosphate buffer (pH 7.0). Then, each warmed enzyme aliquot was incubated at 30 °C with the other ingredients of the standard reaction mixture (Section 2.3) to test for the residual laccase activity. The stability of free and (random and oriented) immobilized laccase preparations against pH inactivation was evaluated by preincubating enzyme preparations at 50 °C for 6 h in different pH values (pH 3.0–9.0) using a Robinson buffer (100 mM). Using the normal test conditions, the remaining laccase activity was assessed at pH 4.5 (Section 2.3). The residual activity was calculated and compared with the beginning activity of each laccase preparation type.

2.9. Thermal Kinetics

To analyze the experimental thermal stability data, the parameters of thermal deactivation were investigated employing nonlinear regression. The obtained data indicate that the enzyme went through one conformational change at high temperatures. This could be in accordance with the inactivation model (Equation (3)) utilized by some investigators in the case of exponential decay [46–49]. The half-life time ($t_{1/2}$) of laccase preparations in both free and immobilized forms is the time required to lose a half portion of the original activity. $t_{1/2}$ was calculated according to Equation (4), where A is the residual enzyme activity, k is the constant of the first-order inactivation rate, and t is the time.

$$A = e(-kt) \tag{3}$$

$$t_{1/2} = \frac{ln\,2}{k} \tag{4}$$

The Arrhenius equation (Equation (5)) was applied to formulate the temperature impact on the inactivation rate constants. The activation energy (E_a) was calculated from the linear regression analysis of the absolute temperature reciprocal alongside the natural logarithm of the rate constant. The free energy (ΔG) of the thermal inactivation was calculated by applying the first-order rate constant (k) at various temperature values employing Equation (6). Activation enthalpy (ΔH) and entropy (ΔS) were calculated through Equations (7) and (8), respectively. Data statistical analysis, plots, and k-value estimations were achieved by employing GraphPad Prism software (Version 6.01).

$$ln(k) = ln(k_0) - \frac{E_a}{RT} \tag{5}$$

$$\Delta G = -RT\,ln\left(\frac{kh}{K_B\,T}\right) \tag{6}$$

$$\Delta H = E_a - RT \tag{7}$$

$$\Delta S = \frac{\Delta H - \Delta G}{T} \quad (8)$$

where k is the inactivation rate constant, k_0 is the Arrhenius constant, T is the absolute temperature (Kelvin), and R is the universal gas constant (8.31 J mol^{-1} K^{-1}). K_B is the Boltzmann constant (1.3806 × 10^{-23} J K^{-1}), and h is the Planck constant (1.84 × 10^{-37} J h).

2.10. Kinetic Constants

Kinetic constants of the free and (random and oriented) immobilized laccase were calculated through the reaction of enzyme forms with the most laccase-specific substrate (ABTS). Serial concentrations of ABTS were considered in the current study to react with laccase forms (in the case of the free enzyme: 0.029 mM–2.9 mM; whereas in the case of both random and immobilized laccase: 0.029 mM–5.8 mM) at the standard reaction conditions (30 °C and pH 4.5; Section 2.3). The Michaelis–Menten constant (K_m) refers to the attraction between the enzyme and its substrate. It was calculated through the determination of the enzyme activity of a fixed amount of enzyme at serial concentrations of the substrate. The K_m and V_{max} (the maximum reaction velocity) values were estimated from the Lineweaver–Burk plot applying GraphPad Prism version 6.01.

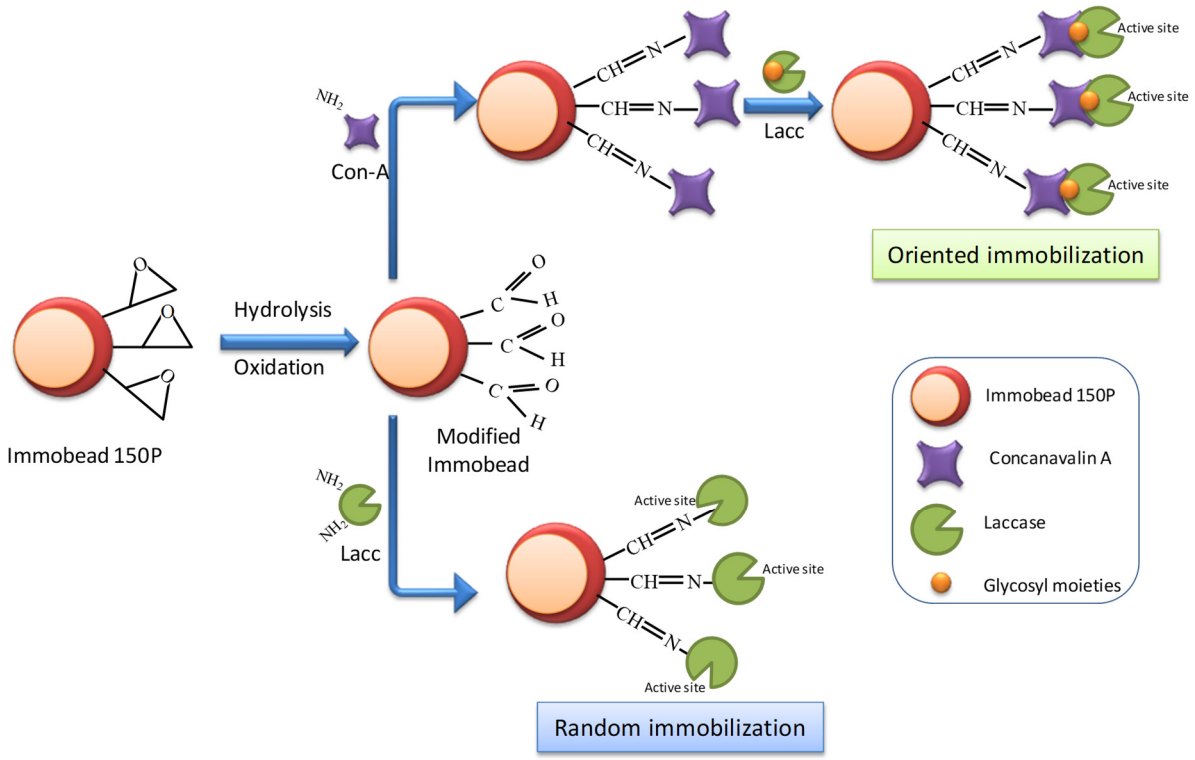
2.11. Biodegradation of Some Phenolic Compounds Using Immobilized Laccase

Under magnetic stirring conditions, *m*-cresol, *p*-nitrophenol, and pyrimethanil, as pollutant phenolic compounds, were separately submitted for biodegradation by the free, random, and oriented laccase in Pyrex vials. The vials were separately fed with a final concentration of 10 ppm from *m*-cresol, *p*-nitrophenol, and pyrimethanil in a potassium phosphate buffer (pH 7.0, 0.1 M) containing 0.1 M KCl, 0.1 mM of $CaCl_2$, and 0.1 mM of $MnCl_2$. One unit of immobilized enzymes (random or oriented) was added to start the biodegradation reactions at 50 °C and pH 7.0 in the presence of 1 mM hydroxybenzotriazole (HBT) in a 5 mL total reaction mixture for 3 h. The biodegradation caused by laccase preparations was estimated as the difference between the initial and final concentrations of tested compounds in the reaction. The control of Immobead 150P was considered to eliminate the adsorption effects of the support. The concentration of phenolic compounds before and through the reaction was estimated via high-performance liquid chromatography (HPLC). For HPLC analysis, a Zorbax Eclipse XD8-C8 reverse-phase column (150 × 4.6 mm i.d. 5 m) was used with an Agilent 1100 HPLC coupled with a diode array detector to quantify the reduction in concentration. A 40:60 water–acetonitrile mobile phase was pumped in isocratic mode at 1 mL/min for 20 min. All samples were filtered via 0.45 m PVDF filters before being subjected to chromatographic analysis.

3. Results

3.1. Bioaffinity Immobilization of Myceliophthora thermophila Laccase Utilizing Con-A onto Immobead 150P

In the current approach, *Myceliophthora thermophila* laccase was aminated to enhance the interaction between the support and amino groups of the enzyme. Furthermore, Immobead 150P was modified to hydrolyze its epoxy groups to aldehyde units. The epoxy groups of the Immobead 150P support were hydrolyzed using sulfuric acid. This step was followed by diol oxidation by sodium periodate to aldehyde moieties. Then, Con-A was covalently attached to the modified Immobead 150P via primary amine functionalities. Using those modified parts, aminated laccase was immobilized using two paths (Scheme 1): for random immobilization (covalent binding without Con-A), the purified or aminated laccases were added to the immobilization mixture that contains modified Immobead 150P. In the case of oriented immobilization, laccase was immobilized via the interaction between Con-A attached to the Immobead 150P surface (Immobead 150P–Con-A complex) and laccase glycosyl moieties at pH 7.0.



Scheme 1. Schematic illustration of random and oriented immobilization of laccase enzyme onto Immobead 150P though Con-A. For random immobilization, laccase was added to immobilization mixture that contains modified Immobead 150P. In the case of oriented immobilization, laccase was immobilized via interaction between Con-A attached to Immobead 150P surface and laccase glycosyl moieties.

The efficiency of immobilization processes of both oriented and random laccase immobilization using Con-A attached to glyoxyl Immobead 150P was evaluated and is cited in Table 1. The obtained results indicate the superiority of immobilized laccase activity (1.25 U/g) over other immobilization trials in the case of applying the oriented immobilization technique using the aminated laccase. Additionally, the results also revealed the exceeding of the oriented immobilization technique using aminated laccase in both protein loading onto the carrier (4.26 mg/g) and immobilization yield (93.57%). This could be due to the enrichment of enzyme molecules with amino groups, as well as the availability of more active sites due to the orientation approach (Table 1 and Figure S1).

Table 1. Efficiency of both oriented and random laccase immobilization using Con-A attached to glyoxyl Immobead 150P.

Immobilization Technique	Enzyme Type	Immobilized Activity (U/g)	Protein Loading (mg/g)	Immobilization Yield (%)
Oriented	Aminated	1.25 ± 0.05	4.26 ± 0.01	93.57 ± 2.75
	Purified	1.17 ± 0.15	3.20 ± 0.08	80.97 ± 3.07
Random	Aminated	1.13 ± 0.03	3.69 ± 0.16	79.42 ± 1.75
	Purified	0.68 ± 0.00	2.14 ± 0.02	55.31 ± 5.02

For random immobilization, 250 mg of Immobead 150P was dispersed in 5 mL of a phosphate buffer (100 mM, pH 7.0 containing 0.1 M KCl, 0.1 mM CaCl2, and 0.1 mM MnCl2). The purified or aminated laccase solutions were added to the immobilization mixture and then were shaken at 200 rpm and 30 °C for 24 h. For oriented immobilization, Immobead 150P was added into a preactivated Con-A solution (0.8 mg mL^{-1}; Con-A was

preactivated in a 0.1 M phosphate buffer at pH 7.0 containing 0.1 M KCl, 0.1 mM CaCl2, and 0.1 mM MnCl2 for 15 h) and shaken at 200 rpm at 30 °C for 3 h. After that, the Immobead 150P–Con-A complex was washed with an excess of distilled water to remove the unbound Con-A. The purified or aminated laccase was added to Immobead 150P–Con-A complexes, and then they were shaken at 200 rpm and 30 °C for 24 h.

3.2. Reusability of the Immobilized Laccase Preparations

Four trials using two different immobilization strategies and two laccase forms were conducted in order to obtain a full description of the operational stability of different applied approaches (Figure 1). This kind of stability concerns the catalytic state of the immobilized enzyme later than its repeated use for different cycles. The data presented in Figure 1 declare the differences between the four presently utilized immobilization approaches. The differences in operational stability depend on the utilized support due to the specific nature of each immobilization carrier and the enzyme coupling sites. Despite the fact that there was a slight decrease in the operational stability of all immobilized laccase preparations, the oriented immobilization approach using aminated laccase preserved 100% and 95% of its original activity after six and ten cycles, respectively, which could be due to the proposed orientation of the enzyme molecule. Additionally, aminated laccase showed enhanced reusability (95%) compared with the purified one (91%) after ten cycles (Figure 1). For that, oriented and random immobilization of the aminated laccase will be further studied through next experiments.

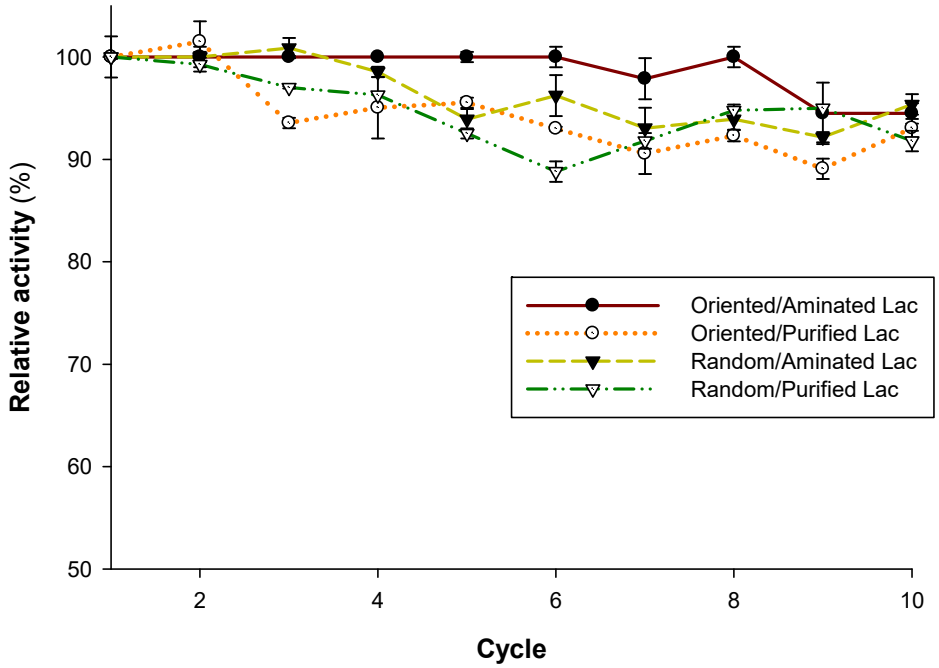


Figure 1. Reusability of laccase immobilized using oriented and random immobilization by Con-A on modified Immobead 150P. ABTS was used as substrate under standard reaction conditions (Section 2.3) at 50 rpm through ten operational cycles (each round is 4.0 min). Rounds of laccase reaction were stopped by removal of all reaction media except immobilized laccase through filtration. Consequently, immobilized enzyme was gently washed by distilled water, and then it was returned to a fresh reaction medium to start a new reactivity cycle.

3.3. Optimum Temperature of Different Laccase Preparations

The laccase activity in free and both random and oriented immobilization forms was regularly increased by the gradual increase in the reaction mixture temperature up to 70 °C, which was recorded as the optimal temperature value for all preparations (Figure 2). This could be due to the improvement of reaction kinetics between the enzyme active sites and substrate molecules. Subsequently, laccase activity starts to decrease as a result of enzyme denaturation at upper reaction temperatures. It is worthwhile to mention that the oriented immobilized laccase is affected to a greater extent by the reaction temperature than the other two laccase forms.

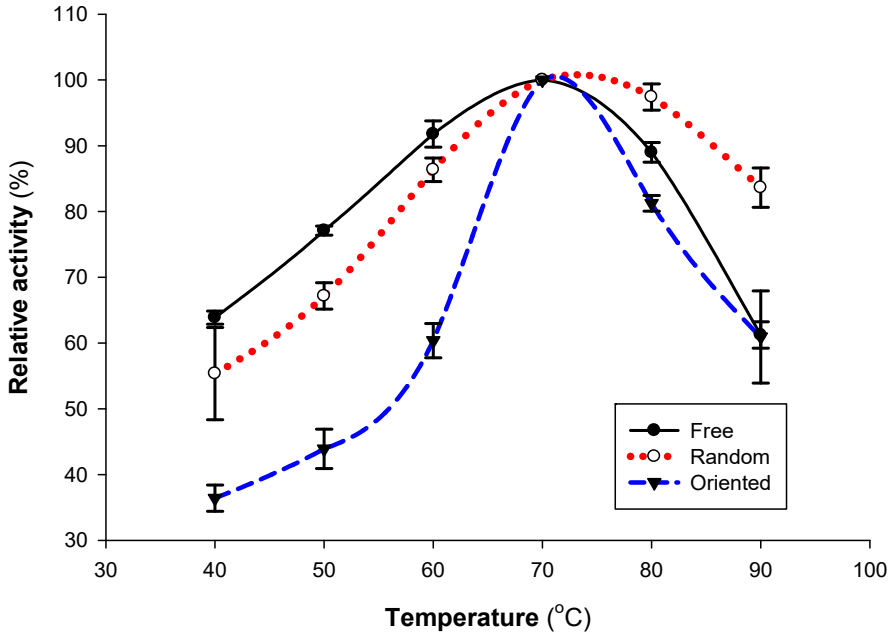


Figure 2. Effect of reaction temperature on activity of free, random, and oriented laccases. Laccase activity was monitored at a wide range of temperatures from 40 to 90 °C.

3.4. Thermal Stability and Thermodynamic Parameters

Different laccase preparations (free, oriented, and random immobilized laccase) were assessed for their thermal stability. All the laccase forms were stable and able to conserve their enzymatic activity at 50 and 60 °C with different stability levels. The thermal stability performance of the oriented immobilized laccase was the best among them, since it was able to conserve 79% and 44% of its initial activity after 6 h at 50 and 60 °C, respectively. On the other hand, free aminated laccase was more stable than the random immobilized laccase and retained 67% of its initial activity after 6 h of exposure at 50 °C, whereas the random immobilized laccase retained only 46% (Figure 3).

The $t_{1/2}$ values of different laccase forms were temperature-dependent. The results obtained in Table 2 verified the superiority of oriented laccase thermal stability, where $t_{1/2}$ values of oriented, random, and free laccase forms were 28.9, 11.2, and 18.01 h at 50 °C. These values were decreased by increasing the incubation temperature to become around 6 h for all laccase forms at 70 °C (Table 2). The values of the laccase inactivation kinetic constant (k) are presented in Table 2, where the k values of the oriented immobilized laccase are the lowest values among the developed laccase forms. The k values were raised by the increase in the incubation temperature, which indicates the incident enhancement of oriented immobilized laccase thermal stability over both free and random immobilized forms.

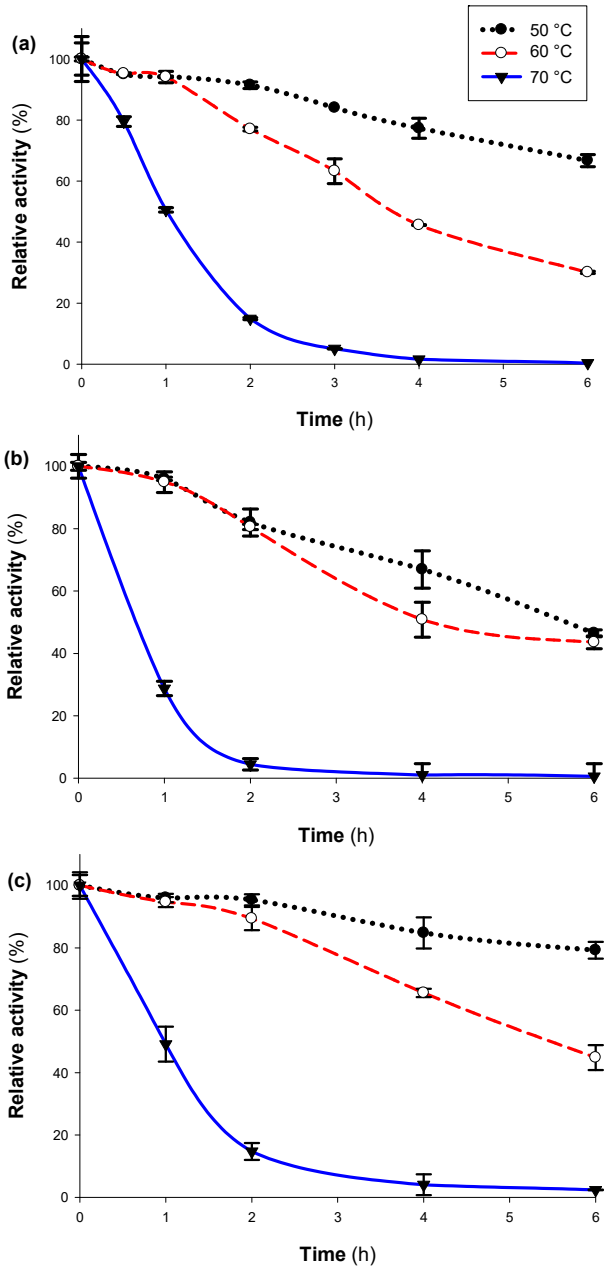


Figure 3. Thermal stability of (**a**) free, (**b**) random immobilized, and (**c**) oriented immobilized laccase. Laccase preparations were preincubated at 50, 60, and 70 °C without substrate (ABTS) for sequence time intervals in 50 mM phosphate buffer (pH 7.0). Tests for remaining laccase activity were then conducted at 30 °C under standard assay conditions (Section 2.3).

Table 2. Kinetic inactivation parameters of free, random, and oriented immobilized laccases at different temperatures.

Temperature (°C)	Half-Life (h)			k (h^{-1})			R^2		
	Free	Random	Oriented	Free	Random	Oriented	Free	Random	Oriented
50	18.01 ± 0.25	11.20 ± 0.13	28.90 ± 1.33	0.038	0.062	0.024	0.765	0.990	0.976
60	8.58 ± 0.73	10.63 ± 0.85	10.87 ± 0.76	0.081	0.065	0.064	0.935	0.964	0.982
70	6.02 ± 0.08	6.03 ± 0.23	6.14 ± 0.55	0.115	0.152	0.113	0.972	0.998	0.993

Parameters of thermal deactivation were investigated employing nonlinear regression. Half-life time ($t_{1/2}$) of laccase preparations in both free and immobilized forms is the time required to lose a half portion of original activity.

The thermodynamic parameters of thermal inactivation of laccase forms are listed in Table 3. The activation energy (E_a) of the oriented immobilized laccase (71.570 kJ/mol) was higher than those of the free laccase (50.678 kJ/mol) and random immobilized laccase (28.296 kJ/mol), which declares the highest thermal stability of the oriented immobilized laccase. That means more required energy to malfunction the oriented form of the immobilized enzyme (Table 3). The positive free energy (ΔG) and standard enthalpy (ΔH) values for free, random, and oriented immobilized laccases prove the endothermic and nonspontaneous process of enzyme thermal inactivation. All the three laccase forms showed ΔS negative values to different degrees.

Table 3. Thermodynamic parameters of thermal inactivation and kinetic constants of laccase activity.

Parameter	T (K)	Form of Laccase Enzyme		
		Free	Random Immobilized	Oriented Immobilized
ΔG (kJ mol^{-1})	323.15	110.14	108.83	111.38
	333.15	111.54	112.15	112.19
	343.15	113.97	113.18	114.02
ΔH (kJ mol^{-1})	323.15	47.99	38.35	68.88
	333.15	47.91	38.27	68.80
	343.15	47.83	38.19	68.72
ΔS (J mol^{-1} K^{-1})	323.15	−192.34	−218.09	−131.51
	333.15	−191.00	−221.76	−130.25
	343.15	−192.77	−218.53	−132.03
Ea (kJ mol^{-1})		50.678	28.296	71.570
K_m (mM)		0.040	0.280	1.951
V_{max} (µmol min^{-1})		1.986	1.723	10.74
V_{max}/k_m		49.650	6.154	5.505

Activation energy (E_a) was calculated from linear regression analysis of absolute temperature reciprocal alongside natural logarithm of rate constant. Free energy (ΔG), activation enthalpy (ΔH), and entropy (ΔS) of thermal inactivation were calculated applying first-order rate constant (k) at various temperature values employing their standard equations. Data statistical analysis, plots, and k-value estimations were achieved employing GraphPad Prism software (Version 6.01). K_m and V_{max} values were estimated from Lineweaver–Burk plot applying GraphPad Prism.

3.5. Optimum pH and pH Stability of Laccase Preparations

Depending on the substrate and its redox potential, laccases have variable optimal pH levels. Using ABTS as a substrate, the influence of pH on the activity of both free and immobilized laccase, randomly or orientally (using Con-A), was studied. Laccase has shown the greatest action on ABTS in acidic conditions at pH 3.0 (Figure 4). The ideal pH of free and random immobilized laccase was 3.0, but the optimal pH of oriented immobilized laccase was altered to 4.0.

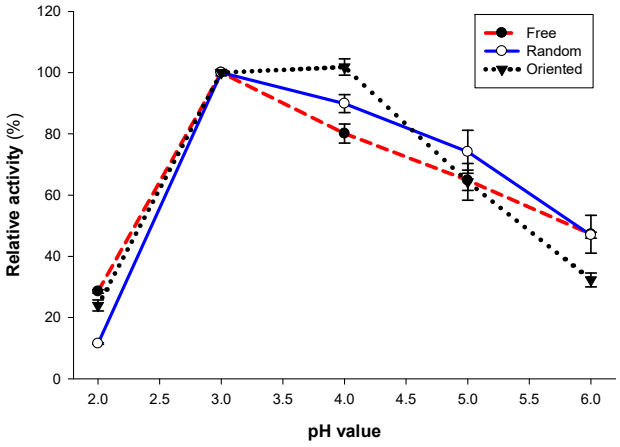


Figure 4. Effect of pH value on activity of free, random, and oriented laccases. pH values ranging from 2.0 to 6.0 were examined using Robinson buffer. ABTS was employed as substrate in pH optimum value estimations.

Figure 5 depicts the pH stability of various laccase preparations (free, orientated, and random immobilized laccases) at pH 3.0, 5.0, 7.0, and 9.0 (at 50 °C). Both free and random immobilized laccases are completely stable at pH 9.0 for 6 h, whereas the laccase residual activity decreased as the pH values decreased toward the acidic pH region, with the lowest residual activity at pH 3.0 (Figure 5a,b). Otherwise, oriented immobilized laccase has a higher percentage of stability at pH 7.0 than at pH 9.0 (Figure 5c), which could be due to the instability of the Immobead 150P–Con-A complex at pH 9.0, but the oriented immobilization process was performed at pH 7.0. It is worth noting that oriented immobilized laccase is more stable than both free and random immobilized laccases at pH 7.0 (Figure 5c).

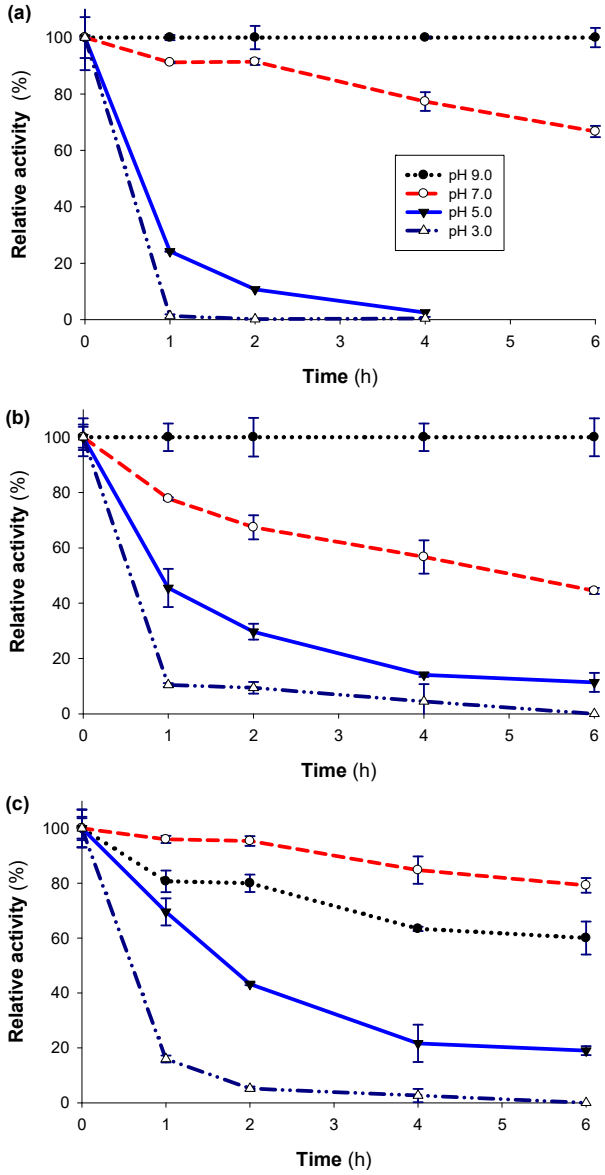


Figure 5. Laccase pH stability; (**a**) free, (**b**) random immobilized, and (**c**) oriented immobilized laccases. Enzyme preparations were preincubated at 50 °C for 6 h in different pH values (pH 3.0–9.0) using Robinson buffer (100 mM). Remaining laccase activity was determined in comparison with initial activity of each laccase preparation form according to standard assay conditions (pH 4.5 and 30 °C).

3.6. Kinetic Constants

Using ABTS as a substrate, the apparent K_m and V_{max} values of free, oriented, and random immobilized laccases were estimated. The Michaelis–Menten relationship was used to calculate the kinetic constants when the starting ABTS concentrations were varied while the laccase enzyme activity remained constant (Figure 6). The slope and intercept of the Lineweaver–Burk relationship were used to calculate the K_m and V_{max} values using GraphPad Prism (version 6.01). Free, random, and orientated immobilized laccases had K_m and V_{max} values of 0.040, 0.280, and 1.951 mM and 1.986, 1.723, and 10.74 µmol/min, respectively (Table 3), where the V_{max} value of the oriented laccase, 10.74 µmol min^{-1}, was the highest among other laccase preparations (free (1.986 µmol min^{-1}) and random immobilized (1.723 µmol min^{-1})) (Table 3 and Figure 6).

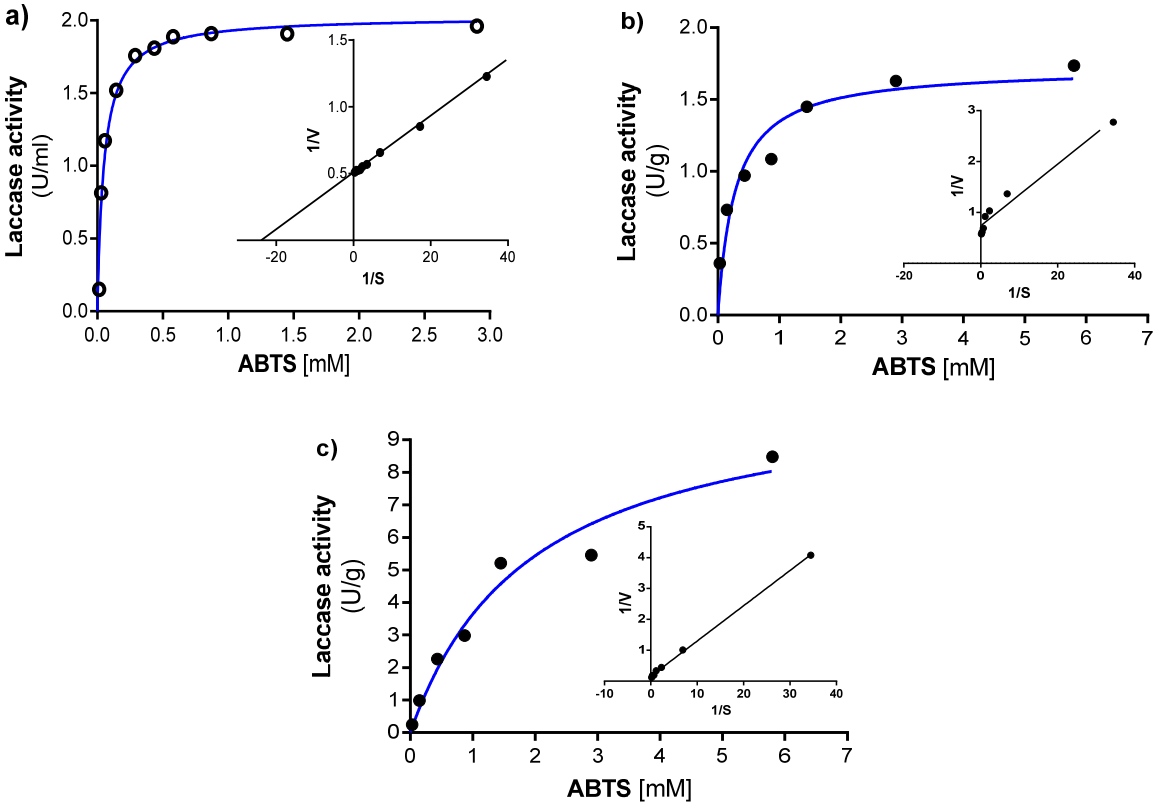


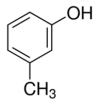
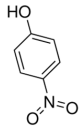
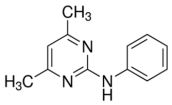
Figure 6. Kinetic constants of (**a**) free aminated, (**b**) random, and (**c**) oriented immobilized laccases. Serial concentrations of ABTS were reacted with laccase forms (in case of free enzyme: 0.029–2.9 mM; whereas in case of both random and immobilized laccase: 0.029–5.8 mM) at standard reaction conditions (30 °C and pH 4.5). K_m and V_{max} values were estimated from Lineweaver–Burk plot applying GraphPad Prism version 6.01.

3.7. Biodegradation of Some Compounds Using Random and Oriented Immobilized Laccase

The biodegradation of *m*-cresol, *p*-nitrophenol, and pyrimethanil as water-pollutant compounds utilizing the two immobilized (random and oriented) laccases was estimated. With a final concentration of 10 ppm, one unit of immobilized enzymes (random or oriented) was added to start the biodegradation reactions at 50 °C and pH 7.0 in the presence of 1 mM HBT for 3 h. The adsorption effect of the support was thought to be eliminated by using modified Immobead 150P as a control. The concentration of residual phenolics was measured using HPLC in an acetonitrile–water mobile phase. Table 4 shows that both immobilized laccases had a good clearance rate against all phenolic chemicals tested. A quick adsorption of phenolics by Immobead 150P was also evidenced, benefiting not just high removal efficiency but also, and more significantly, the comprehensiveness of the

removal, accounting for the removal of *m*-cresol, *p*-nitrophenol, and pyrimethanil by 84%, 92%, and 97%, respectively. The adsorption action of the support has the added benefit of intensifying enzymatic processes, especially the full conversion of a feeble target. The oriented and random immobilized laccase's enzymatic biodegradation action enhanced the proportion of phenolics removed (Table 4). This was visible in the biodegradation of *m*-cresol, where clearance percentages rose from 84% in control to 88% and 87% in random and oriented immobilized laccases, respectively.

Table 4. Biodegradation of some compounds using random and oriented immobilized laccases.

Compound	Structural Formula	Molecular Weight	EC Number	Water Solubility (g/L)	Treatment Type	Residual Contaminants (%)		
						0 h	1 h	3 h
m-Cresol		108.14	203-577-9	23.5 g/L at 20 °C	Random	100.00	11.53 ± 1.26	11.68 ± 0.76
					Oriented	100.00	12.23 ± 0.08	12.40 ± 0.15
4-Nitrophenol		139.11	202-811-7	11.6 g/L at 20 °C	Random	100.00	19.35 ± 2.13	21.22 ± 1.75
					Oriented	100.00	19.23 ± 0.09	19.00 ± 1.33
Pyrimethanil		199.25	414-220-3	0.121 g/L at 25 °C	Random	100.00	2.13 ± 0.08	1.99 ± 0.15
					Oriented	100.00	2.47 ± 0.33	2.10 ± 0.00

One unit of immobilized enzymes (random or oriented) was added to start separate biodegradation reactions against a final concentration of 10 ppm from m-cresol, *p*-nitrophenol, and pyrimethanil in a potassium phosphate buffer (pH 7.0, 0.1 M) at 50 °C in the presence of 1 mM HBT in a 5 mL total reaction mixture for 3 h. The concentration of phenolic compounds before and through the reaction was estimated via high-performance liquid chromatography (HPLC) analysis utilizing the acetonitrile–water mobile phase.

4. Discussion

The bioaffinity immobilization of enzymes is considered a gentle approach that has few harmful outcomes for the immobilized proteins. Furthermore, this course of action awards the enzymes an oriented immobilization approach that favors perfect demonstration of the enzymatic activity and reusability [50,51]. Different types of enzymes could be immobilized through various biospecific interlinkages, such as avidin–biotin, antibody–antigen, and lectin–sugar associations [52]. Laccase enzyme is a glycoprotein molecule, so it can be situated on supports on the basis of the interactivity between the glycosyl moiety of glycoprotein and Con-A [14]. For that reason, the current study aimed to find out the catalytic and stability differences between both random and oriented immobilized laccases in the absence and presence of Con-A, respectively. In the current study, *Myceliophthora thermophila* laccase was aminated to enhance the interaction between the support and amino groups of the enzyme [11]. Additionally, Immobead 150P was modified to hydrolyze its epoxy groups to aldehyde units. Then, Con-A was covalently attached to the modified Immobead 150P via primary amine functionalities [27]. Using those modified parts, aminated laccase was immobilized through random and oriented immobilization techniques. At pH 7.0, Con-A offers four glycosyl binding sites, which enhance the affinity forces between the enzyme and Con-A [44]. The obtained results indicate the superiority of the oriented immobilization technique using the aminated laccase over other immobilization trials. This could be due to the availability of more active sites due to the orientation approach.

Immobilization processes should enhance enzyme properties such as operational stability [15]. The data declare that the differences in operational stability depend on the utilized support due to the specific nature of each immobilization carrier and the enzyme

coupling sites. The oriented immobilization approach using aminated laccase preserved the highest operational stability, which could be due to the proposed orientation of the enzyme molecule. These outcomes are in line with those obtained using cross-linked aminated laccase on glyoxyl modified Immobead, where the immobilized laccase may be employed ten times without losing catalytic activity [18]. This preservation of catalytic competence points to the existence of robust bonds between the glyoxyl Immobead 150P and aminated laccase in the correct orientation, as well as the prevention of biocatalysts from leaking into the reaction system. In comparison with earlier strategies employing different carriers, the present immobilization techniques performed better. For instance, after seven cycles, laccase that had been immobilized on Amberlite IR-120 had an enzymatic activity that was 30% lower than it had initially been [53]. Additionally, after seven cycles of usage, a laccase-activated carbon composite retained between 10% and 30% of its initial laccase activity [54].

The laccase activity was regularly increased by the gradual increase in the reaction mixture temperature up to 70 °C, which was recorded as the optimal temperature value for all preparations. These results are consistent with that of free, adsorbed, and cross-linked immobilized *Thermothelomyces thermophilus* laccases on Immobead 150P, which showed that their peak activity was at 70 °C [18]. This could be due to the improvement of reaction kinetics between the enzyme active sites and substrate molecules. Subsequently, the laccase activity starts to decrease as a result of enzyme denaturation at upper reaction temperatures [55]. It is worthwhile to mention that oriented immobilized laccase is affected to a greater extent by the reaction temperature than the other two laccase forms, which could be due to its more complicated structure and the nature of the bond between Con-A and laccase molecules. Earlier studies regarding laccase optimum temperatures revealed that most fungal laccases have optimal temperatures between 50 and 60 °C [55,56], which declares the superiority of the laccase forms under study here in their higher optimum temperatures and, hence, their stability.

The $t_{1/2}$ values of different laccase forms were temperature-degree-dependent. The results obtained verified the superiority of oriented laccase thermal stability. The inactivation kinetic constant (k) values of oriented immobilized laccase are the lowest values among the developed laccase forms, which indicates the incident enhancement of oriented immobilized laccase thermal stability over both free and random immobilized forms. According to Li et al. [44], the enhanced thermostability of immobilized enzymes on single-walled carbon nanotubes (SWNTs)/n-dodecyl β-D-maltoside (DM)/Con-A carrier is owing to a particular affinity between Con-A on the carrier and the glycosyl on the enzymes' surface, which can stabilize the enzymes' 3D structure. The thermodynamic parameters of the thermal inactivation of laccase forms were studied. The activation energy (E_a) of the oriented immobilized laccase was higher than those of the free and random immobilized laccases, which declares the highest thermal stability of the oriented immobilized laccase. The positive free energy (ΔG) and standard enthalpy (ΔH) values for free, random, and oriented immobilized laccases prove the endothermic and nonspontaneous process of enzyme thermal inactivation [18,57]. ΔH and ΔS are temperature-dependent and range from huge negative values to huge positive values via temperatures appropriate to biology (0–100 °C) [58]. All three laccase forms showed ΔS negative values to different degrees. The negative values of the standard entropy change (ΔS) are an indication of the more ordered transition states (after substrate binding) than the equivalent ground (reactant) states [59].

Depending on the substrate and its redox potential, laccases have variable optimal pH levels [12]. Laccases have shown the greatest action on ABTS in acidic conditions at pH 3.0. This is consistent with the findings of Schückel et al. [60], Othman and Wollenberger [12], and Othman et al. [18], who found that using ABTS as a substrate, the optimal laccase activity from *Marasmius* sp., *Coriolus hirsuta*, and *Thermothelomyces thermophilus* was reported at pH 3.0. Hydroxyl ions block T_2 and T_3 copper domains in alkaline circumstances and prevent electron transport across catalytic sites [61]. The ideal pH of free and random immobilized laccase was 3.0, but the optimal pH of oriented immobilized laccase was

altered to 4.0, suggesting that an electrostatic interaction developed between both the protein and matrix microenvironment. Furthermore, changes in the enzyme's dissociation and ionization state throughout the immobilization process might have resulted in pH alterations [62].

Oriented immobilized laccase has a higher percentage of stability at pH 7.0 than at pH 9.0, which could be due to the instability of the Immobead 150P–Con-A complex at pH 9.0, but the oriented immobilization process was performed at pH 7.0. It is worth noting that oriented immobilized laccase is more stable than both free and random immobilized laccases at pH 7.0. Even though pH stability significantly varies depending on the enzyme source [63], laccases from fungal sources are typically stable at a slightly acidic pH [64]. Laccases from *Monilinia fructicola* [65], *Thielavia* sp. [66], and *Colletotrichum lagenarium* [67] are, therefore, stable in an acidic pH range of 3.0–5.0, whereas *Trametes* sp. laccase functions in an alkaline pH range of 7.0–9.0 [68]. Numerous homologous laccases from *Shiraia* sp. SUPER-H168 (pH 4.0–9.0) [69], *Elaeocarpus sylvestris* (pH 4.0–10.0) [70], and *Chaetomium* sp. (pH 4.0–9.0) [71] showed a wide pH-stability range. Additionally, laccase from *Bacillus* sp. was extremely stable throughout pH 5.0 and 10.0, retaining more than 80% of activity across pH 7.0 and 10.0 for up to 24 h [72].

Using ABTS as a substrate, the obtained K_m values of free and random immobilized laccases toward ABTS were lower than those of laccases from *Cryptococcus albidus* (K_m = 0.8158 mM) [73], *Colletotrichum lagenarium* (K_m = 0.34 mM) [67], and *Kabatiella bupleuri* G3 IBMiP (K_m = 0.58 mM) [63]. The low K_m values suggest that laccase preparations have a strong propensity for ABTS oxidation. The occurrence of steric hindrance as a result of the Con-A protein employed in this type of immobilization is a plausible cause for such high K_m values of orientated laccase. According to Xu et al. [74], this might be due to the hampered transfer of the substrate from the bulk liquid phase to the support saturated with Con-A agglutinated enzyme. On the other hand, the V_{max} value of the oriented laccase, 10.74 μmol min^{-1}, was the highest among other laccase preparations. Using poly(ethylene glycol) dimethacrylate cryogel functionalized by Con-A, Altunbaş et al. [38] showed that the V_{max} value of free enzyme (3.59 μmol/min) was greater than that of immobilized enzyme (0.58 mol/min). They interpreted the reduction in immobilized enzyme V_{max} as a result of the substrate's nonspecific affinity for the cryogel support [38].

The biodegradation of *m*-cresol, *p*-nitrophenol, and pyrimethanil, as pollutant phenolic compounds utilizing the two immobilized (random and oriented) laccases, was estimated. The results show that both immobilized laccases had a good clearance rate against all phenolic chemicals tested. A quick adsorption of phenolics by Immobead 150P was also evidenced, benefiting not just high removal efficiency but also, and more significantly, the comprehensiveness of the removal, accounting for the removal of *m*-cresol, *p*-nitrophenol, and pyrimethanil. The adsorption action of the support has the added benefit of intensifying enzymatic processes, especially the full conversion of a feeble target. The oriented and random immobilized laccase's enzymatic biodegradation action enhanced the proportion of phenolics removed. This strong adsorptive capacity of Immobead 150P was substantially higher than previously observed using immobilized laccase on Con-A-coated activated carbon (AC), where the individual AC particles adsorbed 55% of the phenol, but the AC–Con-A–laccase combination removed 90% of the phenol [74].

5. Conclusions

In conclusion, we presented a two-step procedure to immobilize laccase enzyme on Immobead 150P with the application of Con-A. The distinct advantage of this procedure is that it dramatically reduces both enzyme leakage and enzyme deactivation. This significant change is attributed to the presence of Con-A, which immobilizes glycoproteins via molecular recognition of their glycosidic residues and, in the meantime, shields the unfavorable interactions between enzymes and the support surface and thus prevents enzyme deactivation. Using laccase as a model enzyme, we demonstrated the effectiveness of this immobilization procedure. The immobilized enzyme greatly extends reusability and

operational stability in terms of both temperature and pH. Moreover, the Immobead 150P intensifies the enzymatic process through enhanced substrate uptake, as shown in the case of phenol removal, which is promising for diverse future applications such as conversion of volatile or diluted substrates. Furthermore, the biodegradation ability of the developed immobilized laccase against tested pollutant compounds ensures that it has high removal efficiency, which emphasizes the capability of the developed biocatalyst as an effective ecological technology for sustainable wastewater treatment.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/su142013306/s1, Figure S1: Detection of decrease in protein and activity during oriented and random laccase immobilization using Concanavalin A on modified Immobead 150P.

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