Site-Specific Covalent Immobilization of *Methylobacterium extorquens* Non-Blue Laccase Melac13220 on Fe₃O₄ Nanoparticles by Aldehyde Tag

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Abstract: In the present study, the non-blue laccase Melac13220 from *Methylobacterium extorquens* was immobilized using three methods to overcome problems related to the stability and reusability of the free enzyme: entrapment of the enzyme with sodium alginate, crosslinking of the enzyme with glutaraldehyde and chitosan-, and site-specific covalent immobilization of the enzyme on Fe₃O₄ nanoparticles by an aldehyde tag. The site-specific covalent immobilization method showed the highest immobilization efficiency and vitality recovery. The optimum temperature of Melac13220 was increased from 65 °C to 80 °C. Immobilized Melac13220 showed significant tolerance to some organic solvents and maintained approximately 80% activity after 10 cycles of use. Differential scanning calorimetry (DSC) indicated that the melting temperature of the enzyme was increased (from 57 °C to 79 °C). Immobilization of Melac13220 also led to improvement in dye decolorization such that Congo Red was completely decolorized within 10 h. The immobilized enzyme can be easily prepared without purification, demonstrating the advantages of using the aldehyde tag strategy and providing a reference for the practical application of different immobilized laccase methods in the industrial field.

Keywords: laccase; immobilization; covalent binding; entrapment; thermal stability

1. Introduction

In recent years, with the increasing awareness of environmental protection, the restriction of relevant laws, and increasing scientific knowledge, many industries have devoted themselves to developing green chemical technology via enzymatic methods. The traditional physical and chemical methods widely used in the past have several potential shortcomings, such as high overall cost, low efficiency, and large amounts of sludge and toxic products [1]. In this case, the enzyme is a multifunctional biocatalyst that shows strong chemical selectivity, stereoselectivity, and regioselectivity under mild conditions and is a good substitute for traditional chemical catalysts [2]. The enzyme industry was worth more than $7 billion in 2017 and is expected to reach $10.5 billion by 2024 [3]. Common laccases (EC 1.10.3.2) are four copper-containing polyphenol oxidoreductase enzymes. These four copper ions can be divided into three copper centers according to their spectral properties and are used as “green” biocatalysts for environmental protection and energy saving in many fields. Laccase is considered one of the most powerful biocatalysts and can be used in a variety of biotechnology applications, such as dye decolorization in the textile industry, clarification in the food industry, organic synthesis in the chemical industry, and bioremediation of endocrine disruptors inhibition of the proliferation of cancer cells [4–8]. However, due to the presence of Cu atoms on its catalytic site, the final biocatalyst has low stability, difficult product recovery, and low reusability under industrial conditions,
and the use of laccase is more expensive, resulting in the application of laccase not being expanded. Therefore, improving the stability and reusability of laccase is an important direction for laccase-related research.

With the development of enzyme modification technologies and tailoring techniques, many strategies have been applied to improve the efficiency of enzyme biocatalytic systems and reduce the high cost of enzyme catalysis processes. Among them, the immobilization of enzymes is one of the most promising, economical, and attractive strategies for inducing the stability, reusability, and remarkable resistance of enzymes. The process of enzyme immobilization can promote the recycling of enzymes, which reduces the cost of the process by 50% [9]. Moreover, the immobilization process can reduce enzyme aggregation, producing a favorable microenvironment and enhancing the rigidity of the enzyme structure, thereby protecting the enzyme against self-proteolysis and denaturing agents and thus improving the catalytic performance of the enzyme [10]. Enzyme immobilization limits the enzyme to a phase (matrix or support) different from the substrate and products [11].

Immobilization depends on the choice of support material, biocatalyst, and immobilization methods. Since the first commercialization of enzyme immobilization in the 1960s [12], attempts in this field have realized several methods of enzyme immobilization, such as absorption, entrapment/encapsulation, crosslinking, and covalent binding. The adsorption was the oldest and preferred physical method for biocatalyst immobilization, which refers to the adsorption of laccase to the surface of the support and the interior of the pores through low-energy intermolecular forces, such as ionic bonds, hydrogen bonds, and van der Waals forces [13]. The preparation conditions of the adsorption method are mild and simple, and most supports are cheap and easy to obtain. Entrapment/encapsulation refers to the immobilization of laccases in semipermeable polymer membranes or polyhedral structures, highlighting that the native structure of the enzyme is not damaged. The crosslinking method refers to using a multifunctional reagent to react with the amino or carboxyl groups of the enzyme molecules to associate the enzyme molecules with each other to form water-insoluble aggregates [3]. Covalent binding is one of the most widely used methods and refers to the formation of covalent bonds between functional groups (tyrosine, amino groups in histidine, carboxyl, or aromatic rings) on the surface of enzyme molecules and groups on the surface of the support [13]. If there is a suitable group on the surface of the support, it can be directly covalently bonded to the laccase; if there is no suitable group on the surface of the support, the support can obtain a group by some activation method and then covalently bond with the enzyme molecule. Compared to other methods, this method makes the binding of the support and the enzyme molecule stronger. Covalent immobilization usually includes nonspecific and site-specific covalent binding. Of these, site-specific immobilization introduces some special functional groups through reasonable design and modification to allow site-specific binding between the enzyme and supports. After immobilization, due to changes in the functional structure of proteins around the active site, the activity of enzymes usually decreases [14]. In addition, immobilization of enzymes, especially covalent attachment, may enhance the rigidity of enzymes and lead to the dispersion of enzymes on the surface of the support, thus preventing their aggregation and improving the thermal stability of the enzymes. Therefore, it is necessary to develop site-specific covalent immobilization methods to maintain the inherent functions of enzymes. For any enzyme, however, determining the optimal immobilization method and immobilization support has always been a challenge. The immobilization method is influenced by the different chemical properties and structures of the enzymes.

The early approach to protein modification was to react with excess thiol or amine-based reagents by utilizing the reaction of cysteine and lysine residue functionalization of protein. For example, formylglycine-generating enzyme (FGE) found in eukaryotes catalyzes the oxidation of specific cysteine residues in nascent sulfatase polypeptides into formyl-glycine (FGly) with an aldehyde group by specifically recognizing the conversed sequence CXPXR or LCXPXR (refer to “aldehyde tag”). The aldehyde side chain of FGly can react with the amino active functional group on the carrier to form new chemical bonds,
and the polypeptide sequence CXPXR or LCXPXR can be fused to the amino or carboxyl terminal of the target protein without affecting the protein functions. Therefore, FGE- and aldehyde-based tags have become favorable tools for the site-specific modification of proteins and are widely used, for instance, in protein immobilization [15,16] and the attachment of drugs to antibodies [17].

To develop enzymes for various industrial applications, it is necessary to find more suitable and inexpensive supports with simple immobilization processes, high immobilization yields, and good enzyme recovery rates. Therefore, a large number of various supporting materials, such as organic materials, inorganic natural and synthetic polymers, hybrid materials, and composite materials, have been developed [18]. In recent years, with their unique superparamagnetism, biocompatibility, biodegradability, and low cytotoxicity, magnetic nanoparticles (MNPs) have attracted a great deal of attention, followed by low-energy magnetic isolation [19,20]. The magnetic properties of these materials enable the encapsulated enzymes to be easily separated from the reaction medium, thereby rapidly terminating the enzymatic reaction and recovering the enzyme for further use. The most common biocompatible magnetic nanomaterials are pure iron oxide, such as magnetite ($\gamma$-Fe$_2$O$_3$) and magnetite (Fe$_3$O$_4$) [21]. With a fast response to applied magnetic fields, these compounds can be easily separated and recovered from the reaction system, thus improving the catalytic efficiency of the immobilized enzyme. However, direct immobilization of enzymes on these magnetic nanoparticles is usually limited because bare/naked MNPs tend to aggregate and exhibit high reactivity and are highly sensitive to acid and oxidation conditions. In addition, they are easily oxidized to $\gamma$-Fe$_2$O$_3$, leading to loss of magnetism and dispersibility, and cannot provide good interactions with enzymes owing to the lack of suitable surface functional groups [20,22]. For these reasons, surface functionalization may help prevent the aggregation and oxidation of Fe$_3$O$_4$ nanoparticles and achieve the efficient immobilization of enzymes. Generally, surface functionalization of MNPs can be achieved using binding compounds containing amino (NH$_2$) hydroxide (OH), carboxylic acid (COOH), or phosphate functional groups in their structures, which can immobilize biomolecules, such as proteins, enzymes, or antibodies. On this basis, Fe$_3$O$_4$ magnetic nanoparticles covered by amino-modified SiO$_2$ (SiO$_2$-Fe$_3$O$_4$-NP) with a particle size of 100 nm were used in this study, which provided the conditions for the covalent binding of the enzyme.

Chitosan is a natural biopolymer of a 2-amino-2-deoxy-\(\beta\)-d-glucose unit connected by a \(\beta\)-1,4-glycosidic bond that is widely accepted due to its advantages, such as strong practicability, low cost, and simple modification. It is biorecognizable because it is usually obtained from chitin, which is present in cellular fungi and bones [3]. In addition, chitosan also has structural properties, such as strong mechanical strength and good affinity for proteins. Because the surface of chitosan has an active group, -NH$_2$, it can be used to immobilize enzymes, but to increase the immobilization efficiency, the covalent coupling of biocatalysts to chitosan substrates is usually accomplished by the reaction of the cross-linking agent (glutaraldehyde) with the amino group [23]. Alginate has good biodegradability and economic benefits and is currently a commonly used polymer for immobilizing enzymes. Laccase is encapsulated in an alginate matrix combined with divalent ions, such as Ba$^{2+}$, Ca$^{2+}$, and Cu$^{2+}$, as cross-linking agents [11].

Recently, we effectively expressed the non-blue laccase gene, Melac13220, derived from Methylobacterium extorquens in Escherichia coli (E. coli) carriers and successfully produced extracellular recombinant laccase, avoiding the formation of inactive inclusion and promoting the subsequent purification process. In our previous study [24], the enzyme was endowed with some atypical characteristics different from the general laccase, such as no absorption peak at 610 nm and remaining silent within electron paramagnetic resonance spectra. Additionally, both inductively coupled plasma spectroscopy/optical emission spectrometry (ICP-OES) and isothermal titration calorimetry (ITC) indicated that one molecule of Melac13220 can interact with two iron ions. The optimum temperature of Melac13220 was 65 °C, which indicates that Melac13220 is a thermophilic enzyme, but
its activity was almost reduced to zero because it easily aggregates after being stored. Based on our previous work, we tried to introduce an aldehyde group into Melac13220 from *Methylobacterium extorquens* at the N-terminal, C-terminal, and both terminals with a special carrier system. The immobilized enzyme was also obtained using covalent immobilization of SiO$_2$-Fe$_3$O$_4$-NP with aldehyde-labeled Melac13220 via Schiff base reaction. Three methods—namely entrapment, cross-linking, and covalent binding—were used to immobilize Melac13220 laccase to compare the immobilization effects of different methods. Understanding various immobilization methods would provide an informed choice for the immobilization of Melac13220 and an optional choice for the practical application of different immobilized laccase methods in the industrial field.

2. Results and Discussion

2.1. Expression of the Aldehyde Tag Protein

The aldehyde tag (LCTPSR) was ligated to the C-terminal, the N-terminal, or both terminals of Melac13220 using recombinant plasmid pET28a. The specific activity of modified and origin Melac13220 are listed in Table 1. The C-terminal tag (Melac13220-CQ) showed increased enzyme activity. However, the amino terminal tag (Melac13220-NQ) and the double terminal tag (Melac13220-DQ) exhibited evidently reduced enzyme activities consistent with the results reported by Hui et al. and Lyu et al. [15,16]. The polypeptide sequence tag may slightly alter the structure of Melac13220 during protein folding [16].

Table 1. The specific activity comparison of modified and original Melac13220.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Enzyme Specific Activity (U/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Melac13220</td>
<td>515.52</td>
</tr>
<tr>
<td>Melac13220-DQ</td>
<td>121.20</td>
</tr>
<tr>
<td>Melac13220-NQ</td>
<td>183.03</td>
</tr>
<tr>
<td>Melac13220-CQ</td>
<td>602.87</td>
</tr>
</tbody>
</table>

Additionally, while the C-terminal tag improved protein expression, the amino terminal and double terminal aldehyde tags decreased protein expression (Figure 1) compared to wild-type proteins. In general, protein folding, activity, and expression tend to be easily affected when exogenous sequences are inserted and expressed at the N-terminal of natural enzymes [25]. It can be inferred that the N-terminal aldehyde tag might affect the folding and expression of proteins, then affect their activity. Fusion aldehydes were used for site-specific protein modification and can be identified with an aminooxy-functionalized probe. Aldehyde-labeled proteins were incubated with Alexa Fluor 647 C5-aminooxy-acetamide and reactions analyzed by the Odyssey NIR Laser Imaging System. The wild-type protein without the aldehyde tag showed absence of signal, and the protein signal of the C-terminal aldehyde tag was the strongest, while the protein signals of the N-terminal and double-terminal tags exhibited weaker signals, similarly to the results reported by Hui et al. [15]. Therefore, Melac13220-CQ was selected for subsequent immobilization.

2.2. Optimization of Conditions for Immobilization

Although similar immobilization methods were used in enzyme immobilization, the results of different enzymes may be different. Therefore, the immobilization efficiency results reported by each study are very specific and difficult to replicate unless the same enzyme (i.e., commercial enzyme) or a similar enzyme is used (produced at the laboratory scale from an established microorganism stored in culture) with comparable biochemical profiles. The efficiency of the biocatalyst goes to the immobilization method or to different compounds used. Because of the above reasons, the immobilization method should be adjusted or optimized (assessed by parameters such as initial enzyme activity, pH, temperature, or reaction time), respecting the nature of the natural enzyme, to obtain satisfactory immobilization conditions [26].
Figure 1. Expression and analysis of molecular weight of different modified Melac13220 using SDS-PAGE. Lane M: protein marker; lane 1: Melac13220; lane 2: Melac13220-DQ; lane 3: Melac13220-NQ; lane 4: Melac13220-CQ.

2.2.1. Optimization of Conditions for Site-Specific Covalent Immobilization

Site-specific immobilization refers to the introduction of some special functional group into the enzyme protein through rational design of the protein. The use of these functional groups makes the enzyme specifically bind to the specific site of the carrier. This method has become popular in studies because of its ability to improve enzyme activity, stability or catalytic specificity. In this study, we used the site-specific covalent immobilization method. Through the rational design and modification of the enzyme, a specific functional group—the aldehyde group—was introduced into the enzyme protein, and the functional group was covalently immobilized with the amino carrier. For site-specific covalent immobilization, to optimize the reaction temperature, immobilization was carried out in glycine-HCl buffer (pH 2.5) from 10 °C to 30 °C for 6 h. The immobilized enzyme was separated by a magnet, and its activity was determined using the ABTS oxidation method. Figure 2a shows that the optimum immobilization temperature is 20 °C. To determine the optimal pH value of immobilization, the process was carried out in various buffers with pH range of 1.0–9.0 at 20 °C for 6 h. To determine whether only the pH of the buffer or also its composition affects the activity of laccase, the effect of the same pH values on the activity of the enzyme was measured with different buffer compositions, and the results showed that the buffer composition had little effect on the activity of laccase. The immobilized enzyme was separated by a magnet, and its activity was determined by the ABTS oxidation method. Figure 2b shows that the optimum pH value of immobilization was pH 2.5. The amount of enzyme loaded on the carrier is an important factor in the immobilization process. By keeping the same number of nanoparticles in different samples, this factor was optimized. Thus, every sample contained 5 mg of carriers, and the enzyme (from 0.5 mg to 3.0 mg) was loaded in 2.5 mL of glycine-HCl buffer (pH 2.5) at 20 °C for 6 h. Figure 2c shows the trend between the amount of enzyme added and the relative activity of the immobilized enzyme. The optimal enzyme amount added was 2.5 mg per 5 mg of carrier. In other words, the optimal loading amount of 500 mg/g. A higher enzyme/carrier ratio may cause enzyme aggregation on the carrier and hide some active sites, thus reducing the activity of the enzyme. Figure 2d shows the effect of reaction time on the activity of immobilized Melac13220. The enzyme activity gradually decreased after more than 6 h. This phenomenon may be caused by overcrowding of enzyme molecules on the surface of nanoparticle, as substrate diffusion may be restricted.
2.2. Optimization of Entrapment Conditions for Melac13220

We visually assessed the production of suitable alginate beads and chitosan beads. The correct bead formation process is when the biopolymer is dropped into the solution and gelling occurs immediately in the water bath, obtaining spherical and regular shapes and sinking to the bottom of the container. Bead formation conditions were considered incorrect when the droplets disintegrated upon contact with the cross-linking solution or when the beads formed irregular shapes or floated on the surface of the cross-linking solution. Empty beads without enzymes were formed in the same way without adding enzymes to the polymer solution. The higher the concentration of sodium alginate, the higher the viscosity, and the operation of preparing gel beans were more difficult; the shape was changed from a spherical shape at the beginning to a trailing bean. According to Figure 3a, it showed that the concentration increased from 0.5% to 2.5%; the enzymatic activity of the immobilized enzyme also increased. When the concentration was higher than 2.5%, the immobilized enzyme activity was decreased. As shown in Figure 3b, the optimum concentration of CaCl$_2$ was 2.5%. When the concentration was low, the immobilized gel was relatively loose, the gel pore size was relatively large, which resulted in the enzyme leaking from the beads. However, when the concentration of CaCl$_2$ was too high, the Ca$^{2+}$ distributed on the surface of the gel could cause the reducing activity of immobilized enzymes. As can be seen from Figure 3c, when the amount of enzyme solution and sodium alginate was 1:4 (v/v), the highest immobilization efficiency was obtained. At 1:2 (v/v), the resulting beans were smaller. When the ratio was higher, the immobilization efficiency decreased linearly. Because of the solution having a high viscosity, bean preparation became more difficult, and some trailing beans were seen. For laccase entrapment, Melac13220 laccase was added to sodium alginate solution (2.5% w/v) at a ratio of 1:4 (v/v) and mixed thoroughly by slight shaking. The viscous alginate–enzyme mixture was added dropwise into 0.2 M copper sulfate and (2.5% w/v) calcium chloride, respectively, and stirred with a syringe with a diameter of 0.4 mm to produce beads.

Figure 2. The optimization of site-specific covalent condition for Melac13220; (a) the optimization of temperature; (b) the optimization of pH; (c) the effect of enzyme loading rate on the activity of immobilized Melac13220; (d) the effect of reaction time on the activity of immobilized Melac13220. All experiments were performed in triplicate, and the results represent mean ± standard deviation.
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**Figure 3.** The optimization of entrapment conditions for Melac13220; (a) the optimization of sodium alginate concentration; (b) the optimization of calcium chloride concentration; (c) the optimization of enzyme/alginate ratio. All the results are represented as the means ± standard deviation from at least three independent experiments.

### 2.2.3. Optimization of Crosslinking Conditions for Melac13220

For the crosslinking immobilization of laccase, chitosan was dissolved in 1.5% acetic acid solution by heating and continuous stirring at 50 °C; 2.5% (w/v) chitosan was used to develop beads with better mechanical strength (Figure 4). The chitosan mixture was extruded into 1 M KOH solution drop by drop and hardened for several hours at room temperature then filtered and activated by incubation with 2.0% (v/v) glutaraldehyde solution at room temperature for 3 h, and high-equality beads with uniform size and shape were collected (Table 2). The glutaraldehyde activated chitosan beads provided a biocompatible carrier surface. Finally, the beans were incubated with enzyme solution (1 mg/mL) at 4 °C with 24 h with the highest immobilization efficiency of 74.7%. When glutaraldehyde reacts with chitosan, the aldehyde group on the surface of the support may react with the amino group of the enzyme and other functional groups on the surface such as phenols, thiols, and imidazole [27]. In addition, the reaction of glutaraldehyde with aminoglycan chitosan also promotes the cross-linking of various polymer chains, improves the mechanical resistance of the carrier, and avoids its solubilization in an acidic water environment due to
its cationic nature [28]. The mechanism of the reaction between glutaraldehyde and the amino group of the enzyme may involve Schiff base, Michael addition, and nucleophilic substitution [27]. At the lower concentrations of glutaraldehyde, fewer aldehyde groups were formed, resulting in lower immobilization efficiency. However, in the higher concentrations, especially beyond 2.5%, immobilization efficiency was decreased, which may be due to steric hindrance caused by the large number of cross-linking points between the chitosan surface and the enzyme molecules [23].

![Figure 4. Influence of different chitosan concentrations on Melac13220 immobilization efficiency of chitosan beads. All the results are represented as the means ± standard deviation from at least three independent experiments.](image)

**Table 2. The optimization of glutaraldehyde concentration and activation time for laccase immobilization on chitosan beads.**

<table>
<thead>
<tr>
<th>Glutaraldehyde (%)</th>
<th>Activation Time (h)</th>
<th>Immobilization (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.0</td>
<td>3.0</td>
<td>51.0 ± 3.16</td>
</tr>
<tr>
<td></td>
<td>6.0</td>
<td>48.2 ± 1.87</td>
</tr>
<tr>
<td>1.0</td>
<td>3.0</td>
<td>57.4 ± 2.66</td>
</tr>
<tr>
<td></td>
<td>6.0</td>
<td>58.0 ± 3.15</td>
</tr>
<tr>
<td>2.0</td>
<td>3.0</td>
<td>74.7 ± 2.78</td>
</tr>
<tr>
<td></td>
<td>6.0</td>
<td>69.4 ± 3.29</td>
</tr>
<tr>
<td>3.0</td>
<td>3.0</td>
<td>66.3 ± 2.69</td>
</tr>
<tr>
<td></td>
<td>6.0</td>
<td>62.0 ± 1.58</td>
</tr>
<tr>
<td>4.0</td>
<td>3.0</td>
<td>60.2 ± 3.18</td>
</tr>
<tr>
<td></td>
<td>6.0</td>
<td>56.0 ± 2.19</td>
</tr>
</tbody>
</table>

### 2.3. Characterization of Three Immobilized Enzymes

Activity recovery is an important factor governing the cost of immobilization. As shown in Table 3, the activity recovery of SiO$_2$-Fe$_3$O$_4$-Melac13220-CQ was up to 76.18%, followed by chitosan-bound Melac13220 (~50%), and entrapment led to the lowest recovery rate of approximately 37.81%. Previous studies have reported similar declines in activity recovery [29,30], indicating that the enzyme structure was not affected during the immobilization process. However, several studies have shown an increase in activity recovery [31]. Ibrahim et al. showed that compared to physical adsorption, covalent immobilization has higher immobilization efficiency and activity recovery, but the activity of immobilized enzymes was lower than that of free enzymes [32]. Covalent binding provides a strong linkage between the enzyme and the carriers, preventing the release of the enzyme into the reaction environment.
Table 3. The immobilization vitality recovery rates of immobilized laccases.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Immobilization Efficiency(%)</th>
<th>Activity Recovery Rate (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SiO2-Fe3O4-Melac13220-CQ</td>
<td>89.93</td>
<td>76.18</td>
</tr>
<tr>
<td>E-Melac13220</td>
<td>78.53</td>
<td>50.30</td>
</tr>
<tr>
<td>C-Melac13220</td>
<td>74.70</td>
<td>37.81</td>
</tr>
</tbody>
</table>

The optimum pH, optimum temperature and thermal stability of immobilized Melac13220 were evaluated under the optimum conditions. The pH determines the ionized state of the amino acid and therefore significantly affects the structure and activity of the enzyme. On one hand, solution pH affects the surface charge of the enzyme, and on the other hand, it affects the expression of enzyme activity. Improper pH environment will lead to the loss of enzyme activity even to enzyme inactivation. The optimum pH value of the free laccase was 1.5, and the optimum pH value of the immobilized laccase was 2.5 (Figure 5a). The immobilized laccase has a wide range of pH activity compared with the free laccase. At pH 5.0, the free laccase was almost completely inactivated, but the residual activity of immobilized laccases exceeded 60%, and the activity of entrapped laccase was the highest. At low pH, the enzyme molecule is usually positively charged, and the distribution of the charge on the surface of the enzyme molecule is uniform. At higher pH values, the enzyme molecules are negatively charged. Alginate fibers in aqueous solutions are characterized by a negative charge, which is due to the interaction of the microfibrils with water molecules and the presence of a set of OH bonds on the fiber surface. Therefore, a lower pH can increase the immobilization rate of laccase in the alginate carrier, which is a result of the electrostatic charge interaction of laccase with alginate fibers. The greater the pH value is, the greater the structural change and diffusivity of sodium alginate and the faster the enzyme leaching process [33].

The optimum temperatures of free laccase and site-specific immobilized Melac13220 were 65 °C and 80 °C, respectively (Figure 5b); the site-specific covalent immobilization caused an increase in the optimum temperature of 15 °C. The thermal stability of free and immobilized laccase was studied at 70 °C. After incubation for 4 h, the activity of the immobilized enzyme remained over 50%. However, the free enzymes retained less than 20% of their enzyme activity after 4 h (Figure 5c). The comparison of the thermal stability of different immobilized enzymes clearly showed that the thermal stability of the covalently immobilized enzyme was significantly improved (Figure 5c). The improved thermostability was attributed to the conformational mobility of Melac13220 being restricted after immobilization.

Considering the immobilization efficiency and vitality recovery effect, SiO2-Fe3O4-Melac13220 was selected for the following study. The higher-order structure of the protein will be destroyed under high-temperature conditions, and the catalytic activity of the enzyme depends on its higher-order structure. The protein is denatured at high temperatures, and laccase immobilization protects the tertiary structure of the enzyme protein and keeps it active at high temperature, proving that immobilization plays an important role in the stabilization of the enzyme conformation. The increase in thermal stability through the immobilization of proteins can also be reflected in increasing the thermal transition temperature \( T_m \) (the maximum \( T_m \) corresponding temperature of each thermal spectrum curve leads to protein unfolding), and the transition of the protein from its native denaturation state is accompanied by the rupture of intramolecular and intermolecular bonds. Figure 6a shows that Melac13220 denatured since 40.6 °C. After immobilization, the thermal stability of Melac13220 increased, the \( T_m \) was 78.7 °C (Figure 6b). Two main differences were observed between the thermograms of free and immobilized Melac13200. (1) The immobilized Melac13220 showed a wider thermal denaturation curve than the native enzyme; (2) all the immobilized enzymes denatured above the \( T_m \) of the free enzyme up to over 90 °C. These results indicate that immobilized Melac13220 appears to be more stable than free Melac13220 and that immobilization can prevent the aggregation of enzyme during heat
treatment, thus preventing the denaturation of enzymes. Generally, immobilization can improve the thermal stability of enzymes by preventing subunit dissociation, reducing aggregation, autolysis or proteolysis, enhancing the rigidity of enzymes and providing a favorable microenvironment [10]. Additionally, no more than one aldehyde was labeled at the end of the enzymatic peptide chain, and the site was used up when the recombinant aldehyde-tagged enzyme was covalently coupled to a carrier. Without an excess of the active group for the enzyme, there was no possibility of enzyme inactivation because the subsequent reaction between the enzyme and the carrier was slower. The biocompatible amino group on the carrier cannot combine with the group of the recombinant enzyme to destroy its structure, resulting in the good residual activity of the immobilized enzyme [34].

Figure 5. Effect of pH (a), temperature (b) on the activity and thermostability (c) of the free and immobilized Melac13220. All the results are represented as the means ± standard deviation from at least three independent experiments.
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![Graph of DSC](image)

**Figure 6.** The curve of DSC. The melting temperature ($T_m$) and enthalpy of melting ($\Delta H_l$) of free (a) and immobilized laccase (b).

Organic solvents are the most commonly used nonaqueous biocatalyst media [35]. Therefore, a better understanding of the activity profile of laccases in organic solvents can contribute to identifying the most suitable reaction media for a given biocatalyst. The effects of organic reagents on the activity of the two enzymes were different, and the results are shown in Figure 7. For free Melac13220, enzyme activity was inhibited by all tested organic solvents, while for immobilized enzymes, DMF and acetonitrile showed no inhibitory effects. The organic solvent peels off the tightly bound water layer from the enzyme, which leads to the disturbance of the enzyme structure and has a harmful effect on laccase activity [36]. Therefore, the improvement of the resistance of immobilized laccase indicates that magnetic carriers can enhance the rigidity of the enzyme and prevent the conformational transformation of laccase in organic solvents [37]. The polarity of acetonitrile and DMSO was greater than that of methanol, ethanol, and acetone. Therefore, the effects of acetonitrile and DMSO on the activity of free laccase were higher than that of other solvents. In contrast, the adverse effect of solvent on the activity of immobilized laccase was not obvious. This may be because the removal of aprotic polar solvent from the water surface of laccase was prevented by the immobilization of the enzyme, thus reducing the folding and subsequent denaturation of laccase [36].
Although there are common concerns about reduced enzyme flexibility, steric hindrance, enzyme deactivation, and protein leakage when shaking in reaction and recycling, the enzyme retained 81.1% of its initial activity (Figure 8). The loss of activity may be due to enzyme deactivation and protein leakage after the completion of ten substrate oxidation operations. After 10 cycles, the immobilized laccase showed residual activity of 96.8% and 92.6% after three and five cycles of reuse, respectively. Notably, more than 80% of the activity of the immobilized laccase was observed even after the completion of ten substrate oxidation operations. After 10 cycles, the immobilized enzyme retained 81.1% of its initial activity (Figure 8). The loss of activity may be due to enzyme deactivation and protein leakage when shaking in reaction and recycling. The enzyme did not easily fall off by covalent immobilization, and the immobilized enzyme could be conveniently collected by a magnet. The solution environment may cause folding and subsequent denaturation of laccase [36]. It is evident that immobilization can sometimes improve the catalytic activity of laccases, although there are common concerns about reduced enzyme flexibility, steric hindrance, and diffusion limitation [40]. With the use of the aldehyde tag in the immobilization, there was no subsequent slow reaction between the enzyme and the excessive active groups on the carrier surface, which usually results in destruction of the structure, activity, and stability of the enzyme occurring in conventional covalent immobilization.

**Table 4.** The kinetic parameters of free and immobilized Melac13220.

<table>
<thead>
<tr>
<th></th>
<th>$K_m$ (mM)</th>
<th>$V_{\text{max}}$ (mM s$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Free Melac13220-CQ</td>
<td>$7.39 \times 10^{-2}$</td>
<td>0.378</td>
</tr>
<tr>
<td>Immobilized Melac13220</td>
<td>$9.33 \times 10^{-2}$</td>
<td>0.439</td>
</tr>
</tbody>
</table>

**2.4. Recycling of Immobilized Melac13220**

In addition to high thermal stability, reusability is also very important due to cost considerations and the practical application of biocatalysts in different industries. The recycling of SiO$_2$-Fe$_3$O$_4$-Melac13220 was studied using the ABTS oxidation method. The residual activity of laccase was 96.8% and 92.6% after three and five cycles of reuse, respectively. Notably, more than 80% of the activity of the immobilized laccase was observed even after the completion of ten substrate oxidation operations. After 10 cycles, the immobilized enzyme retained 81.1% of its initial activity (Figure 8). The loss of activity may be due to enzyme deactivation and protein leakage when shaking in reaction and recycling. The enzyme did not easily fall off by covalent immobilization, and the immobilized enzyme could be conveniently collected by a magnet. The solution environment may cause some conformational changes of enzyme molecules during the continuous reprocessing process, resulting in the degradation of enzyme catalytic performance. Thus, it can be inferred that immobilization of Melac13220 onto SiO$_2$-Fe$_3$O$_4$-NPs can provide substantial enzyme resistance and thus maintain its catalytic stability over many continuous oxidation cycles. Reza et al. showed 70% activity after 10 cycles [41]. Compared to other immobilization methods, the proposed covalent immobilization method obviously showed better potential for application.
Dyes are widely used in the textile industry, posing a serious threat to the environment and aquatic organisms. There are some disadvantages when using free laccase in the decolorization process, such as loss of enzymatic activity, excessive sensitivity to environmental conditions, and low stability during storage, greatly limit the efficiency of laccases and their ability to be practically reused. To study the biodegradation of pollutants by immobilized laccase, Congo Red, Remazol Brilliant Blue R, Crystal Violet, and Indigo Carmine were selected as model pollutants. Considering the immobilization efficiency, activity correction effect, and enzyme stability, SiO$_2$-Fe$_3$O$_4$-Melac13220 was used as the immobilized enzyme for dye decolorization and degradation research. As shown in Table 5, immobilization of Melac13220 also led to improvements in dye decolorization such that Congo Red was completely decolorized within 10 h. However, there was little difference in decolorization rate between the free enzyme and the immobilized enzyme, possibly because of the faster reaction speed of the enzyme for this reaction. For the decolorization of other dyes, the decolorization efficiency of the immobilized enzyme is obviously better than that of the free enzyme.

### Table 5. Decolorization of various synthetic dyes by immobilized Melac13220.

<table>
<thead>
<tr>
<th>Dyes</th>
<th>$\lambda_{\text{max}}$ (nm)</th>
<th>Laccase</th>
<th>Decolorization Rate (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Remazol Brilliant Blue R</td>
<td>595</td>
<td>Immobilized</td>
<td>41.3 ± 1.6</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Free</td>
<td>13.9 ± 0.7</td>
</tr>
<tr>
<td>Crystal Violet</td>
<td>590</td>
<td>Immobilized</td>
<td>51.4 ± 1.1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Free</td>
<td>10.7 ± 1.1</td>
</tr>
<tr>
<td>Indigo Carmine</td>
<td>610</td>
<td>Immobilized</td>
<td>87.9 ± 1.3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Free</td>
<td>54.0 ± 0.9</td>
</tr>
<tr>
<td>Congo Red</td>
<td>488</td>
<td>Immobilized</td>
<td>100 ± 0.9</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Free</td>
<td>99 ± 0.7</td>
</tr>
</tbody>
</table>

### 3. Materials and Methods

#### 3.1. Materials

Fe$_3$O$_4$ monodispersed magnetite microspheres (100 nm) covered with SiO$_2$ and amino groups were purchased from Beisile (Tianjin, China). A Rv0712 (FGE) plasmid, Escherichia coli BL21(DE3) pLysS, was maintained in our laboratory. Samples of 2,2’-Azino-bis(3-ethylbenzothiazoline-6-sulfonate) (ABTS), isopropyl-$\beta$-d-thiogalactopyranoside (IPTG), L-arabinose, and the synthetic dyes Remazol Brilliant Blue R, Crystal Violet, Indigo Carmine, and Congo Red were commercially obtained from Sigma-Aldrich (St. Louis, MO, USA). All the solutions required for the experiment were prepared in deionized water, and the reagents and chemicals with the highest purity levels were used. Absorption spectra were recorded using a SHIMADZU UV-2700 spectrophotometer (Shimadzu, Kyoto, Japan). The BCA protein assay kit was purchased from Biotek Company (Beijing, China). TECAN infinite F200 Pro (Tecan, Männedorf, Switzerland) was performed for the protein assay.
3.2. Experimental Setup

3.2.1. Acquisition of Melac13220 with Aldehyde Tags

The polypeptide sequence LCTPSR was fused to the amino terminus (NQ), carboxy terminus (CQ), or double terminus (DQ) of the target enzyme. The recombinant expression vectors pET-28aNQ-Melac13220, pET-28aCQ-Melac13220, pET-28aDQ-Melac13220 and pET-28a-Melac13220 were transformed into E. coli BL21 (DE3), and the formylglycine modified enzyme expression system myc-his A Rv0712 (FGE) was cotransformed into expression-engineered bacteria. For culturing and identification, monoclonal colonies with a double-expression system were screened using a solid medium containing both kanamycin and ampicillin and shaken at 37 °C until the optical density (OD₆₀₀) reached 0.6. Then, 0.2% L-arabinose was used to induce the expression of FGE at 37 °C for 1 h. Afterward, different recombinant strains with aldehyde tags were induced with 0.5 mM IPTG at 16 °C for 12 h before harvesting by centrifugation at 4000 × g at 4 °C for 30 min, and the collected cells were resuspended in Tris-HCl buffer (pH 7.5). Subsequently, the samples were disrupted by ultrasonication until the bacterial solution was no longer viscous. Then, the supernatant and precipitate were separated by centrifugation at 13,000 × g at 4 °C for 10 min. The expression of the different proteins was analyzed by 12% (w/v) sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE, Waltham, MA, USA) and stained with Coomassie Brilliant Blue R250 (Waltham, MA, USA).

3.2.2. Enzyme Assay

Using ABTS (ε₄₂₀nm = 36000 M⁻¹ cm⁻¹) as a substrate, the free and immobilized laccase activities were determined by spectrophotometry. The change in absorbance is measured to determine whether the reaction occurs, and then it can be concluded whether the expressed laccase has activity. The reaction mixture contained a suitable amount of rational free enzyme solution or immobilized enzyme suspension and 0.5 mM ABTS in 20 mM sodium acetate–acetate acid buffer solution (pH = 4.5) for a total volume of 1.0 mL. The unit of enzyme activity (U) was defined as the amount of enzyme required to catalyze the oxidation of 1.0 µmol substrate per minute.

3.2.3. Immobilization of Melac13220

Three methods were used to immobilize laccase. The immobilization parameters were optimized to maximize enzyme activity recovery. For site-specific covalent immobilization (SiO₂-Fe₃O₄-Melac13220), Melac13220 was dissolved in glycine-HCl buffer (20 mM, pH 2.5). The SiO₂-Fe₃O₄-NPs were washed twice with deionized water and glycine-HCl buffer (pH 2.5) and dispersed via ultrasonication before immobilization. Carrier nanoparticles (approximately 5 mg) were mixed with a proper amount of enzyme solution (1 mg/mL), shaken at 20 °C for 6 h and then reduced with 1% (w/w) NaCNBH₃ for 1 h. Immobilized enzymes were collected with magnets and washed with 1 M NaCl, saturated (NH₄)₂SO₄, and deionized water. The BCA protein assay kit and supernatant were used to measure the immobilization efficiency. The activity of the immobilized enzyme was detected by the method mentioned in Section 3.2.2.

For the entrapment of laccase (E-Melac13220), sodium alginate was used as an effective carrier material, which was dissolved in distilled water at a concentration of 2.5% (w/v). Enzyme solution (1 mg/mL) was thoroughly mixed with sodium alginate by slight shaking on a rotary shaker, and the mixture was dropped into 2.5% (w/v) CaCl₂ by using a syringe with a diameter of 0.4 mm to produce beads. The alginate beads were collected by filtration and washed with distilled water several times until laccase activity was not detected in the washing water.

For the crosslinking of Melac13220 (C-Melac13220), chitosan was well dissolved in 1.5% acetic acid solution by mild heating at 50 °C with continuous stirring. Chitosan (2.5% w/v) was used to develop beads with better mechanical strength. The resultant chitosan mixture was extruded dropwise into 1 M KOH solution with a syringe and hardened in this solution for 4 h at room temperature. High-quality beads of uniform size and shape
were collected, filtered, and activated via incubation with 2% (v/v) glutaraldehyde solution at room temperature for 3 h. The activated chitosan beads were thoroughly washed with buffer solution to remove any unattached glutaraldehyde on the surface of the beads. Finally, the beans were incubated with enzyme solution (1 mg/mL) at 4 °C with 24 h.

\[
\text{Immobilization efficiency} = \frac{(C_1 - C_2)}{C_1} \times 100\%
\]

where \(C_1\) is the concentration of Melac13220 solution added in the immobilization volume (mg/mL), and \(C_2\) is the concentration of supernatant after immobilization of Melac13220 (mg/mL). The activity recovery rate (%) is the ratio of the activities of the immobilized Melac13220 and initial Melac13220.

3.2.4. Characterizations of the Immobilized Melac13220

In glycine-HCl buffer solution (pH 2.5), the optimum temperature was determined from 30 °C to 90 °C. The effect of pH on laccase activity was between pH 1.0 and 9.0. The thermal stability test was analyzed by incubating the enzyme at 90 °C for 1 h, and the pH stability of the enzyme was tested by incubating laccase in different buffer solutions with pH values from 1.0 to 5.5.

The structural stability and melting point of free and immobilized laccase were determined via differential scanning calorimetry (DSC) using a VP-DSC microcalorimeter (MicroCal, LLC, Northampton, MA, USA). Since the application of the DSC to inhomogeneous systems has its own characteristics and limitations, the enzyme SiO\(_2\)-Fe\(_3\)O\(_4\)-Melac13220 immobilized on Fe\(_3\)O\(_4\) nanoparticles by aldehyde tag was chosen because the carrier Fe\(_3\)O\(_4\) has a fast response to the applied magnetic fields and can be easily separated and recover from the reaction system. The scanning began at 30 °C and heated to 90 °C at a heating rate of 1 °C per min, and each sample was scanned twice. Prior to measurement, samples and buffer were thoroughly degassed under vacuum at room temperature and loaded into a calorimeter cell. The DSC curve was corrected by subtracting a buffer–buffer scan as the instrument baseline. Routine data analysis within OriginTM was performed during DSC runs without regard to compromising the real-time operations. The postcycle thermostat was set to 25 °C, the pre-scan thermostat was set to 15 min, and the current pressure of the cells was maintained at 36 psi to prevent the formation of gas bubbles during the heating. Free and immobilized Melac13220 were diluted in Tris-HCl buffer (20 mM, pH 7.5) to a final concentration of 0.1 mg/mL for the scan. Before the protein scan, a buffer scan was obtained to subtract the substrate from the protein signal, and Origin 7 was used to fit the two-state model to determine the maximum melting point (\(T_m\), peak temperature) of the enzyme.

The effect of various organic solvents on enzyme activity was studied by adding various organic solvents to the enzyme solution for 1 h, and all measurements were performed in triplicate.

3.2.5. Recycling of Immobilized Melac13220

To determine the recycling stability, immobilized Melac13220 was collected after each reaction, washed twice with fresh buffer, and then used for the next cycle. The percentage of residual activity of immobilized enzymes was monitored periodically using the standard assay protocol. The process was repeated for 10 consecutive cycles.

3.2.6. Application of Immobilized Melac13220 in Dyes Decolorization

Dyes belonging to the azo-, anthraquinone-, and anthraquinone-based groups are the main pollutants discharged from industrial effluents. Therefore, by incubating enzymes with these different types of dyes, including azo dyes (Congo Red, \(\lambda_{\text{max}} = 488\) nm), anthraquinone dyes (Remazol Brilliant Blue R, \(\lambda_{\text{max}} = 595\) nm), triphenylmethane dyes (Crystal Violet, \(\lambda_{\text{max}} = 590\) nm), and indigo dyes (Indigo Carmine, \(\lambda_{\text{max}} = 610\) nm), the dye decolorization ability of immobilized Melac13220 was evaluated and compared to that of the free laccase. The decolorization process was carried out in the dark at 40 °C under mild
shaking conditions for 10 h. The reaction mixture contained 1.0 U/mg immobilized or free enzyme and 80 mg/mL dyes in 20 mM Tris-HCl buffer (pH 7.5) for Congo Red and 20 mM glycine-HCl buffer (pH 1.5) for other dyes. In the absence of immobilized or free enzyme, the parallel control contained all components.

The decolorization effect of the dye is determined by the decrease in the absorbance of the dyes at the maximum wavelength of 10 h compared with that at time 0 h, expressed as a percentage. All measurements were taken three times.

4. Conclusions

Three types of immobilized laccase, immobilized by entrapment, crosslinking, and covalent binding methods, were prepared and compared. In particular, Melac13220 was site-specific and covalently immobilized on Fe$_3$O$_4$ magnetic nanoparticles covered by aminated modified SiO$_2$ with the highest immobilization efficiency activity recovery rates, 89.93% and 76.18%, and characterized by scanning electron microscope (SEM) (Figures S1 and S2). Regarding free enzyme, covalently immobilized laccase showed enhanced thermal stability, operational stability, and reusability, which may because this immobilization method prevents the aggregation of proteins and thus improves the characterization of enzymes. In this study, Fe$_3$O$_4$ MNPs were considered ideal immobilized carriers because of their excellent recyclability. The modified Melac13220 is covalently bonded to nanoparticles without purification because the aldehyde tag of the modified Melac13220 specifically reacts with the -NH$_2$ covering on the carriers. Therefore, it is convenient to produce immobilized enzymes without purification, which shows the benefits of using the aldehyde tag strategy. The more environmentally friendly method without harmful chemicals for one step purification and immobilization of enzyme using a genetically encoded “aldehyde tag” can be used for numerous other enzyme purification and immobilization applications. This study showed that the immobilization process improved the thermal stability and operational stability of laccase and can apply these biocatalysts in the case of mixture of dyes (industrial wastewater), which indicated that the immobilization of laccase on functional MNPs through covalent binding was suitable for various industrial applications.

**Supplementary Materials:** The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/catal12111379/s1, Figure S1: SEM of immobilized Melac13220, Figure S2: SEM of Fe$_3$O$_4$ nanoparticles.

**Author Contributions:** Conceptualization, R.G. and A.A.; methodology and investigation, A.A., A.L., X.Z., Y.X. and S.H.; writing—original draft preparation, A.A.; writing—review and editing, R.G.; supervision, S.H. and R.G. All authors have read and agreed to the published version of the manuscript.

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