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Immobilization of Horseradish Peroxidase and Myoglobin Using Sodium Alginate for Treating Organic Pollutants

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Abstract: Removing organic pollutants from wastewater is crucial to prevent environmental contamination and protect human health. Immobilized enzymes are increasingly being explored for wastewater treatment due to their specific catalytic activities, reusability, and stability under various environmental conditions. Peroxidases, such as horseradish peroxidase (HRP) and myoglobin (Mb), are promising candidates for immobilized enzymes utilized in wastewater treatment due to their ability to facilitate the oxidation process of a wide range of organic molecules. However, the properties of the carrier and support materials greatly influence the stability and activity of immobilized HRP and Mb. In this research, we developed immobilized HRP and Mb using support material composed of sodium alginate and $CaCl_2$ as carriers and glutaraldehyde as a crosslinking agent. Following this, the efficacy of immobilized HRP and Mb in removing aniline, phenol, and p-nitrophenol was assessed. Both immobilized enzymes removed all three organic pollutants from an aqueous solution, but Mb was more effective than HRP. After being immobilized, both enzymes became more resilient to changes in temperature and pH. Both immobilized enzymes retained their ability to eliminate organic pollutants through eight treatment cycles. Our study uncovered novel immobilized enzyme microspheres and demonstrated their successful application in wastewater treatment, paving the way for future research.

Keywords: immobilization; peroxidase; sodium alginate; reusability; organic pollutants

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1. Introduction

Enzymes are biochemical catalysts that are very selective for the substrates on which they act [1]. Peroxidase is a class of enzyme capable of catalyzing the oxidation of aromatic compounds [2]. The roots of horseradish plants (*Armoracia rusticana*, syn. *Cochlearia armoracia*) contain horseradish peroxidase (HRP), which is utilized extensively in environmental protection, wastewater treatment, and biotechnology [3,4]. Myoglobin (Mb) is an abundant iron-containing heme protein in vertebrate cardiac and skeletal muscle [5] which has peroxidase activity [6]. Both HRP and Mb catalyze organic compounds, including phenolic compounds, aniline, and dyes [7–10]. HRP is a multifunctional peroxidase that oxidizes substrates in the presence of hydrogen peroxide [11]. The efficiency of HRP in breaking down a wide range of contaminants has been demonstrated, thereby making a valuable

contribution to environmental remediation [12]. Mb can bind and transport oxygen, suggesting it has potential applications in enzymatic degradation [13]. Due to its strong affinity for contaminant binding, Mb can be exploited to efficiently remove pollutants [14].

Both HRP and Mb are highly substrate-specific, environmentally friendly, and require mild catalytic conditions and low energy [15], making them ideal for enzymatic wastewater treatment and organic pollutant removal [9]. Nevertheless, the application of unbound HRP and Mb for wastewater treatment is not cost-effective [16]. In addition, unbound HRP and Mb are unstable and sensitive to denaturants, such as temperature, pH, metal ions, and surfactants [16–18]. Additionally, free enzymes, particularly those that are more costly and difficult to purify, have a restricted capacity for a single application [19]. Enzyme immobilization is a potent method for overcoming these drawbacks and providing superior features for practical applications of peroxidases [20–23]. Immobilization improves the stability, storage, recovery, and recycling of peroxidases [22].

The stability and activity of immobilized enzymes are largely determined by the characteristics of the carrier, reaction conditions, the nature of binding (e.g., the number of bonds formed), the microenvironment of the enzyme molecule, and the characteristics of the spacer connecting enzyme molecules to the carrier [24]. An ideal immobilization carrier needs to be cost-effective, biodegradable and non-toxic [25]. Various carriers, including silica nanoparticles [26], mesoporous carbon [27], carbon nanotubes [28], and alginate microspheres [29], have been employed for enzyme immobilization. Alginate is a naturally derived biocompatible and non-toxic biopolymer which provides a suitable environment for enzyme immobilization [30]. Alginate is frequently combined with calcium chloride ($CaCl_2$) and glutaraldehyde for enzyme immobilization. Calcium chloride is employed in the synthesis of alginate gels or microspheres due to its low toxicity [31]. Glutaraldehyde is a cross-linking agent that forms covalent bonds between amino groups of enzymes and functional groups in alginate [32,33] to efficiently impeded enzyme leaching, hence prolonging the functionality of immobilized enzymes [34–38].

Wastewater, particularly industrial wastewater, often contains organic contaminants such as aniline, phenol, and p-nitrophenol, as these compounds are frequently used in manufacturing and can be released into water supply through industrial discharges [39]. Aniline is an aromatic amine which is used in the production of various chemicals, dyes, and pharmaceuticals [40]. Phenol, chemically referred to as carbolic acid, is a crystalline solid that possesses a white coloration and is predominantly employed in the manufacturing processes of pharmaceuticals, polymers, and resins [41]. p-Nitrophenol is a substituted phenol and is primarily utilized in the synthesis of dyes, pesticides, and pharmaceuticals [42]. When remain untreated, these three organic substances are capable of disturbing the balance of microbial communities and harming aquatic organisms, both of which are detrimental to aquatic ecosystems [39]. In addition, because of their potential environmental and public health risks, it is essential to properly manage and treat wastewater containing these organic contaminants before they are released into the environment.

While HRP has been extensively studied and applied in wastewater treatment, the potential of Mb in wastewater treatment is an emerging area of research, and limited investigation has been devoted to the synthesis of immobilized Mb. In addition, the efficacy of Mb in removing aniline, phenol, and p-nitrophenol compared to HRP has not been well-understood. Here, we developed immobilized HRP and Mb using a support material composed of sodium alginate and $CaCl_2$ as carriers and glutaraldehyde as a crosslinking agent to remove these organic contaminants from wastewater. The stability and efficacy of the synthesized immobilized enzymes in removing all three organic contaminants in wastewater were also evaluated under various storage periods, as well as pH and temperature conditions. To the best of our knowledge, this is the first report on the removal of aniline, phenol, and p-nitrophenol from wastewater using HRP and Mb immobilized with sodium alginate, $CaCl_2$, and glutaraldehyde.

2. Materials and Methods

2.1. Materials

Sodium alginate (SA), calcium chloride ($CaCl_2$), sodium dihydrogen phosphate (NaH_2PO_3), disodium hydrogen phosphate (Na_2HPO_3), citric acid, sodium citrate, sodium chloride (NaCl), phenol (C_6H_5OH), aniline ($C_6H_5NH_2$), and p-nitrophenol ($C_6H_4(NO_2)OH$) were purchased from Beijing chemical Co., Ltd., Beijing, China (analytical grades). Horseradish peroxidase (HRP), myoglobin (Mb), and 2,2′-azinobis-(3-ethylbenzthiazoline-6-sulphonate) (ABTS) were purchased from Aladdin Industrial Co., Ltd., Shanghai, China. All chemicals used in this research were of an analytical grade.

2.2. Preparation of Immobilized HRP and Mb

The procedure for immobilizing HRP and Mb in calcium alginate microspheres is shown in Figure 1. The HRP and Mb microspheres were prepared using sodium alginate and glutaraldehyde as the carrier and crosslinking agent, respectively. For this, 5 mL of each enzyme (1 mg/mL) were mixed with 10 mL sodium alginate (3.5% *w*/*v*), and the mixture was added dropwise to 50 mL of calcium chloride (3.0% *w*/*v*) and allowed to harden for 6 h. Subsequently, the hardened material was neutralized and crosslinked in 100 mL of 0.2% glutaraldehyde solution for 1 h. The immobilized HRP and Mb microspheres were stored at 4 °C for later use.

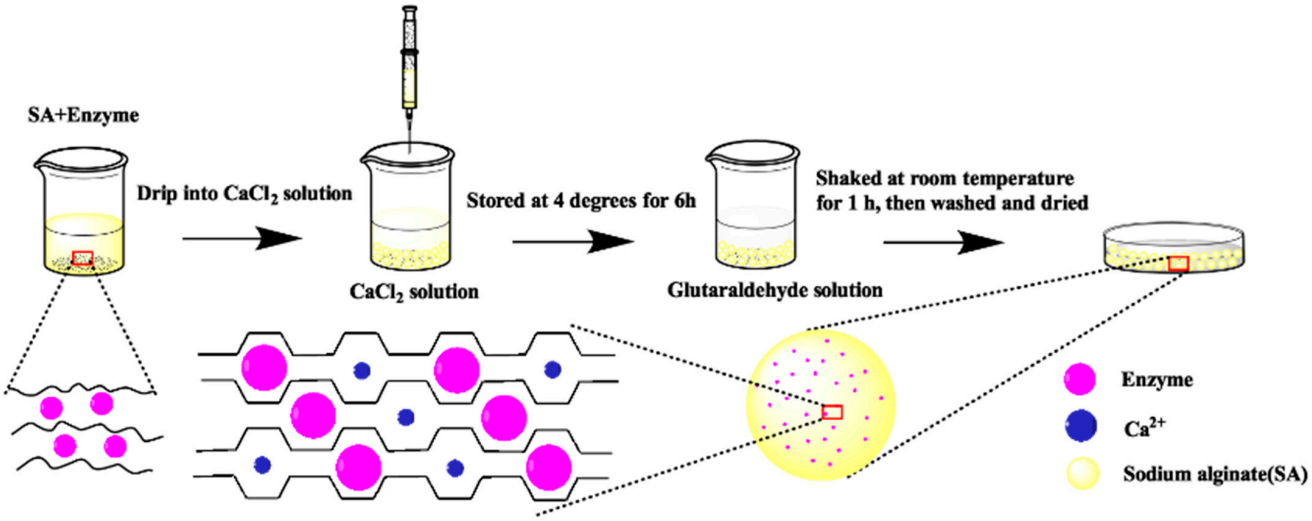

Figure 1. The process involved in the immobilization of myoglobin (Mb) and immobilized horseradish peroxidase (HRP) in calcium alginate microspheres.

2.3. Characterization of Immobilized HRP and Mb

A field emission scanning electron microscope (SEM) (Hitachi, Tokyo, Japan) equipped with an energy dispersive X-ray spectrometer su8010 (FESEM-EDS) was used to examine the structure and appearance of immobilized Mb and HRP microspheres (FESEM-EDS). The element distribution of the microspheres of both immobilized enzymes was analyzed using FESEM-EDS with the following parameters: electron beam energy = 20 kV, and electron beam current = 80.0 μA

2.4. Enzymatic Activity and Stability of Immobilized HRP and Mb

The activity of immobilized HRP and Mb was evaluated in the presence of hydrogen peroxide with ABTS as a substrate using the method described by Andrade et al. [43]. Briefly, 20 immobilized HRP and Mb microspheres were added to a mixture of 0.12 mmol·L^{-1} of ABTS, 25 mmol·L^{-1} of H_2O_2, and 0.03 mol·L^{-1} phosphate buffer solution (pH 6.0) and reacted at 25 °C for 3 min. Subsequently, the oxidation of ABTS was evaluated at 420 nm using a spectrophotometer (UV2550, Shimadzu, Kyoto, Japan). The amount of immobilized

HRP and Mb needed to generate 1 nmol of ABTS+ per minute was defined as one unit of activity. To assess the stability, the activity of both immobilized HRP and Mb was assessed under a wide range of temperatures (i.e., 15 to 55 °C), pH (i.e., 3.0 to 8.0) and storage periods (i.e., 0 days to 32 days at 25 °C), using the same ABTS assay outlined above.

2.5. Removal Efficiency and Reusability of Immobilized HRP and Mb

The efficiency of the immobilized HRP and Mb microspheres to remove organic pollutants from wastewater was assessed and compared with enzyme-free sodium alginate microspheres (control), in the presence of 15 mg/L H_2O_2. For this, we established single-factor assays to investigate the effect of treatment time (0.5 to 6 h), the initial concentration of organic compounds (5 to 50 mg/L), and amounts of microspheres (5 to 25) on the removal efficiency of phenol, aniline, and p-nitrophenol. To prepare the aqueous solution of organic contaminants, a stock solution of each contaminant was prepared by adding 50 mg of each compound to 1 L of water. Subsequently, by employing serial dilution, different concentrations of each organic compound were produced (i.e., 5 mg/L, 10 mg/L, 20 mg/L, 30 mg/L, 40 mg/L, 50 mg/L). To determine the removal efficacy, the concentration of the organic compounds in treated aqueous solutions was evaluated with a UV–Vis spectrophotometer (UV2550, Shimadzu, Kyoto, Japan) at 270, 251, and 371 nm for phenol, aniline, and p-nitrophenol [44].

To assess the reusability of the immobilized microspheres, the microspheres were applied to aqueous solutions containing 5 mM of each phenol, aniline, and p-nitrophenol. The microspheres were then removed from aqueous solutions using filters, and washed with purified water before using in the next cycle. The reusability experiment consisted of eight cycles, and the residue of each organic compound was assessed using the spectrophotometric method outlined above to examine the reusability of the immobilized enzymes. To ensure for the maximum removal of organic compounds, the recovery of microspheres and the assessment of residuals were performed at 6 h intervals.

2.6. Statistical Analyses

All enzymatic assays were performed using a randomized design with three replicates for each treatment. All experiments were repeated in time, and as the variance of the data from separate experiments were equal according to a Levene's test, the data were pooled. Experiments measuring enzymatic activity and stability were subjected to a *t*-test at a significance level of 5%. For studies testing removal efficiency and reusability, we used one-way ANOVA, and the means were compared using Tukey's test at a 5% level of probability. All statistical analyses were performed with SPSS v.21.

3. Results and Discussion

3.1. Characterization of Immobilized HRP and Mb

Myoglobin and HRP solutions (0.1 mmol·L^{-1}) were mixed with calcium alginate (3.5% *w*/*v*) in order to synthesize the microspheres, as shown in Figure 1. The capsulation of HRP and Mb enzymes yielded 3-mm microspheres with a uniform size and a smooth surface (Figure 2).

The SEM analysis of the microspheres revealed that their inner structure was collapsed and damaged, likely as a result of water loss during freeze-drying, which led to the formation of a lamellar structure (Figure 3a,c). It was noted that the inner structure of immobilized microspheres contains evenly distributed small particles (Figure 3b,d). These small particles likely represent the crystals of immobilized enzymes formed after the freeze-drying treatment [23], as no particles were observed in the empty microspheres of sodium alginate carries (Figure 3e,f). This result indicates that HRP and Mb were successfully immobilized in the microspheres.

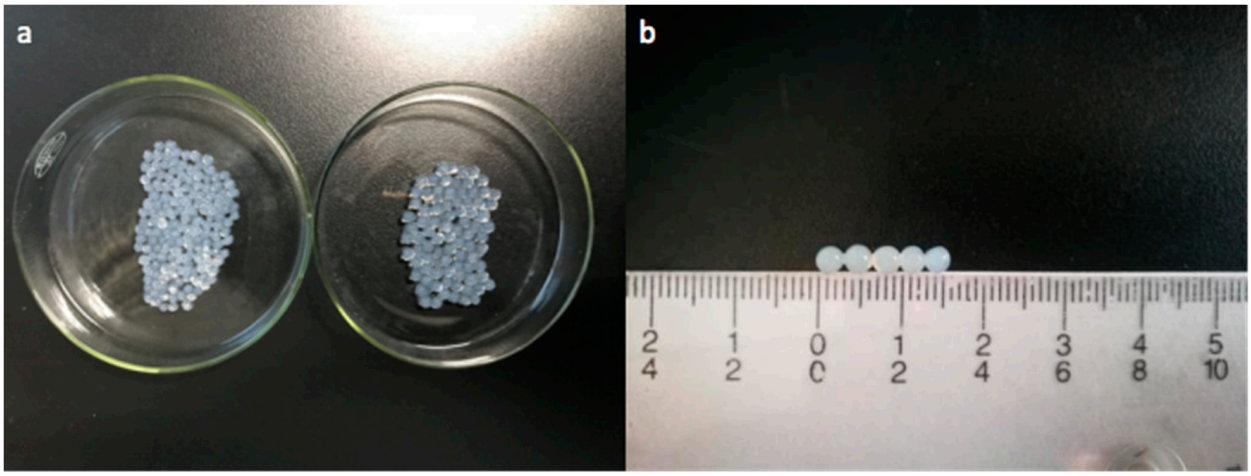

Figure 2. (**a**) Synthesized sodium alginate microspheres, (**b**) size of sodium alginate microspheres.

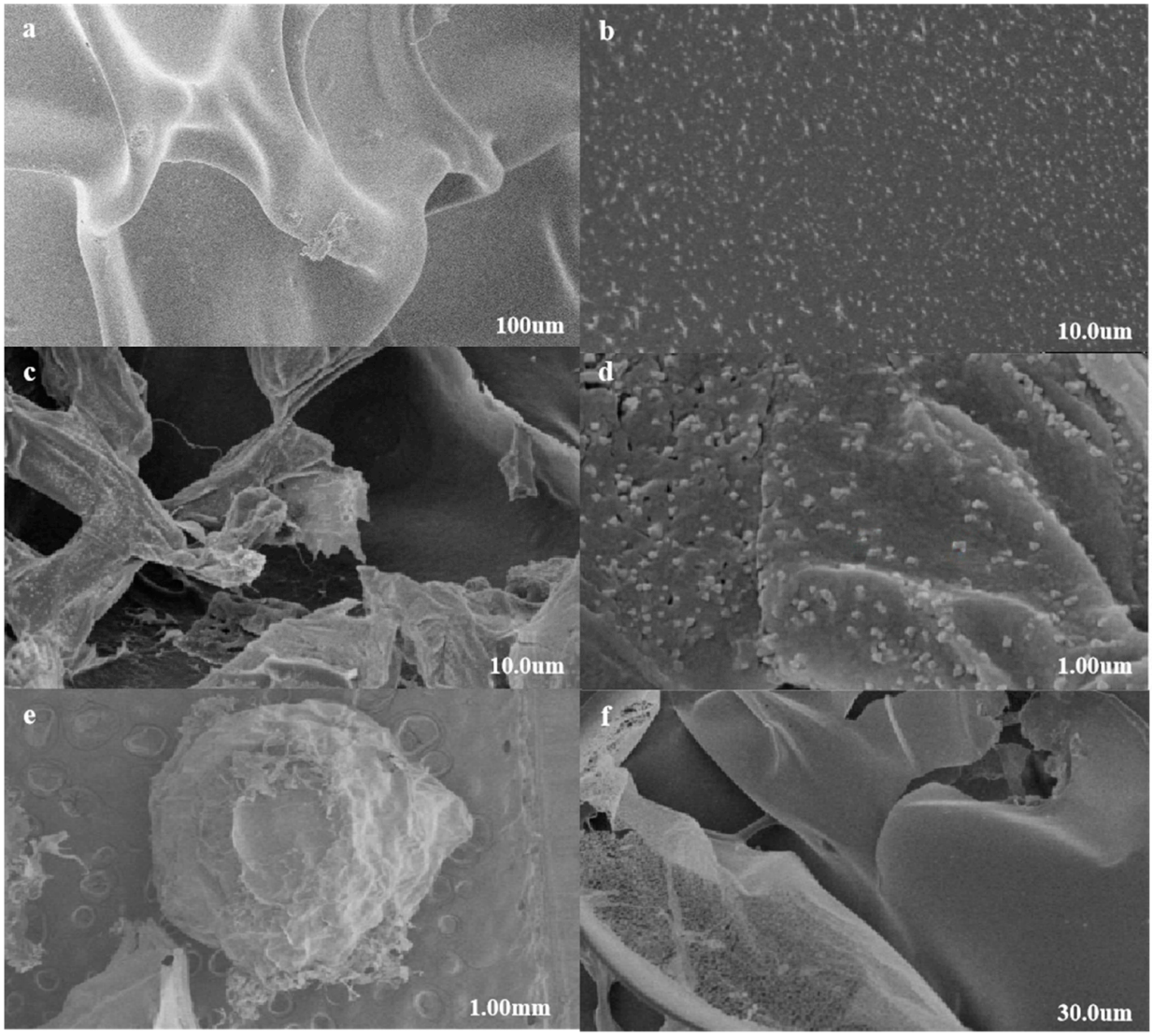

Figure 3. SEM images of morphology of microspheres (**a**,**b**), SEM images of immobilized myoglobin (**c**,**d**), SEM images of immobilized horseradish peroxidase (**c**,**d**), and SEM images of empty morphology (**e**,**f**) of sodium alginate microspheres.

To further prove that HRP and Mb were successfully entrapped in the carries, the content of nitrogen was compared between the immobilized sodium alginate carrier and the empty ones (Table 1). The results revealed that the nitrogen content of immobilized microspheres was much higher than that of empty microspheres. The nitrogen content of immobilized Mb and HRP was 14.1% and 11.9%, respectively.

Table 1. Elemental analysis of empty sodium alginate and immobilized myoglobin (Mb), and horseradish peroxidase (HRP).

Sample	Nitrogen Content (%)	Carbon Content (%)
Empty sodium alginate	0.1	29.0
Immobilized Mb	14.1	32.8
Immobilized HRP	11.9	36.2

As nitrogen is a crucial component of enzymes [22], this result provides further evidence for the immobilization of HRP and Mb in sodium alginate carriers. The peroxidase activity of immobilized HRP and Mb was investigated using an ABTS assay, and the results revealed that the enzyme activity of the immobilized Mb and HRP was 123.78 and 85.20 U/mg, respectively (Figure 4). This indicates that an immobilized HRP concentration of nearly 1.5 times that of Mb is required to achieve similar absolute enzymatic activity. These results are in agreement with a previously published study [45].

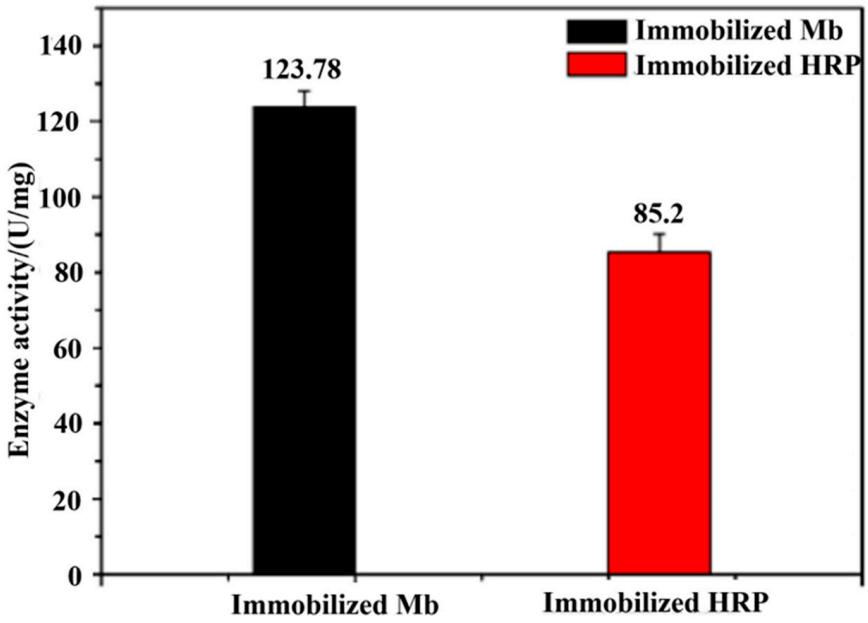

Figure 4. Absolute enzyme activity of immobilized myoglobin (Mb) and immobilized horseradish peroxidase (HRP).

3.2. Stability of Immobilized Enzyme

The effect of temperature, pH, and storage periods on the stability of immobilized HRP and Mb was assessed (Figure 5). According to the results, the activity of immobilized Mb and its corresponding free enzymes showed a generally increasing trend with increasing the temperature, with their highest activity peaking at 35 °C (Figure 5a). However, a downward trend was recorded for the activity of both immobilized Mb and its native enzyme at temperatures greater than 35 °C. The immobilized HRP activity likewise increased with increasing the temperature up to 35 °C, whereas the free HRP activity peaked at 45 °C (Figure 5b). Overall, the results showed that immobilization of both enzymes improve their thermal stability when compared to their corresponding native enzymes, suggesting the immobilization increased the rigidity of the HRP and Mb structures, hence limiting conformational changes in both enzymes at elevated temperatures [46]. However, the

activity of immobilized Mb appeared to be less affected at low temperatures compared to the immobilized HRP. It is probable that the immobilization of Mb utilizing the methodology implemented in this study provided Mb with greater resistance to low temperatures than HRP. Immobilization offers a level of protection for enzymes, enhancing their stability and minimizing their susceptibility to environmental influences, ensuring a more sustained enzyme activity and improving processing effects [47].

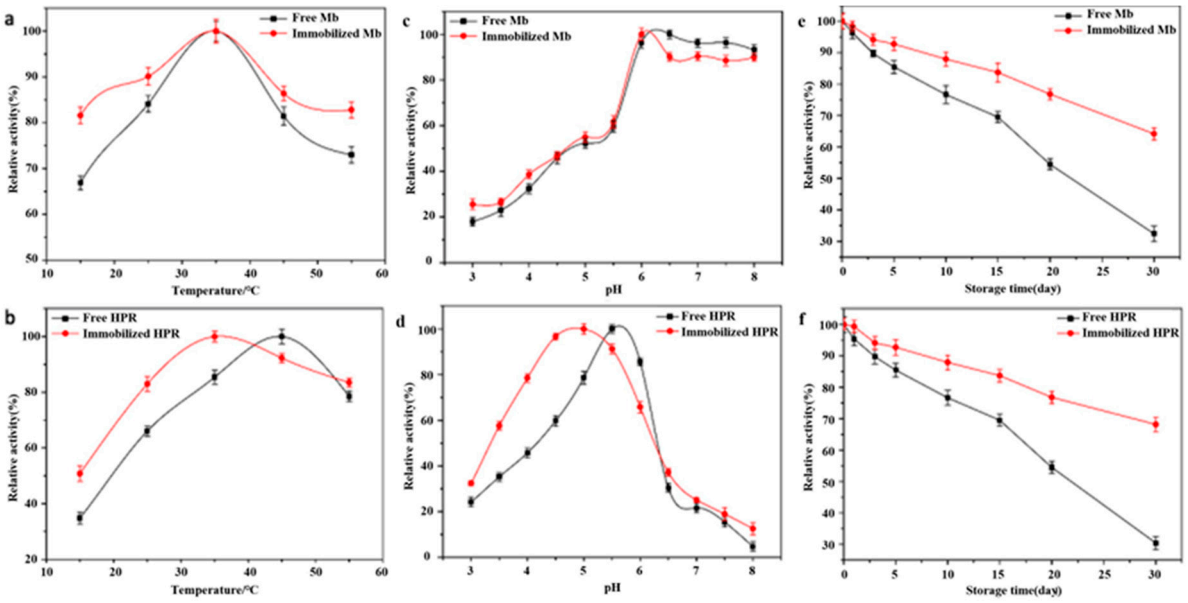

Figure 5. The stability (**a**,**b**), various temperatures (**c**,**d**), and pH levels (**e**,**f**) of free and immobilized myoglobin (Mb) and horseradish peroxidase (HRP) storage periods.

The effect of pH on the activity of both immobilized Mb and HRP was investigated and compared to their corresponding free enzymes. Generally, a different trend was recorded between the response of both immobilized enzymes with increasing the level of pH. The results showed that increasing pH progressively increased the activity of free and immobilized Mb, and both responded similarly when pH increased from 3 to 6 (Figure 5c). However, the greatest activity of the immobilized Mb was recorded at pH of 6, while the free Mb was the most active at pH of 6.5. Despite this small difference, the activity of the immobilized Mb and its native form remained at above 80% at a pH range of 6 to 8. On the other hand, a different pattern in response to increasing the level of pH was recorded for the immobilized HRP and its native enzyme. The results showed that the activity of the immobilized HRP and its native form peaked at pH of 5 and 5.5, respectively. However, there was a general downward trend in their activity at higher pH levels (Figure 5d). In addition, the activity of immobilized HRP appeared to be greater at a pH range between 3 to 5, suggesting that immobilization improved acidic pH tolerance of HRP, possibly owing to the hydroxyl and carboxyl groups on the surface of the support acting as buffer to create a favorable environment for the enzyme. Overall, it appears that the immobilization method employed in this research conferred broader pH tolerance to Mb than HRP, making Mb preserve its activity at a wider range of pH [29,48].

The effect of storage on the stability of immobilized HRP and Mb was evaluated, and the results showed that the activity of both immobilized enzymes and their corresponding native forms progressively reduced with increasing the storage period (Figure 5e,f). However, the activity of the immobilized enzymes was less affected during storage compared to their native forms. For instance, both immobilized enzymes retained over 85% of their initial activity when they were stored for 15 days at 4 °C, while less than 70% activity was recorded for the free enzymes over the same storage period. Additionally, after 30 days of storage, the activity of free HRP and Mb dropped to 30% of their initial activity, while the activity of immobilized HRP and Mb was greater than 65% after 30 days. These results

indicated that immobilization provided a relatively stable microenvironment for HRP and Mb molecules, making them less prone to inactivation during storage [31,49]. This finding is consistent with prior research indicating that calcium alginate does not react with the by-product and can effectively prolong the enzyme's durability, hence resulting in enhanced removal efficiency [50]. It has been suggested that the "egg-box" structure formed by sodium alginate and calcium chloride can protect the secondary and tertiary structures of embedded enzymes from damage during long-term storage periods [51].

3.3. Treatment of Organic Wastewater by Immobilized HRP and Mb

3.3.1. Single Factor Exploration

The effect of reaction time, initial concentration of organic pollutants, and the number of microspheres on the efficiency of immobilized HRP and Mb in eliminating aniline, phenol, and p-nitrophenol was evaluated. According to the results, the immobilized HRP and Mb showed improved aniline, phenol, and p-nitrophenol removal efficacy with longer reaction times (Figure 6). Immobilized Mb, however, consistently showed higher removal efficiency of all organic pollutants than immobilized HRP. For instance, at 4 h after treatment, almost 65%, 85%, and 32% of aniline, phenol, and p-nitrophenol, respectively, was removed by the immobilized Mb, while the immobilized HRP only removed 40%, 75%, and 8% of aniline, phenol, and p-nitrophenol, respectively, over the same reaction period. The greater removal efficacy recorded for the immobilized Mb under prolonged reaction time is probably due to a greater synergistic effect between the Mb and the support material compared to the immobilized HRP [52].

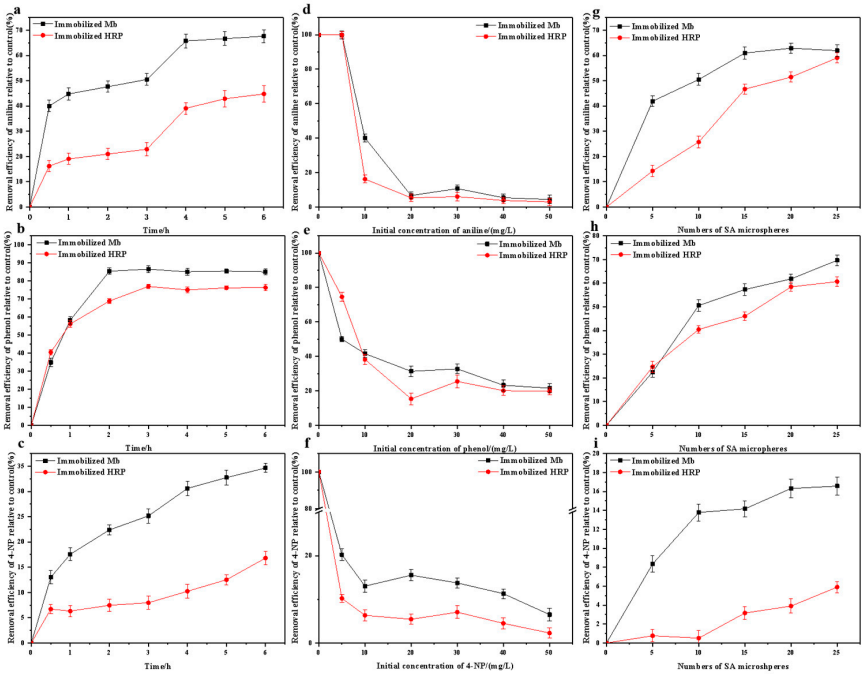

Figure 6. The effect of different reaction periods (**a**–**c**), concentration of pollutants (**d**–**f**), and number of microspheres (**g**–**i**) on the removal efficacy of aniline, phenol, and p-nitrophenol by immobilized myoglobin (Mb) and horseradish peroxidase (HRP) relative to control (enzyme-free sodium alginate microspheres).

Increasing the initial concentration of organic pollutants negatively influenced the removal efficiency of both immobilized enzymes. According to the results, almost 100% of aniline was removed by both immobilized enzymes when the concentration of this organic pollutant was 5 mg/L (Figure 6d). However, increasing the concentration of aniline to 10 mg/L dramatically reduced the removal efficiency of the immobilized Mb and HRP to 40% and 18%, respectively. The removal efficiency of the immobilized HRP and Mb was less than 10% when the concentration of aniline was greater than 20 mg/L. The greatest removal

efficiency of both immobilized enzymes was achieved at 5 mg/L of phenol, and it was 74% and 50% for the immobilized HRP and Mb, respectively (Figure 6e). The removal efficiency of the immobilized HRP was reduced dramatically with increasing the concentration of phenol, and it was 40% and 12% at 10 and 20 mg/L of phenol, respectively. Increasing the concentration of phenol also reduced the removal efficiency of the immobilized Mb, though not as prominent as that of the immobilized HRP. According to the results, the removal efficiency of the immobilized Mb at 10 and 20 mg/L of phenol was 41% and 30%, respectively, which was greater than that recorded for the immobilized HRP. The immobilized Mb was also found to be less affected by increasing the concentration of p-nitrophenol compared to the immobilized HRP (Figure 6f). The results showed that at 5 mg/L of p-nitrophenol, the immobilized Mb and HRP removed almost 20% and 10% of this organic pollutant, respectively. At a concentration range of 10 to 50 mg/L of p-nitrophenol, the removal efficiency of the immobilized HRP notably reduced to less than 10%, in contrast to the immobilized Mb, which had a greater removal efficiency at this concentration range. Overall, these results indicate that the removal efficacy of both immobilized enzymes is highly dependent on the concentration of pollutants, which is in agreement with prior research [53,54].

A positive relationship was recoded between increasing the number of microspheres and removal efficiency of the immobilized HRP and Mb, though the removal efficiency of the immobilized Mb was always greater than that of the immobilized HRP, regardless of the type of organic pollutants (Figure 6g–i). For instance, when the solutions containing organic pollutants were treated with 15 microspheres, 60%, 55%, and 14% of aniline, phenol, and p-nitrophenol, respectively, were removed by the immobilized Mb. While only 45%, 42%, and 3% of aniline, phenol, and p-nitrophenol, respectively, were removed by the immobilized HRP, using the same number of microspheres.

The removal efficiency of both immobilized enzymes was assessed when the initial concentration of organic pollutants was 10 mg/L, the number of microspheres was 10 and 15 for the immobilized Mb and HRP, respectively, and the reaction time was 2 h. The results showed that the immobilized Mb had greater removal efficiency than the immobilized HRP, regardless of the type of organic pollutants, as shown in (Figure 7). It was also noted that organic pollutant removal efficiency followed the order of p-nitrophenol < aniline < phenol for both immobilized Mb and HRP.

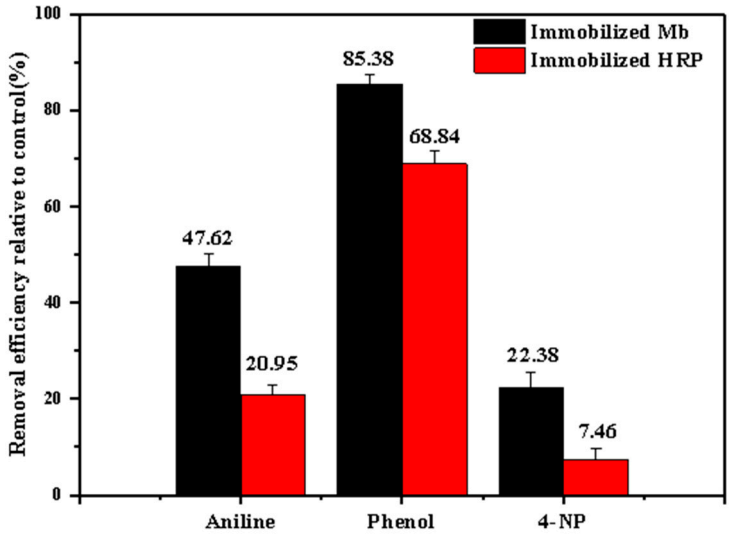

Figure 7. The removal efficiency of aniline, phenol, and p-nitrophenol (4-NP) by myoglobin (Mb) and horseradish peroxidase (HRP) relative to control (enzyme-free sodium alginate microspheres) when the initial concentration of organic pollutants was 10 mg/L, the number of microspheres was 10 and 15 for immobilized Mb and HRP, respectively, and the reaction time was 2 h.

3.3.2. Reusability

The reusability of the immobilized Mb and HRP was assessed when the initial concentration of organic pollutants was 10 mg/L, the number of microspheres was 10 and 15 for the immobilized Mb and HRP, respectively, and the reaction time was 1 h. The results showed that both immobilized enzymes retained their removal efficiency of all three organic pollutants up to eight cycles of treatment (Figure 8). According to the results, the removal efficiency of the immobilized Mb after eight cycles of treatments remained at 48%, 55%, and 14% for aniline, phenol, and p-nitrophenol, respectively. The removal efficiency of the immobilized HRP after eight cycles of treatment was 22%, 49%, and 2% for aniline, phenol, and p-nitrophenol, respectively. Although the activity of Mb may not be as high as that of HRP, the enhanced stability it achieved through immobilization resulted in more efficient catalytic performance. Taken together, these results indicate that both immobilized enzymes can be recycled up to eight times with a negligible loss in their activity during the recycling process. The recyclability and stability of immobilized enzymes play an important role in their applicability. Immobilized enzymes with improved stability and reusability can make wastewater treatment procedures cost-effective [23,48,55].

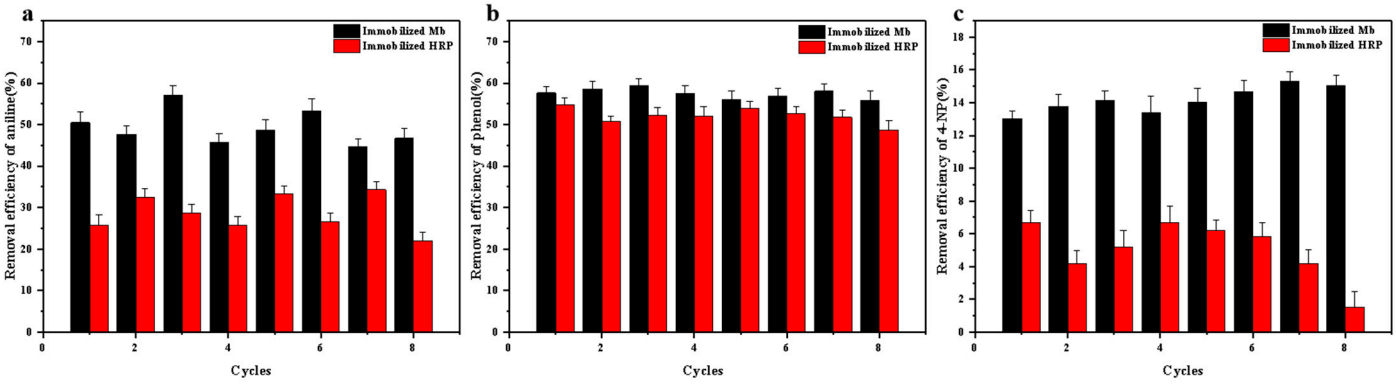

Figure 8. Reusability of myoglobin (Mb) and horseradish peroxidase (HRP) for treating solutions containing (**a**) aniline, (**b**) phenol, and (**c**) p-nitrophenol.

4. Conclusions

In this study, we immobilized HRP and Mb on microspheres made of sodium alginate and calcium chloride, with glutaraldehyde as a crosslinking agent. The efficiency of the immobilized HRP and Mb in eliminating aniline, phenol, and p-nitrophenol, three commonly found organic pollutants in industrial wastewater, was subsequently evaluated. Both immobilized enzymes could remove all three organic pollutants from aqueous solutions, but the immobilized Mb was more effective than the immobilized HRP. Both enzymes were more stable and rigid after immobilization against temperature and pH changes. After 30 days of storage at 4 °C, the immobilized enzymes retained higher activity than free enzymes, and their ability to remove organic pollutants was maintained through eight treatment cycles. Our research has led to the discovery of novel immobilized enzyme microspheres and their application in wastewater treatment, which opens up new avenues for further investigation. The findings of this research revealed that the immobilized Mb performed better as the concentration of organic compounds and reaction period increased, however, the underlying mechanism for this improvement is still unknown and needs additional research. The limitation of our study is that it did not assess the efficacy of both immobilized enzymes using industrial wastewater containing aniline, phenol, and p-nitrophenol. In the future, a large-scale treatment system for organic wastewater will be used to test the removal efficiency of immobilized HRP and Mb. In addition, further research is required to facilitate the retrieval of microspheres from treated water by the implementation of magnetic material techniques. Important to consider in biotechnological applications is the environmental impact of immobilized enzymes. Although immobilization frequently improves the stability and reusability of enzymes, the environmental

repercussions of the materials and techniques utilized can differ. Therefore, in order to obtain a thorough understanding of the trade-off between the effectiveness of the immobilized Mb and HRP, and sustainable environmental practices, a holistic environmental impact assessment should be employed.

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Data Availability Statement: The data presented in this study are available on request from the corresponding author. The data are not publicly available due to privacy reasons.

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