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Research Article

Effects of Temperature and pH on Immobilized Laccase Activity in Conjugated Methacrylate-Acrylate Microspheres

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Immobilization of laccase on the functionalized methacrylate-acrylate copolymer microspheres was studied. Poly(glycidyl methacrylate-*co*-n-butyl acrylate) microspheres consisting of epoxy groups were synthesized using facile emulsion photocuring technique. The epoxy groups in poly(GMA-*co*-nBA) microspheres were then converted to amino groups. Laccase immobilization is based on covalent binding via amino groups on the enzyme surface and aldehyde group on the microspheres. The FTIR spectra showed peak at 1646 cm^{-1} assigned to the conformation of the polymerization that referred to GMA and nBA monomers, respectively. After modification of the polymer, intensity of FTIR peaks assigned to the epoxy ring at 844 cm^{-1} and 904 cm^{-1} was decreased. The results obtained from FTIR exhibit a good agreement with the epoxy content method. The activity of laccase-immobilized microspheres increased upon increasing the epoxy content. Furthermore, poly(GMA-*co*-nBA) microspheres revealed uniform size below 2 μm that contributes to large surface area of the microspheres to be used as a matrix, thus increasing the enzyme capacity and enzymatic reaction. Immobilized enzyme also shifted to higher pH and temperature compared to free enzyme.

1. Introduction

The selection of a suitable carrier material is a key factor in enzyme covalent immobilization [1]. Poly(glycidyl methacrylate) (PGMA) containing epoxy group has been widely applied due to its attractive properties and discovered as an ideal support for enzyme immobilization [2]. It has the ability to form strong linkages with amino, hydroxyl, and thiols group under mild condition [3, 4]. The modifications of epoxy ring with amine groups endow PGMA with excellent affinity to a variety of proteins, which makes it applicable in many areas and easily available for immobilization of the enzyme [5]. On the other hand, poly(n-butyl acrylate) (PnBA) provides hydrophobic property of copolymer. As the microspheres are hydrophobic, laccase immobilization will be confined to the surface of the spheres thus allowing the enzymatic reaction of enzyme and analyte to occur at the surface [6]. PGMA based materials are used as supports in various forms, such as powder, membrane, and gel with different geometrical configurations for immobilization of several enzymes [7]. Recently, PGMA supports were investigated for enzyme immobilization using physical and chemical immobilization techniques. It is because smaller diameter of the microspheres provides more active sites for enzymes reaction thus improving immobilization efficiency.

Immobilization is always a successful strategy for stabilizing enzymes or improving their performance [8]. Bezerra et al. (2015) gave guidelines on the selection of suitable solid supports and immobilization conditions [9]. Durán et al. (2002) comprehensively reviewed the different immobilizations of laccase, including a variety of supports available, immobilization techniques, and potential industrial applications [10]. Several approaches have been reported for the immobilization of laccase such as entrapment into polymer

support [11, 12], absorption method [13], or covalent linkages [9, 14]. Among various immobilization methods, covalent attachment is the most frequently used because it provides strong and stable enzyme attachment and, in some cases, reduces enzyme deactivation rates. It was reported that the immobilized enzyme by covalent linkage is usually more stable in the reaction system [15]. Immobilization via covalent binding prevents enzyme leaching and improves enzyme stabilization [5]. As biomolecules, their structures, functions, and biological activities have to be maintained. Besides that, they must be tightly bound to the surface to prevent leaching [16]. Physical properties of polymeric support such as surface area, porosity, and functional group density can easily be tailored in accordance with their specific needs. Among them, polymeric microspheres and nanospheres have attracted much attention due to their large-scale applications including ion exchange, bioseparation, biosensor, and bioreactor [6].

In this study, we have successfully synthesized poly(glycidyl methacrylate-*co*-n-butyl acrylate) referred to as poly(GMA-*co*-nBA) microspheres by a rapid method of emulsion photopolymerization. The prepared polymer microspheres were used for laccase immobilization via covalent linkage. Among the great variety of enzymes, laccase has gained much attention for being capable of catalyzing a wide range of popular reactions such as delignification, waste detoxification and decontamination, decolourization of dyes, degradation of polycyclic aromatic hydrocarbons, and bioremediation and as bioreceptor in biosensors application [17, 18]. Thus, laccase was chosen as an enzyme to understand the interaction of epoxy-immobilized laccase conjugated methacrylate-acrylate copolymer microspheres. The efficiency of laccase immobilization was evaluated in the aspects of size distribution of polymer microspheres, pH effect, and thermal property. This article is an extension version from the previous work reported by Mazlan and Hanifah [17]. To the best of our knowledge, poly(GMA-*co*-*n*BA) microspheres have never been used as laccase immobilization support in order to improve the stability of immobilized laccase.

2. Experimental

2.1. Material. The following chemicals were obtained from commercial sources: glycidyl methacrylate, GMA (Sigma-Aldrich), *n*-butyl acrylate, nBA (Merck), ethylene glycol dimethacrylate, EGDMA (Sigma-Aldrich), sodium dodecyl sulphate, SDS (Systerm), 2,2-dimethoxy-2-phenylacetophenone, DMPP (Sigma-Aldrich), glutaric dialdehyde (Sigma-Aldrich), Bradford reagent (Sigma-Aldrich), and bovine serum albumin, BSA (Sigma-Aldrich). Deionized water was used for making aqueous solution during the experiments.

2.2. Synthesis of Poly(GMA-co-nBA) Microspheres. Poly(GMA-*co*-nBA) microspheres were prepared via photopolymerization in the form of emulsion. Two compositions of GMA and nBA monomers were prepared in sample bottles to produce GN91 and GN82, respectively. For GN91 copolymer, a mixture of 90% v/v of GMA monomer, 10% v/v of nBA monomer, 0.09 g DMPP, 400 μL EGDMA, 0.1 g SDS, and 10 mL deionized water was prepared in a sample bottle. The same amount of DMPP, EGDMA, SDS, and deionized water was used for the preparation of GN82, except that it contained 80% v/v GMA monomer and 20% v/v nBA monomer. The mixture turned into milky white solution after five-minute sonication. The solution was photocured for five minutes under continuous purging with nitrogen gas in an ultraviolet exposure unit (R.S. Ltd.) of 15-watt light intensity at a wavelength of 350 nm. Poly(GMA-*co*-nBA) microspheres were isolated by centrifugation (4,000 rpm, KUBOTA) for 8 min and then thoroughly washed a few times with methanol. Finally poly(GMA-*co*-nBA) microspheres were dried at room temperature and kept at 4°C when not in use [18].

2.3. Surface Modification of Poly(GMA-co-nBA) Microspheres. The epoxy groups of the poly(GMA-*co*-nBA) microspheres were aminated with 0.5 M ammonia at 65°C in a beaker containing 25 g of microspheres and magnetically stirred for 5 h. After reaction, the aminated poly(GMA-*co*-nBA) microspheres were washed with deionized water. The aminated poly(GMA-*co*-nBA) microspheres (5 g) were equilibrated in phosphate buffer (10 mL, 50 mM, pH 7.0) for 18 h and transferred to the medium containing glutaric dialdehyde (20 mL, 0.01% v/v). The activation reaction was carried out at 25°C for 12 h, while the medium was continuously stirred. After the reaction period, excess glutaric dialdehyde was removed by washing sequentially with distilled water, acetic acid solution (100 mM, 100 mL), and phosphate buffer (100 mM, pH 7.0). The product of modified poly(GMA-*co*-nBA) microspheres was dried in a vacuum oven at 40°C and stored at room temperature [19].

2.4. Immobilization of Laccase onto Poly(GMA-co-nBA) Microspheres. Immobilization of laccase on the modified poly(GMA-*co*-nBA) microspheres was carried out at room temperature. An amount of 1 ml from 20 mg/ml laccase solution was added to 100 mg beads in the presence of 5 mg sodium borohydride ($NaBH_4$) and stirred for 1 hour. After 1 hour, physically bound enzyme was removed by rinsing the supports with phosphate buffer (50 mM, pH 7.0). It was stored at 4°C in the phosphate buffer until use [10].

2.5. Microspheres Characterization. The composition of copolymer microspheres was analyzed by Attenuated Total Reflection-Fourier Transform Infrared (ATR FT-IR) (model FTIR-8400S, Shimadzu, Japan) equipped with a MCT-A detector at a resolution of 4 cm^{-1}. The morphology and size of copolymer microspheres were observed by scanning electron microscopy (SEM, LEO 1450VP) at resolution voltage of 20 kV. Dry copolymer microspheres were placed on a piece of glass slide and then deposited within a thin layer of gold to reduce the charge effect from primary electron beam, which may cause scanning error [20]. Size and distribution of copolymer microspheres were determined based on a random selection of 300 microspheres from a scanning electron micrograph [6]. The amount of available

surface functional epoxy groups content of the copolymer microspheres was determined by pyridine–HCl method as described previously [18].

2.6. Laccase Optimization. Bradford protein assay was conducted to determine laccase concentration and to ascertain the optimum amount of glutaric dialdehyde required in the preparation of poly(GMA-*co*-nBA) microspheres [21]. For this purpose, 1.4 g of poly(GMA-*co*-nBA) microspheres for each glutaric dialdehyde (GA) was used (GA = 0.001; 0.010; 0.020; 0.030%) and placed on a screen-printed electrode (SPE) and dried at 4°C. After 24 hrs, 2 μL laccase solution (0.05 mg μL^{-1}) was dropped onto the surface of poly(GMA-*co*-nBA) microspheres deposited on SPE and left at 4°C for 24 hrs. Finally the SPE with immobilized laccase on the spheres was immersed in 3 mL of 0.05 M phosphate buffer solution at pH 5 for 30 min. In order to determine the amount of laccase present in the solution of microspheres, a mixture of 100 μL phosphate buffer for washing, 100 μL NaOH, and 800 μL Bradford reagent were mixed and incubated for 6 min. The mixture was then measured using a spectrophotometer (Cary 50) at 595 nm. For the calibration of Bradford microassay, a series of BSA standard solutions were prepared (0, 10, 20, 30, 40, and 50 μg mL^{-1}) in 0.05 M phosphate buffer at pH 5 [6].

Laccase activity was determined by the oxidation of ABTS method [22]. The assay mixture contained 5.1 mM ABTS, 1 mM sodium phosphate (pH 5), and a suitable amount of enzyme. Oxidation of ABTS was monitored by determining the increase at 414 nm. Absorbance was read at 414 nm in a spectrophotometer (Perkin Elmer Lambda 900 UL/VIS/NIR) against a suitable blank. One unit was defined as the amount of the laccase that oxidized 1 μmol of ABTS substrate per min. The effect of pH on the activity of free and immobilized laccase was carried out over pH range 2.0–7.0 and at 30°C. Concentration of laccase solution used was 2.0 mM and was prepared in 0.05 M sodium phosphate in the pH range 2.0–7.0. The effect of temperature on laccase activity was studied in the range 20–50°C with a laccase concentration of 2.0 mM in 50 mM phosphate buffer pH 5. Results for pH and temperature are presented in a normalised form with the highest value of each set being assigned the value of 100% activity [22, 23].

3. Results and Discussion

3.1. Structural Analysis of Poly(GMA-co-nBA) Microspheres. In the present study, poly(GMA-co-nBA) microspheres were prepared from GMA and nBA via emulsion photopolymerization. The copolymer was then modified at the epoxy ring to allow covalent binding of laccase with the copolymer. The proposed mechanism of the poly(GMA-co-nBA) microspheres modification is presented in Figure 1.

The theoretical epoxy group in copolymer microspheres was dependent on GMA content in the monomer form. The theoretical epoxy was defined as the proportion of epoxy in total polymer by assuming that all of the monomers were converted to the polymer. It was found that the theoretical value of poly(GMA-co-nBA) microspheres was 5.33 mmol/g and based on the determination of epoxy content by titration was 3.44 mmol/g. Percent conversion of epoxy groups was approximately 65% and this value was equivalent to other finding [24]. Some parts of the epoxy groups were hidden in the inner copolymer as they were not chemically reactive under the titration conditions [10].

The activation of aminated poly(GMA-*co*-nBA) microspheres was achieved by the reaction with glutaric dialdehyde under mild condition. Laccase was then covalently immobilized via amino groups to activate poly(GMA-*co*-nBA) microspheres. In this coupling reaction, alkylated functionalization of poly(GMA-*co*-nBA) microspheres may occur after the aldehyde functionalization step. Furthermore, the glutaric dialdehyde can readily react with an amino group in mild condition; thus, aldehyde group content should be closed to the content of the amino group on the microspheres [25].

FTIR spectra were used to ensure the presence of epoxy groups in poly(GMA-*co*-nBA) microspheres and the conjugated poly(GMA-*co*-nBA) microspheres as presented in Figure 2. Both FTIR spectra of polymer microspheres gave characteristic peaks at 1722 cm^{-1} and 2956 cm^{-1} due to C=O of ester group and methyl vibration, respectively, as shown in Figure 2. The most important adsorption bands at 1156 cm^{-1} and 910 cm^{-1} represent epoxy group. The transmittance was significantly decreased after ammonia was introduced into poly(GMA-co-nBA) microspheres.

During synthesis of polymer microspheres, glutaric dialdehyde was chosen for its reactive aldehyde groups to bind with amino-bearing laccase [26]. Long chain alkyl sulphate surfactant, which is amphiphilic, was used to stabilize the emulsion system and to prevent monomers from forming larger droplets by allowing small droplets to remain stable during emulsion phase [27, 28]. Photopolymerization caused droplets that contain GMA and nBA monomers in the presence of photoinitiator to be converted into poly(GMA-*co*-nBA) microspheres at room temperature. This method can be simply terminated by removing the light source [6].

Surface morphology of methacrylate-acrylate microspheres was investigated using SEM and the micrographs are presented in Figure 3. The micrographs revealed that the synthesized copolymers were of spherical shape. However, when nBA composition was increased from 10% to 20%, the microspheres tend to be more aggregated as exhibited by the micrograph (Figure 3(d)). This may be explained by the fact that a higher quantity of nBA leads to the merging of the monomer into larger droplets thus resulting in an increase in the size of the microspheres formed after photopolymerization. The increase of sticky properties is contributed by nBA [29].

Figure 4 demonstrates narrow size distribution with the highest percentage of microspheres diameter in the range of 0.1 μm to 2.0 μm for GN91 copolymer compared to the range of 1.0 to 4.0 μm for GN82. Methacrylate-acrylate microspheres in this study, particularly GN91, produced smaller particles size in comparison to previous findings [29, 30]. As a result, the total surface area will be increased and improve enzyme binding onto the polymer microspheres as reported by other studies [24, 30].

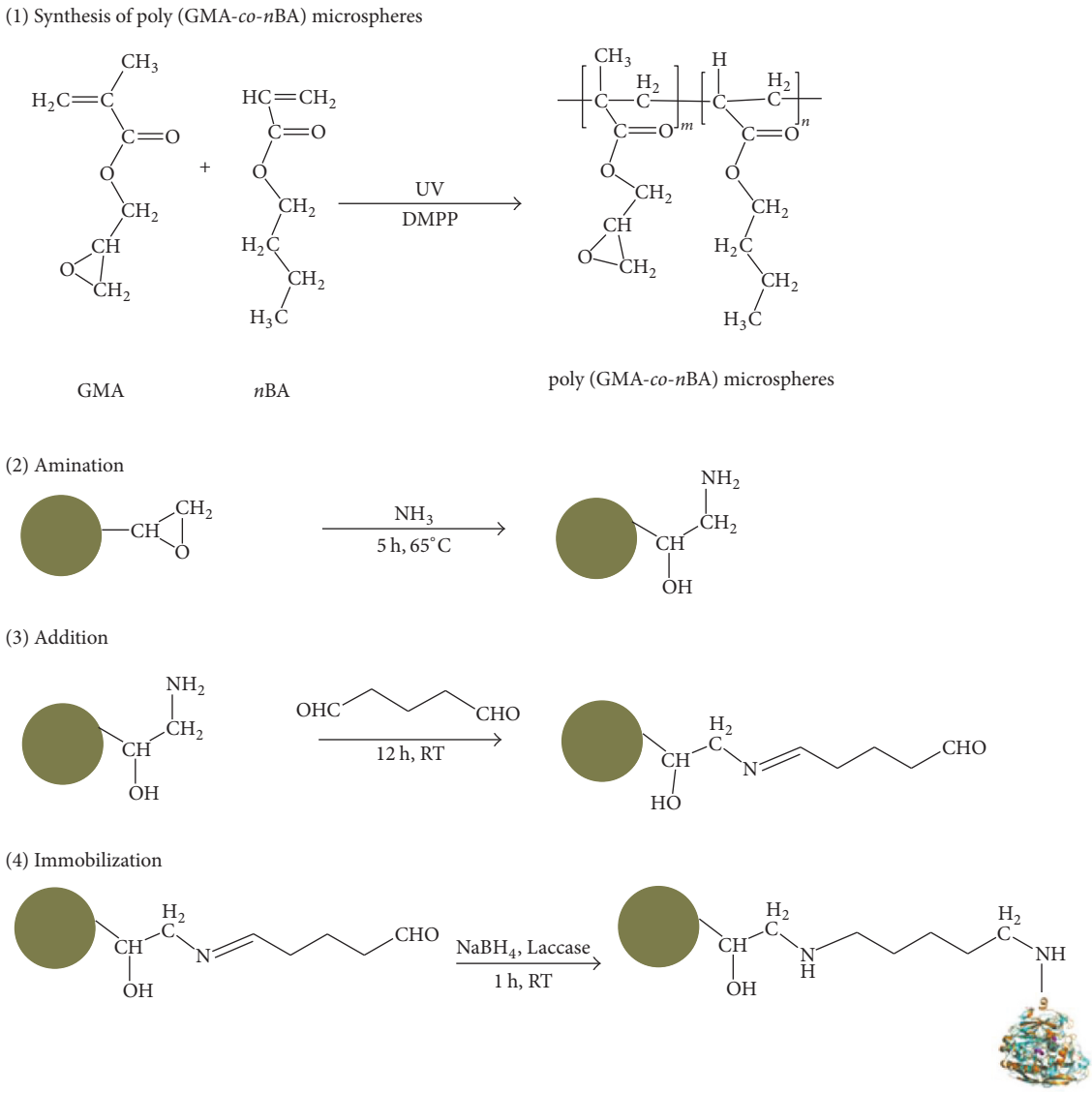

Figure 1: Proposed schematic reaction of poly(GMA-*co*-*n*BA) microspheres for laccase immobilization by covalent binding.

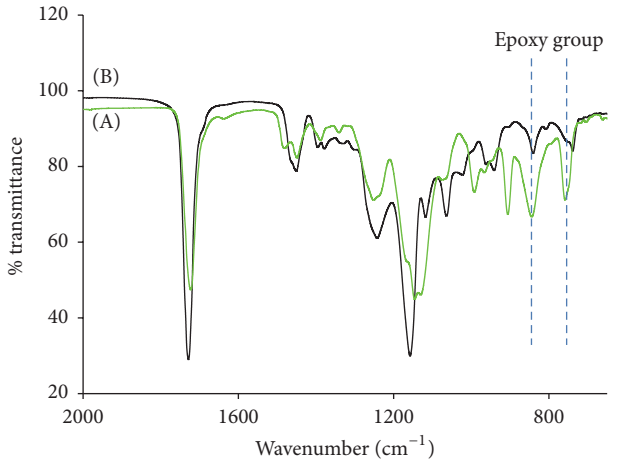

Figure 2: FTIR spectra of poly(GMA-*co*-nBA) microspheres (A) and poly(GMA-*co*-nBA) microspheres (B) after conjugation. FTIR spectra showed the decreasing peak intensity of epoxy group before and after polymer conjugation.

3.2. Effect of Glutaric Dialdehyde as Cross-Linking Agent. The amount of immobilized laccase on the GN91 copolymer microspheres with varying compositions of glutaric dialdehyde as an active functional group was determined using Bradford assay. Figure 5 shows that the amount of laccase immobilized on GN91 copolymer microspheres increased when the concentration of glutaric dialdehyde increased from 0.001 to 0.030% but decreased after 0.010%. It may be due to an excessive cross-linking between the immobilization materials where the bulk of the immobilized enzyme blocked further access by the free enzyme [25]. This limitation of binding capacity is attributed to the stearic effect between free and immobilized enzyme as shown in Figure 6.

Poor strength of cross-linked microspheres may occur when low concentration of glutaric dialdehyde was used. When glutaric dialdehyde concentration was increased, the amount of free aldehyde groups on the microspheres' surface will be increased thus enhancing the laccase loading capacity. However as the concentration of glutaric dialdehyde was

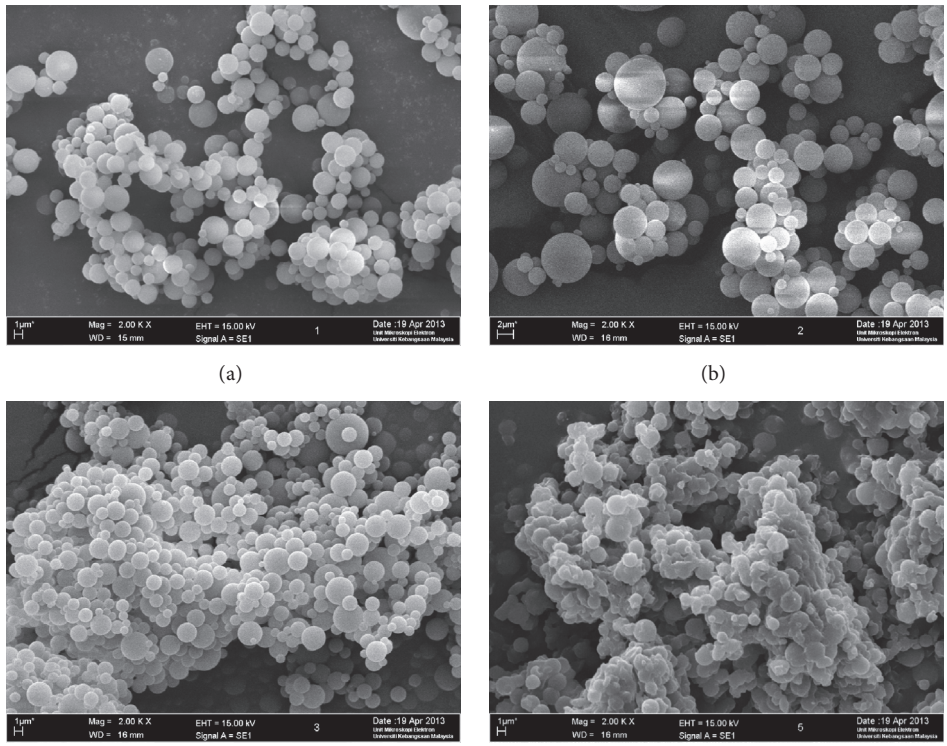

Figure 3: SEM micrographs of poly*n*BA (b), GN91 copolymer (c), and GN82 copolymer (d) at 2.00 Kx magnification and size distribution of poly(GMA-*co*-*n*BA) microspheres in various compositions.

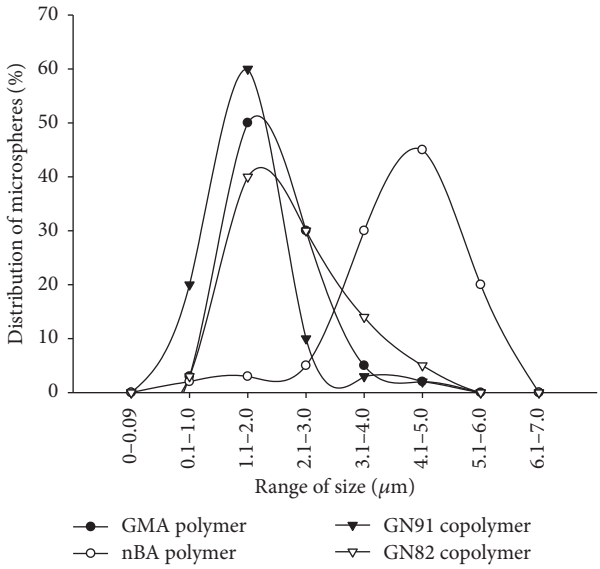

Figure 4: Size distribution for each of conjugated polymers.

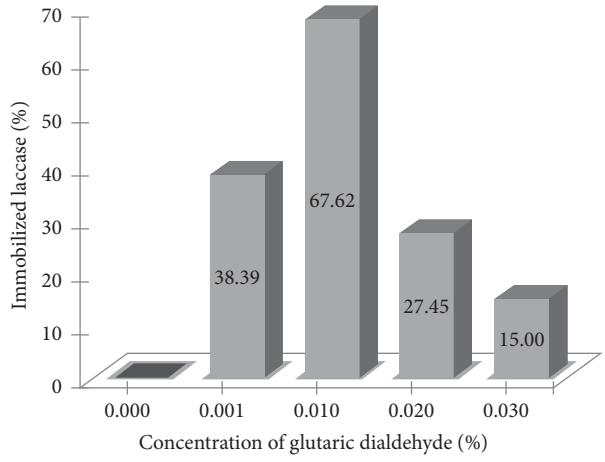

Figure 5: Effect of glutaric dialdehyde concentrations toward enzyme binding capacity.

more than 10%, the excessive cross-linking within the immobilization matrix may be blocked and change the enzyme conformation, hence resulting in the declination in the activity of the immobilized enzyme [27].

3.3. Temperature and pH Effects. The effect of temperature on the free and immobilized laccase activities is shown in Figure 7. The temperature profile of the immobilized laccase improved the stability of the optimum temperature value in comparison to the free laccase. It means that the immobilization method preserved the enzyme activity. The temperature profile of immobilized laccase was also broader than free laccase. The optimum temperature for free laccase appeared at 40°C and 50°C for immobilized laccase. It shows that immobilized laccase could withstand higher temperature conditions compared to free laccase. The

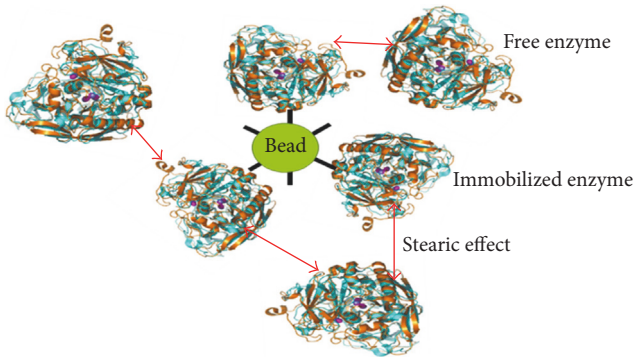

Figure 6: Schematic representation of stearic effect (red arrow) between free and immobilized enzyme.

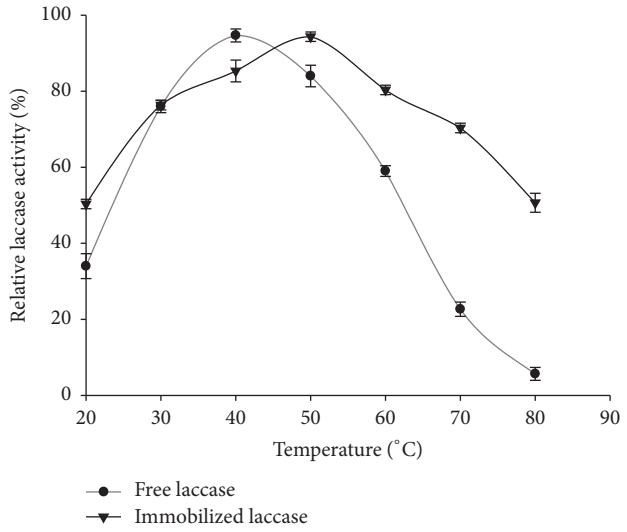

Figure 7: Effect of temperature on enzyme activity in comparison of immobilized laccase with free laccase.

shifting in temperature is caused by changing the physical and chemical properties of immobilized enzyme. The covalent bond formation via amino groups of immobilized laccase might also reduce conformational flexibility and result in higher activation energy for the molecule to reorganise proper conformation of substrate binding to substrate.

The pH effect on the activity of free and immobilized laccase was also examined in the pH range 2.0–7.0 at 30°C and the result is presented in Figure 8. The optimum pH for free laccase was found at pH 4.0 which was similar to that reported previously [22]. On the other hand, the optimum pH for immobilized laccase was shifted to pH 5.0. The microenvironment of the immobilized enzyme and bulk solution usually has unequal partitioning of H^+ and OH^- concentrations due to electrostatic interactions with the matrix, which often leads to the displacements in the pH activity profile [25, 27]. Furthermore, pH profiles of the immobilized laccase are broader than free enzyme, which means that the immobilization method preserved enzyme activity in a wider pH range [31, 32]. These results could probably be attributed to the stabilization of laccase resulting

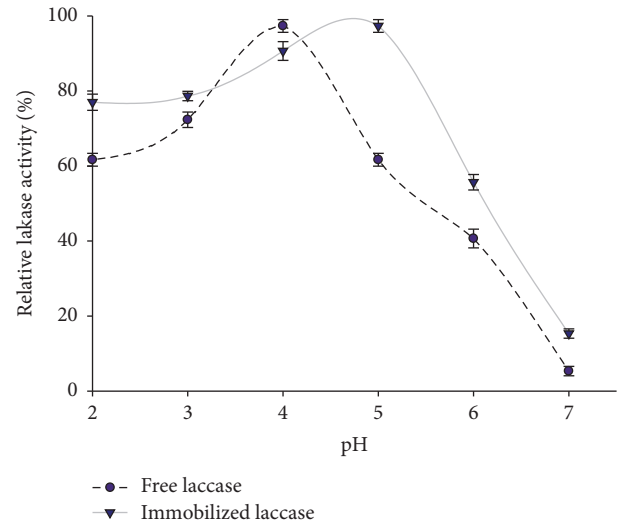

Figure 8: Effect of pH on enzyme activity between immobilized and free laccase.

from its multipoint attachments on the surface of poly(GMA-*co*-nBA) microspheres.

4. Conclusion

The conjugated copolymer microspheres of glycidyl methacrylate (GMA) with n-butyl acrylate (nBA) were successfully synthesized by using emulsion photopolymerization. The micrographs revealed that the synthesized copolymers were of spherical shape. Uniform size distribution of microspheres offers higher surface area thus enhancing laccase binding capacity. The immobilization procedure for laccase that used covalent binding has improved its temperature and pH stability.

Conflicts of Interest

The authors declare that there are no conflicts of interest regarding the publication of this paper.

Acknowledgments

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