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Enzymatic removal of phenol and *p*-chlorophenol in enzyme reactor: Horseradish peroxidase immobilized on magnetic beads

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Abstract

Horseradish peroxidase was immobilized on the magnetic poly(glycidylmethacrylate-co-methylmethacrylate) (poly(GMA-MMA)), via covalent bonding and used for the treatment of phenolic wastewater in continuous systems. For this purposes, horseradish peroxidase (HRP) was covalently immobilized onto magnetic poly(GMA-MMA) beds using glutaraldehyde (GA) as a coupling agent. The maximum HRP immobilization capacity of the magnetic poly(GMA-MMA)-GA beads was 3.35 mg g^{-1}. The immobilized HRP retained 79% of the activity of the free HRP used for immobilization. The immobilized HRP was used for the removal of phenol and *p*-chlorophenol via polymerization of dissolved phenols in the presence of hydrogen peroxide (H_2O_2). The effect of pH and temperature on the phenol oxidation rate was investigated. The results were compared with the free HRP, which showed that the optimum pH value for the immobilized HRP is similar to that for the free HRP. The optimum pH value for free and immobilized HRP was observed at pH 7.0. The optimum temperature for phenols oxidation with immobilized HRP was between 25 and 35 °C and the immobilized HRP has more resistance to temperature inactivation than that of the free form. Finally, the immobilized HRP was operated in a magnetically stabilized fluidized bed reactor, and phenols were successfully removed in the enzyme reactor.

Keywords: Horseradish peroxidase; Immobilization; Phenol removal; Wastewater treatment; Enzyme reactor

1. Introduction

Chemicals are produced by several industries and the impact of only a small fraction of them on both the nature and on human health are completely understood at this stage. The major industries that contribute to chemical compound and waste production are petrochemical (fuels and monomers), agrochemical (pesticide, fertilizer), chemicals (paint, explosive industries), electronics (conductors, resist materials), textile (dyes, fixers), paper (pulp, bleaches), pharmaceutical and medical (drugs) [1–4]. Among them, phenolic compounds are one of the major pollutants of industrial wastewaters and, at the same time, these compounds have high toxicity to the organisms. Since, these aromatic chemical could cause adverse effects on digestive system, nervous system and respiratory system, as well as leading to cancer and interfering with the normal function of thyroid, based on the researches of International Program on Chemical Safety (IPCS) [5–7], it is necessary to find an effective method to remove these phenols from waste or polluted waters. In recent years the use of oxidoreductive enzymes to catalyze the removal of aromatic compounds from wastewaters has been investigated [8–16] and the removal of phenols [17–19], chlorophenols [20,21] and other substituted phenols [22,23] were discussed.

Peroxidases (EC 1.11.1.7) have been known to oxidase aromatic compounds in the presence of hydrogen peroxide and form polymeric products. These polymers have a high molecular weight and are generally insoluble in aqueous medium, thus, they can be easily precipitated and removed from the treated water body. HRP has been used in many studies for removal of phenolic compounds from aqueous media. Because the enzyme is less costly to produce, inactivates less rapidly, or acts on a wider variety of substrates than other oxidoreductive enzymes [9,11,24,25]. Therefore, the HRP-catalyzed process has potential applications in the treatment of waters and wastewaters contaminated with phenolic pollutants.

Immobilization can be defined as the fixation of the biocatalysts (e.g. enzymes, microorganisms, organelles) to insoluble

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solid supports, and this is especially useful in the bio-treatment of industrial and agricultural wastes [26–29]. On the other hand, the use of magnetic particles for the immobilization of enzymes and cells has received attention for large scale applications. These are mainly based on the magnetic properties of the solid-phase that enables to achieve a rapid separation in a magnetic field. Enzyme immobilized magnetic supports can be used in a magnetically stabilized fluidized bed reactors (MSFBR) and can make the removal of contaminants at high rates possible [30–34]. Moreover, the magnetic supports can be easily operated by applying an external magnetic field, and they can also reduce the capital and operation costs [30,35].

In this study, magnetic beads were used for the immobilization of horseradish peroxidase. Magnetic beads were obtained by dispersion polymerization, and epoxy groups of the magnetic beads were converted into amino groups during thermal magnetization. The aminated magnetic beads were then activated with glutaraldehyde and the enzyme "HRP" was covalently linked through the amino groups [34]. The immobilized HRP was characterized under various experimental conditions in a batch system. The optimum pH and temperature for the free and immobilized HRP were investigated. Finally, the application of immobilized HRP for the removal of phenols from aqueous solutions was studied in a magnetically stabilized fluidized bed reactors at different flow rates.

2. Experimental

2.1. Materials

Peroxidase (donor; hydrogen peroxide oxidoreductase; E.C.1.11.1.7) Type II from horseradish (200 purpurogallin units/mg solid), catalase enzyme (EC 1.11.1.6), bovine serum albumin (BSA), *o*-dianisidine, 4-aminoantipyrine (4-AAP), potassium ferricyanide, polyvinyl alcohol (PVA), glutaraldehyde, phenol and *p*-chlorophenol were purchased from Sigma Chemical Company (USA) and was used without further purification. Glycidyl methacrylate (GMA), methylmethacrylate (MMA), ethyleneglycol dimethacrylate (EGDMA) and α-α'-azoisobisbutyronitrile (AIBN) were obtained from Fluka AG (Switzerland). All other chemicals were of analytical grade and were purchased from Merck AG (Darmstadt, Germany). The water used in the following experiments was purified using a Barnstead (Dubuque, IA, USA) RO pure LP reverse osmosis systems.

2.2. Preparation of activated magnetic poly(GMA-MMA) beads

The magnetic beads were prepared as described previously [34,35]. Briefly, ferric-poly(GMA-MMA) beads were prepared via dispersion polymerization, which was carried out in an aqueous dispersion medium containing $FeCl_3$ (0.3 M, 400 mL; it was used as a precursor for the thermal iron oxide precipitation in the beads). The organic phase contained GMA (7.5 mL; 5.68×10^{-2} mol), MMA (7.5 mL; or 7.01×10^{-2} mol), EGDMA (7.5 mL; or 3.98×10^{-2} mol as cross-linker) and 5.0% PVA (20 mL, as stabilizer) were mixed together with 0.2 g of AIBN as initiator in 20 mL of toluene. The polymerization reaction was maintained at 70 °C for 2.0 h and then at 80 °C for 1.0 h. Magnetization of beads structure was provided by conventional co-precipitation reaction of iron oxide in the beads as described previously. During the thermal precipitation reaction, the epoxy groups of the magnetic beads were also converted into amino groups via ammonolysis reaction due to the presence of ammonia in the medium [35]. Glutaric dialdehyde activation of the amino groups of the supports was carried out as described previously [34]. Schematic representation of the chemistry of the preparation of magnetic beads is presented in Fig. 1.

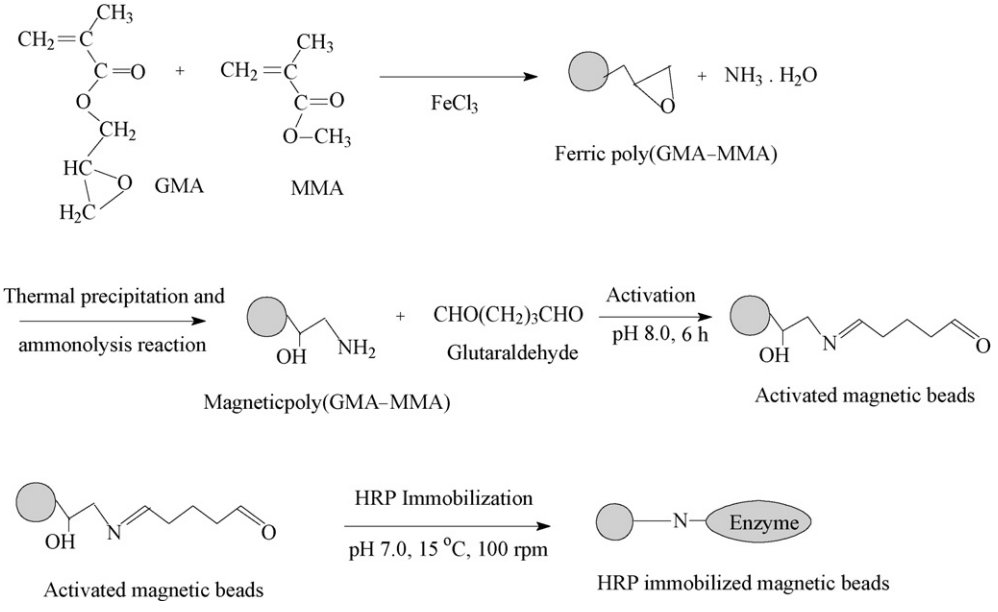

Fig. 1. Schematic representation of the chemistry of the preparation of magnetic beads.

2.3. Characterization of magnetic poly(GMA-MMA) beads

The amount of amino group of the magnetic beads was determined from elemental analysis device (CHNS-932, Leco, USA). The surface area of the magnetic poly(GMA-MMA) beads was measured with a surface area apparatus (BET method). The surface morphology of the magnetic beads was observed by scanning electron microscopy (SEM). The dried beads were coated with gold under reduced pressure and their scanning electron micrographs were obtained using a JEOL (Model JSM 5600; Japan).

2.4. HRP immobilization

The activated beads (1.0 g) were equilibrated in phosphate buffer (50 mM, pH 7.0; 5.0 mL) for 4 h, was then transferred to the enzyme solution (1.0 mg/mL) at 15 °C. The immobilization of enzyme was carried out at 15 °C with a stirring rate of 100 rpm. In order to effectively facilitate the covalent bounding and prevent enzyme inactivation at longer reaction time, the reaction coupling time was varied between 2 and 12 h. After the predetermined reaction period, the enzyme immobilized magnetic beads were removed from the medium by a magnetic separation device. Physically bound enzyme was removed first by washing the supports with saline solution (10 mL, 1.0 M NaCl) and then acetate buffer (50 mM, pH 4.0) and was stored at 4 °C in same fresh buffer until use.

The amount of HRP immobilized on the magnetic poly(GMA-MMA) beads was determined by measuring the initial and final concentration of protein in the immobilization medium using the Bradford protein assay method [36]. A calibration curve constructed with BSA was used in the calculation of enzyme concentration and the calibration curve with correlation coefficients (R^2) less than 0.990 was repeated.

2.5. Activity assays of free and immobilized HRP

Hydrogen peroxide is decomposed by peroxidase, liberating an oxygen atom to oxidase *o*-dianisidine, which has a red color in the oxidized state.

$$H_2O_2 + \underset{\text{(colorless)}}{o\text{-dianisidine}} \xrightarrow{\text{HRP}} \underset{\text{(red)}}{o\text{-dianisidine}} + H_2O \quad (1)$$

HRP activity was measured by following the increase in the amount of oxidized *o*-dianisidine. A stock assay mixture (50 mL) contained *o*-dianisidine (6.6 mg; 1.35×10^{-6} mM) in phosphate buffer (50 mM, pH 7.0). A 2.9 mL assay medium was transferred to a cuvette and the reaction was started by adding enzyme solution (10 μL, 1.0 mg/mL), and then H_2O_2 solution (60 mM; 0.1 mL). The reaction rate was determined by measuring the increases in absorbance at 530 nm resulting from the formation of a colored compound, of oxidized *o*-dianisidine with an UV–vis spectrophotometer (Shimadzu, Model 1601) equipped with a temperature controller.

For the immobilized HRP, assay medium (5.8 mL), prepared as before was put into 0.1 mg of HRP immobilized beads. The reaction was started by addition of hydrogen peroxide solution (0.1 mL). The enzyme activity was determined as described above after removing of magnetic beads with a magnetic device.

One unit of HRP activity was defined as the amount of enzyme, which catalyzes the formation of 1.0 μmol of oxidized product of *o*-dianisidine in the presence of hydrogen peroxide per minute at 35 °C and at pH 7.0.

In order to determine the effect of immobilization on the activity of HRP, substrate concentrations were varied between 10 and 300 μM in the reaction medium and the concentration of H_2O_2 was kept constant as 2 mM. The initial velocities were calculated and a Lineweaver-Burk plot (1/V versus 1/[S]) was constructed. K_m and V_{max} were determined from the intercepts at *x* and *y* axes, respectively. Storage stability experiment was conducted to determine the stabilities of free and immobilized HRP after storage in phosphate buffer (50 mM, pH 7.0) for 56 days.

2.6. Determination of phenol and p-chlorophenol

Phenol and *p*-chlorophenol concentrations were determined in the samples using a colorimetric assay in which the phenolic compounds within a sample react with 4-AAP in the presence of potassium ferricyanide reagent under alkaline conditions [37–39]. The phenolic compound assay contained sample solution (1.0 mL), 4-AAP (2.0 mM, 1.0 mL), and potassium ferricyanide reagent (1.0 mL, 6 mM). The latter two reagents were prepared in phosphate buffer solution (100 mM, pH 10). The absorbance of the assay mixture was measured at 510 nm using a spectrophotometer after 15 min of incubation at room temperature. A blank containing distilled water, 4-AAP, and potassium ferricyanide reagent was used in each set of the assay.

Reaction of phenol and *p*-chlorophenol with peroxidase in the presence of hydrogen peroxide were also performed in a batch system with the free and the immobilized enzymes. Each phenolic compounds solution (2.0 mM) was transferred to a flask contained free and/or immobilized enzymes and the reaction was started by addition of H_2O_2 solution (4 mM). These experiments were carried out over the pH range 4.0–9.0 and temperature range 15–55 °C to determine the effect of pH and temperature on the enzymatic degradation rates. After a predetermined time, the sample was withdrawn and assayed for phenols as described above. The rate of non-enzymatic reactions was ascertained by monitoring the rate of phenols degradations after addition of hydrogen peroxide to the respective phenolic compound.

The results were converted to relative activities (percentage of the maximum activity obtained in that series). The residual activity was defined as the fraction of total activity recovered after covalent immobilization of HRP on the magnetic beads compared with the same quantity of free enzyme. Each set of experiments was carried out in triplicate, the arithmetic mean values and standard deviations were calculated and the margin of error for each data set was determined according to a confidence interval of 95% using the statistical package under Excel for Windows.

2.7. Magnetically stabilized fluidized bed reactor operation

The enzymatic phenol and *p*-chlorophenol removal were carried out in a magnetically stabilized fluidized bed reactor. The magnetically stabilized reactor with a water jacket was constructed from Pyrex glass (height: 80 mm; inner diameter: 12 mm, total volume about 9.0 mL). It was equipped with Helmholtz coils (Model HC-1.5, Magnetic Instrumentation, Indianapolis, USA) and the HRP immobilized magnetic beads about 6 g was transferred into MSFB reactor. The void volume of the reactor was about 3.0 mL. The feed solution (containing 2.0 mM H_2O_2 and 1.0 mM phenol and/or *p*-chlorophenol) was pumped through the bottom inlet part of the reactor by using a peristaltic pump at different flow rate between 20 and 120 mL/h. The reactor temperature was controlled to ±0.2 °C by circulating water through the temperature control jacket. A sufficient amount catalase enzyme solution was added into these collected samples to remove remaining hydrogen peroxide in the sample solution. This quenched the reaction by rapidly converting hydrogen peroxide to oxygen and water. The remaining concentrations of the phenol and *p*-chlorophenol in these solutions were measured as described above.

To determine operational stability of the immobilized HRP for phenols degradation, the MSFB reactor was operated at 25 °C for 48 h. The feed solution contained 1.0 mM phenol and *p*-chlorophenol and 2.0 mM H_2O_2 in phosphate buffer (50 mM, pH 7.0) at a flow rate of 60 mL/h. The remaining phenols content of the solutions leaving the reactor was determined as described above.

The first-order inactivation rate constant, K_i, was calculated from the following equation:

$$\ln A = \ln A_0 - K_i t \quad (2)$$

where A_0 and A are the initial activity and the activity after time t (min).

3. Results and discussion

3.1. Properties of poly(GMA-MMA) beads

In the present study, amino groups-containing magnetic poly(GMA-MMA) beads were prepared from GMA and MMA in the presence of a cross-linker EGDMA via dispersion polymerization. The polymerization yield of the beads was about 87%. The beads were sieved and 75–150 μm size of fraction was used in the further reactions. Elemental analyses of the aminated magnetic poly(GMA-MMA) beads were performed, and the amounts of the linked amino groups were found to be 2.14 mmol/g from the nitrogen stoichiometry. The specific surface area of the magnetic beads was measured by the BET method and was found to be 21.6 m^2/g beads. The SEM micrograph shows that the beads have a porous surface structure (Fig. 2). The porous surface properties of the poly(GMA-MMA) beads would favor higher immobilization capacity for the enzyme due to increase in the surface area. The water content is very important when use of matrix in enzyme immobilization is contemplated. The water content of the magnetic poly(GMA-MMA) beads was determined as 47%. This is a moderate swelling degree for the cross-linked acrylate based hydrogel beads and the water content of beads is suitable in use as column packing material for application of immobilized enzyme in a continuous flow system. The magnetic properties of the poly(GMA-MMA) beads were discussed in our previous studies [33,34].

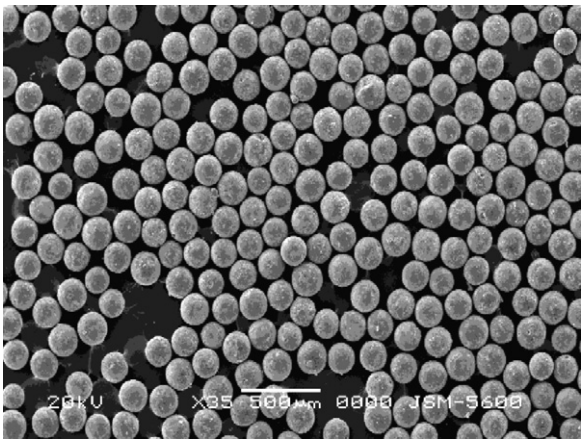

Fig. 2. SEM micrographs of poly(GMA-MMA) magnetic beads.

3.2. Immobilization of HRP onto magnetic poly(GMA-MMA) beads

HRP was covalently immobilized after activation of amino groups of the magnetic beads with glutaraldehyde. HRP was bonded on the magnetic poly(GMA-MMA) beads via a coupling reaction between the free amino groups of enzyme and aldehyde groups of supports. The covalent immobilization of enzyme via coupling reaction could be considered via shift-base formation. Glutaric dialdehyde can readily react with an amino group, therefore, the aldehyde groups content should be close to the content of the amino group on the beads. The –SH groups of HRP can also react with glutaraldehyde after amine groups have been used. It is important to notice that $–NH_2$ groups are more susceptible than –SH groups to react with glutaraldehyde.

The effects of HRP coupling time on the immobilization capacity and on the enzyme activity was studied with the magnetic beads and is presented in Fig. 3. An increase in the coupling duration time led to an increase in the immobilization efficiency (from 1.12 to 3.35 mg/g beads) but this relation leveled off at around 8.0 h. Further increase in the coupling duration time (up to 12 h) did not lead to a significant change in the immobilization capacity.

It should be noted that the coupling agent glutaraldehyde has a 5-carbon length and it should be acted as a spacer-arm in this immobilization method. In the remaining experiments, the immobilization reaction was carried out with a 5 mg of HRP (or 795 U) in immobilization medium (5 mL) and 1.0 g activated magnetic beads and was incubated for 8 h. The amount of immobilized enzyme was about 3.35 mg/g (or 67% of the added enzyme was immobilized) on the magnetic beads. In this proto-

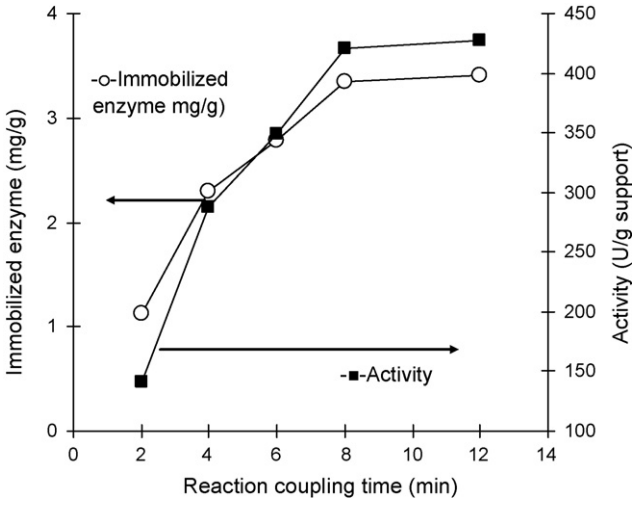

Fig. 3. Effect of reaction coupling time on the immobilization efficiency and enzyme activity.

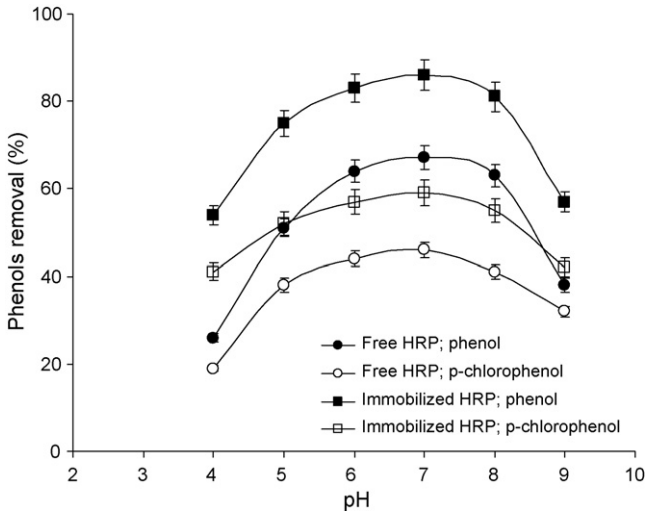

Fig. 4. Effect of pH on the activity of free and immobilized HRP on the magnetic poly(GMA-MMA) beads.

col retained activity of the enzyme was 79% and the measured activity of the immobilized HRP was about 421 U/g beads.

3.3. Biochemical properties of free and immobilized HRP

Kinetic constants of the free and immobilized HRP, i.e., K_m and V_{max} values, were determined by using H_2O_2 as substrate. The apparent K_m values of the free and immobilized HRP were found to be 223 and 368 μM, respectively. The K_m value of the immobilized HRP was increased about 1.65 fold compared to that of the free form. This indicates an alteration in the affinity of the enzyme towards to the substrate upon covalent immobilization on the magnetic beads. The apparent V_{max} values of the free and immobilized HRP were found to be 167 and 128 U/mg, respectively. The V_{max} value of the immobilized enzyme was decreased significantly upon covalent immobilization on the magnetic beads. These variations in the K_m and V_{max} values of the enzyme upon immobilization are attributed to several factors such as the non-covalent interactions of the immobilized enzyme molecule with the modified polymer surface might have induced an inactive conformation to the enzyme molecules. It should be noted that the immobilization process does not also control the proper orientation of the immobilized enzyme on the support [40–42].

3.4. Optimum pH and temperature for enzymatic removal of phenols

The optimization for the removal of phenol and *p*-chlorophenol was performed by measuring the initial rates of enzymatic phenols degradation at different values of pH using the same quantity of free and immobilized HRP, 2.0 mM phenol and/or *p*-chlorophenol and 4.0 mM of H_2O_2. The concentration of the H_2O_2 was about 2 fold higher than those of the phenolic compounds. A conventional bell shaped curves are obtained within the pH range tested for the free and immobilized HRP. Furthermore, the pH profiles of the immobilized HRP are broader than that of the free enzyme, which means that the immobilization methods preserved the enzyme activity in a wider pH range. The optimum pH value for degradation of phenolic compounds using both the free and immobilized HRP was observed at around pH 7.0 with maximum removal of about 86% and 59% for phenol and *p*-chlorophenol, respectively in 2 h (Fig. 4). On the other hand, the free enzyme was less effective than that of the immobilized counterpart, 67% and 46% phenol and *p*-chlorophenol were removed, respectively. In addition, phenol may have more affinity to free and immobilized HRP than that of the *p*-chlorophenol under same experimental conditions. These findings also suggest that the immobilization is stabilized the enzyme against to various forms of denaturation such as pH and heat. Several earlier investigators have described that the covalent immobilization of enzymes on hydrogel supports resulted in the stabilization of enzymes against various forms of denaturation [19,40,43]. It should be noted that the addition of hydrogen peroxide on each phenol solution alone did not give a significant phenol reduction. In the remaining experiment, degradation studies were carried out using immobilized HRP.

The phenol and *p*-chlorophenol degradation was studied under the same phenols and H_2O_2 concentrations with the immobilized HRP at a temperature range of 15–55 °C and the results were expressed as percentage of the phenols removal rates (Fig. 5). For the temperatures between 25 and 35 °C, the reaction rates are similar and a plateau was observed at these temperatures. There are not any significant differences in the degradation rates at these temperatures. As seen in this figure, the reaction rate was less stepper at 15 °C, However, for the temperatures 45 and 55 °C, the phenols degradation rates were much slower compared to those of the others studied temperatures and degradation rate of phenol and *p*-chlorophenol were further decreased up to 47% and 21% after 2 h (Fig. 5). The decrease in degradation rates can be due to the loss of immobilized enzyme activity at high temperature.

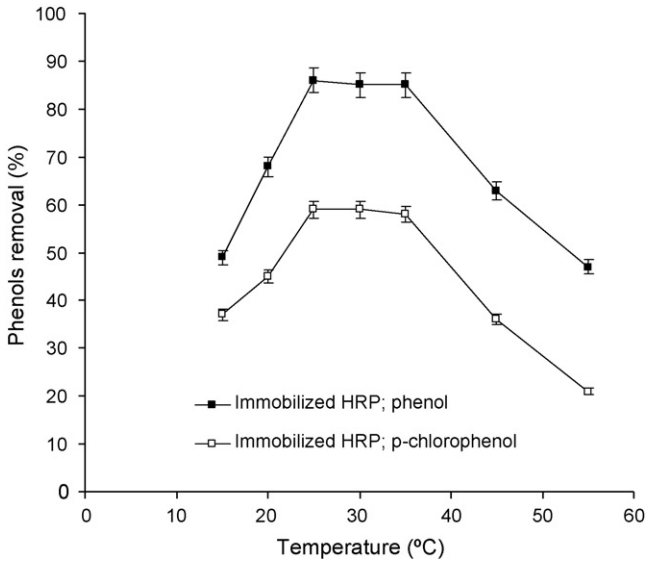

Fig. 5. Effect of temperature on the activity of the immobilized HRP.

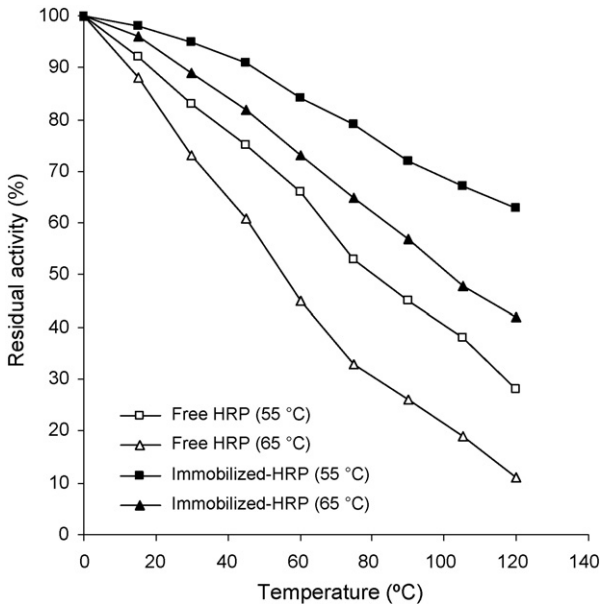

Fig. 7. Thermal stabilities of free and immobilized HRP on the magnetic poly(GMA-MMA) beads.

3.5. Storage and thermal stabilities of the free and immobilized HRP

In general, if an enzyme is in solution, it is not stable during storage, and the activity is gradually reduced. Free and immobilized HRP were stored in phosphate buffer solution (50 mM, pH 7.0) at 4 °C for 56 days and the remaining activity of the enzymes were determined using H_2O_2 and phenol as substrates. Under the same storage conditions, the activity of the immobilized HRP decreased slower than that of the free HRP. The free enzyme lost all its activity within 42 days. The immobilized HRP maintained about 84% of its initial activity during 56 days storage period (Fig. 6). Thus, the immobilized HRP exhibits a higher stability than that of the free HRP. On the basis of this observation, modified magnetic support should provide a stabilization effect, minimizing possible distortion effects imposed from aqueous medium on the active site of the immobilized enzyme. Thus, the modified hydrophilic support and the immobilization method provide higher shelf life compared to that of its free counterpart [44,39].

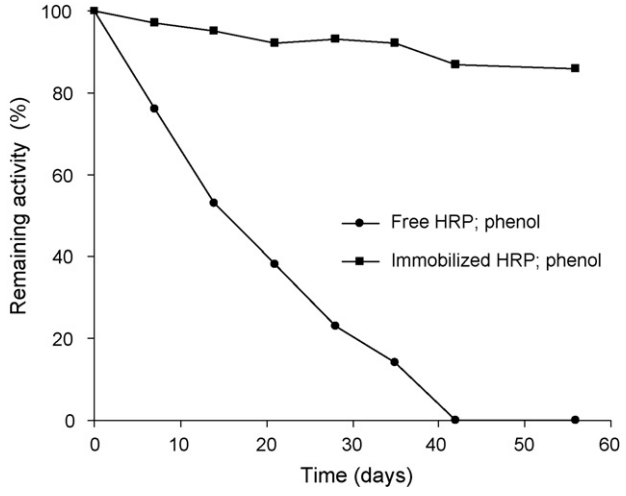

Fig. 6. Storage stabilities of free and immobilized HRP on the magnetic poly(GMA-MMA) beads.

Thermal stability experiments were carried out with free and the immobilized HRP, which were incubated in the absence of substrate at various temperatures (Fig. 7). At 55 °C, the free and immobilized HPR retained their activity to a level of 28% and 63% during a 120 min incubation period. At 65 °C, the free and the immobilized enzymes retained their activity about to a level of 11% and 42%, respectively. The immobilized HPR was inactivated at a much slower rate than that of the native form. These results suggest that the thermo stability of immobilized HPR increased considerably.

3.6. Operation of MSFB reactor for removal of phenols

For the removal of phenolic compounds from the aqueous solution via enzymatic oxidations, the most convenient continuous system configuration can be that of a magnetically stabilized fluidized bed reactor. In this system, (i) the continuous degradation reaction can be also carried out in solutions containing insoluble materials [33,34]; (ii) a large volume of water can be continuously treated using a defined quantity of adsorbent in the reactor system. As a result of the magnetic properties of the adsorbents, they can be selectively removed from the medium under the applied magnetic force. The continuous system operation parameter is presented in Section 2.7, and the residence time corresponding to the given flow rates is calculated by the following equation:

$$D = \frac{\nu_o}{\varepsilon V} \tag{3}$$

where D is the dilution rate (h^{-1}), ν_o is the volumetric flow rate of the phenolic compounds solution (mL h^{-1}), V is the total volume

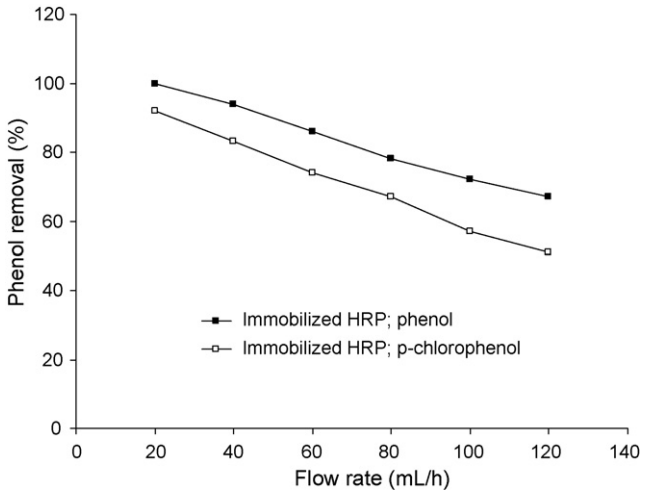

Fig. 8. The effect of residence time on the degradation rates of phenol and *p*-chlorophenol by the immobilized HRP in the enzyme reactor.

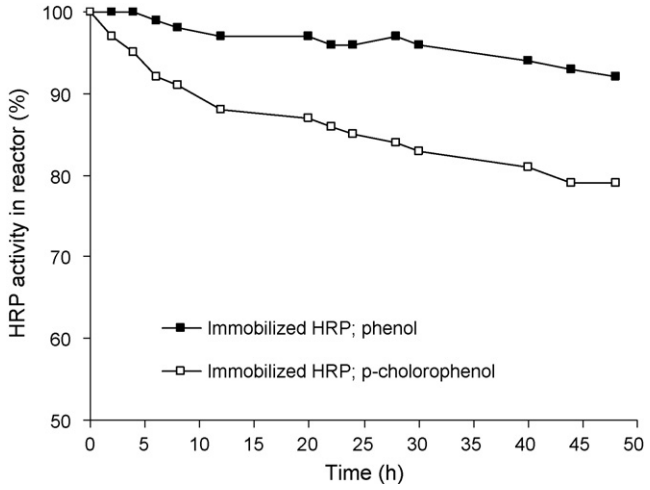

Fig. 9. Operational stability of the immobilized HRP during degradation of phenol and *p*-chlorophenol.

of the continuous system (mL) and ε is the void fraction of given as ratio of void volume to the total volume of the continuous system. Residence time (τ) is the reciprocal of dilution rate.

The effect of flow rate on the phenol and *p*-chlorophenol degradation rates by the immobilized HRP on the magnetic beads was investigated by fixing initial phenol and *p*-chlorophenol concentration of 1.0 mM phenols and keeping of flow rate between 20 and 120 mL/h. Fig. 8 shows the effect of residence time on the degradation rates of phenol and *p*-chlorophenol by the immobilized HRP. As seen in Fig. 7, the phenol and *p*-chlorophenol degradation rates with the immobilized HRP decreased significantly from 100% and 92% to 67% and 51%, respectively, with the decrease in the residence time from 0.15 to 0.025 h. These results show that the phenols degradation rates were dependent on the residence time. As seen in the figure, the residence time is increased; the removal efficiencies of phenol are also increased. At the highest flow rate (i.e., lowest residence time), the lowest degradation rates were observed for both phenolic compounds. This behavior can be due to the insufficient contact time between the phenols and the immobilized HRP.

It is important for economical use of an immobilized enzyme, as a means for the mass production of the desired product, that the enzyme reaction is continuous. One of the problems in continuous enzymatic reaction is the operational stability of the immobilized enzyme on the support. The operational stability of the immobilized HRP was studied at 25 °C for 48 h (Fig. 9). The enzyme reactor was continuously fed with 1 mM phenol and/or *p*-chlorophenol solution. After 48 h continuous operation, the enzyme reactor lost its initial activity about 8% and 21% with phenol and *p*-chlorophenol, respectively. The first-order operational inactivation rate constant, K_i, of the immobilized HRP was calculated from above data as $K_i = 7.70 \times 10^{-5}$ min^{-1} for phenol and $K_i = 2.16 \times 10^{-4}$ min^{-1} for *p*-chlorophenol. This observation may be attributed to some sort of enzyme inactivation and/or inhibition by the free radicals or by phenolic polymer produced during enzymatic oxidation of phenols [43,39]. It should be noted that operational inactivation rate of the HRP was much pronounced during *p*-chlorophenol oxidation. Chlorine ions are known to be released during the enzymatic coupling process of chlorophenols [38] and the released chlorine ions might be responsible of the high inactivation rate of the immobilized HRP during *p*-chlorophenol oxidation in the enzyme reactor.

4. Conclusion

In this study, HRP was covalently immobilized on magnetic beads via glutaraldehyde coupling in order to study the use of this enzyme for the removal of phenolic compounds from aqueous solutions. The immobilized enzyme retained a high activity on the magnetic beads, and it was found that the optimum pH for immobilized HRP was around 7.0 during phenol oxidation reactions. In general, temperature affects the initial reaction rates greatly, in this case, it does not have great influence on the final phenols removal efficiency, and the optimum temperature for the polymerization reaction was found to be between 25 and 35 °C for the immobilized HRP. The immobilized HRP was more stable during operation and during storage compared to free counterpart. So, higher phenol conversions were obtained with the same concentration of immobilized HRP as free counterpart. Finally, a high operational stability obtained with the immobilized HRP in the enzyme reactor indicates that the immobilized HRP could successfully be used in a large scale continuous system for the enzymatic degradation of phenols.

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