Use of Immobilized Enzyme In Granular Activated Carbon And Chitosan Beads For Phenol Removal From Effluents

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Abstract

Tyrosinase enzyme present in a crude extract was immobilized in granular activated carbon (GAC) and activated chitosan beads (ACB). It was possible to immobilize up to 70.0 % of the enzymes in GAC in the conditions of 10.0 g of support, 15.7 rad/s of agitation and 90 minutes of contact time, and 100.0 % of enzymes in ACB when using 5 g of support, agitation of 15.7 rad/s and contact time of 120 minutes. In enzymatic oxidation tests, tyrosinase immobilized in GAC was able to achieve a final phenol concentration below the limit required by Brazilian law, 0.5 mg/L for phenol solutions with an initial concentration up to 20.0 mg/L while the enzyme immobilized in ACB was able to adapt solutions with initial concentrations of phenol up to 40.0 mg /L. It was possible to reuse the enzyme immobilized in GAC 2 times, maintaining the same phenol removal efficiency, while the enzyme immobilized in ACB maintained up to 98.0 % of its efficiency in 5 cycles of enzymatic oxidation of solutions with 10.0 mg/L of phenol initially. It was possible to maintain the same phenol removal efficiency as immobilized enzymes when stored for up to 2 weeks.

Introduction

One of the primary pollutants found in effluents is phenolic compounds commonly found in aqueous effluents from various industrial activities, such as the petrochemical, textile, plastics, resins, cellulose, and paper industries (Mohammadi et al. 2015). Based on the high toxicity of phenolic compounds, trace concentrations are already sufficient to alter the organoleptic properties of water (Ilavský et al. 2017), suggesting the creation of efficient and inexpensive techniques for treating these effluents.

In Brazil, the National Environmental Council (Conselho Nacional do Meio Ambiente - CONAMA) legislation imposes low phenol concentration limits in treated industrial effluents. CONAMA Resolution 430/2011 of May 30, 2011 (MMA 2011) is very restrictive and limits the maximum content of total phenols (4-amino-antipyrine reacting substances) to 0.5 mg/L for release in freshwater bodies.

In general, conventional methods of removing phenolic compounds from effluents have several limitations, increasing interest in developing more efficient and cheaper routes, such as the degradation of phenol and phenolic compounds through the use of enzymes such as polyphenol oxidases (PPO), among them tyrosinase and polyperoxidases. The main advantages of using enzymes for phenol degradation and its derivatives are their high selectivity and efficiency, even at low concentrations (Pandey et al. 2017; Xu and Yang 2013).

One way to reduce the cost of using enzymes in industrial processes, a significant limitation for their large-scale use, is to use enzymes derived from crude extracts, which are cheaper than commercial enzymes, and the use of the immobilized enzyme, which allows their reuse (Eş et al. 2015; Yamada et al. 2006).

The present work aims to study the use of granular activated carbon and activated chitosan beads as supports for the immobilization of the tyrosinase enzyme present in a crude enzyme extract, obtained

from the mushroom *Agaricus bisporus*, optimizing the immobilization by studying the influence of different parameters (mass support, agitation, contact time), for its future use in phenol removal from a synthetic effluent, in order to obtain an efficient alternative for phenol removal from industrial effluents.

Materials And Methods

Materials

The crude enzyme extract used containing the enzyme tyrosinase (EC 1.14.18.1) was produced from *Agaricus bisporus* macrofungus, purchased in lots at a local market.

For the phenol immobilization and enzymatic oxidation assays, two types of support were used, the commercial Carbotrat AP Granular Activated Carbon, supplied by Indústria Química Carbonífera Criciuma S.A, and chitosan beads, produced in the laboratory. All chemicals containing analytical purity and were purchased from Sigma-Aldrich.

Tyrosinase Enzyme Extraction Methodology

The enzymatic crude extract was obtained following the methodology developed by Kameda et al. (2006), in which the fruiting bodies of *Agaricus bisporus* are ground in 1:1 (g/mL) chilled acetone, with a maximum of 500.0 g at a time. The mixture was filtered under vacuum, and the residue was stored at 273K for 24 h. Then the mushroom paste was re-suspended with distilled water and stored again at 273K for 24 h to obtain the first enzyme extract by centrifugation at 418.9 rad/s for 10 minutes. The process of re-suspension and centrifuging the slurry was performed again to obtain a second extract. The measurement of tyrosinase enzymatic activity was determined following the adapted procedure of Santos et al. (2013).

Supports

Carbotrat AP is a granular activated carbon produced in Brazil and supplied by Criciuma S.A Carboniferous Chemical Industry.

Chitosan beads were prepared based on the modified procedure of Zhou et al. (2013), where 2.0 g of medium molar weight chitosan was added in 100.0 mL of 5.0 % (v/v) acetic acid solution under stirring for 30 minutes. The solution was then dripped through an approximately 1mm plastic tip coupled to a peristaltic pump into 1000.0 mL of 2.0 mol/L sodium hydroxide solution and ethanol (4:1) for 24 hours. The formed beads were kept in contact with distilled water for 24 hours to remove traces of sodium acetate, sodium hydroxide, and acetic acid and were filtered and washed with distilled water until reaching a neutral pH value.

The beads were kept in a watch glass for 24 hours to be dehydrated and, consequently, have their mechanical strength increased.

For more efficient immobilization, the chitosan beads were activated with glutaraldehyde (GA). Activation was performed by keeping the chitosan beads in contact with a solution containing up to 3.0 % GA (m/V) in gentle agitation for 90 minutes. After this time, beads were vacuum filtered and stored under refrigeration.

Immobilization Of Enzyme Present In The Crude Extract

Adsorption immobilization on granular activated carbon

The adsorption method used for immobilization on granular activated carbon as support was developed by Silva et al. (2005). For it to occur, a determined amount of support and 50.0 mL of enzymatic crude extract solution, which contained, on average, an enzymatic activity of 3000.0 U (in 0.1 mol/l phosphate buffer, pH 7.0), were placed in an erlenmeyer. It was in contact for 120 minutes, in constant agitation. After that, the enzymes immobilized were vacuum filtered.

The enzyme immobilization rate in the activated carbon was made indirectly from data of the residual enzyme activity (U) of the enzymatic solution about to with concerning the initial enzymatic activity of the enzymatic crude extract solution. This method is used because it is not possible to determine the enzymatic activity of the enzyme immobilized on the support (Kennedy et al. 2007; Kumar et al. 2010; Silva et al. 2005).

Covalent binding immobilization on activated chitosan beads

For covalent immobilization, 5.0 g of activated chitosan beads and 20.0 mL of enzymatic crude extract solution, which contained, on average, an enzymatic activity of 2000.0 U (in 0.1 mol/L phosphate buffer, pH 7.0), were placed in an erlenmeyer. Enzyme solution and beads were in contact for up to 120 minutes, with constant stirring and temperature, in 15.7 rad/s. After the contact period, vacuum filtration was performed to separate the residual enzyme solution from the support containing the adsorbed enzyme. The enzyme immobilization rate was indirectly determined, as described in previous item.

Enzymatic oxidation of phenol using immobilized enzyme

The enzymatic oxidation assay was performed by adding 10.0 g of immobilized enzyme on granular activated carbon with an average activity of 1500.0 U, or 5.0 g of immobilized enzyme on activated chitosan beads, with an average activity of 2000.0 U, in aqueous solutions of phenol, at concentrations ranging from 10.0 to 100.0 mg/L, at pH 7.0, maintained at a constant stirring of 15.7 rad/s. The Assays took 120 minutes and occurred at a room temperature of 298 K. Samples were collected every 15 to analyze the amount of remaining phenol in the solutions.

Phenol removal was calculated considering the percentage of initial concentration and final phenol concentration in the aqueous solution. The phenol concentration of the solutions was determined by the direct colorimetric method, described in Standard Methods (APHA 1995), applying 4-amino-antipyrine reagent as a complexing agent. This method allows the dosing of single phenol and substituted phenols, except in the para position.

Characterization

The characterization of Carbotrat AP granular activated carbon surface, before and after immobilization, and chitosan beads, before GA activation, after activation, and after enzyme immobilization, was performed by Scanning Electron Microscopy (SEM) analysis, providing the surface photographs and by analyzing the Fourier transform infrared (FT-IR) spectroscopy.

Results And Discussions

Support Characterization

Figure 1 (a) and 1 (b) shows the scanning electron microscopy of the Carbotrat AP granular activated carbon before immobilization at magnifications of 10000 and 2500 times, and Figure 1 (c) and 1 (d) present the scanning electron microscopy of granular activated carbon Carbotrat AP after immobilization, also at magnifications 10000 and 2500 times.

The image analyzes of Fig. 1 (a) and (b), most clearly at the 10000 magnification, show a homogeneous and regular structure of activated carbon. Considering the images in Fig. 1 (c) and (d), it is observed the existence of other components on the surface of the granular activated carbon, in other words, the adsorption of the enzyme appears to be superficial.

Figure 2 shows the comparison between the FT-IR spectrum of activated carbon before immobilization (a) and after immobilization of the tyrosinase enzyme (b).

Some differences can be observed between the Carbotrat AP granular activated carbon FT-IR spectrum before and after immobilization of the enzyme. The spectrum of infrared support after immobilization presents peaks indicating the presence of histidine, which is part of the tyrosinase structure. It is also observed that the adsorption of the enzyme did not change the activated carbon structure. The appearance of two bands on 3700 - 3600 cm^{-1} region in FT-IR spectrum of Carbotrat AP granular activated carbon infrared after immobilization (b), corresponding to the stretching frequency of the N-H binding, is due to the interaction of tyrosinase with the activated carbon matrix (Kennedy et al. 2007). In addition, the band that appears near 1400 cm^{-1} in the activated carbon spectrum after immobilization (b) is indicative of C-N bonding, which confirms the presence of tyrosinase immobilized on the matrix. It is noted that this band was absent in the activated carbon spectrum before immobilization (a).

In immobilization using chitosan support, the enzyme tyrosinase was immobilized on the outside of the beads by covalent bonding with amino groups support followed by crosslinking with the bifunctional agent, GA. Beads had diameters between 3.31 mm and 4.15 mm. Fig. 3 (a) and 3 (b) show the scanning electron microscopy of the chitosan beads before and after activation with GA, and Fig. 3 (c) shows the scanning electron microscopy activated chitosan bead after immobilization with the tyrosinase enzyme contained in a crude enzyme extract.

The analysis of the images obtained by scanning electron microscopy provided the beads surface morphology. Chitosan beads before activation with GA had a rougher and non-uniform surface, whereas activation makes the bead surface more uniform and smooth.

Figure 4 presents the comparison among FT-IR spectrum of a chitosan bead before activation (a), after activation (b), and after enzyme tyrosinase immobilization (c).

The infrared spectrum of chitosan beads is characterized by the presence of a large band between 3400 and 3200 cm^{-1}, which is attributed to the axial deformation of the O-H group associated with other polar groups via intra and intermolecular and axial deformation N-H, normally obscured by hydrogen bonding with OH groups. The activation with GA is confirmed with the appearance of a peak in the region around 2900 cm^{-1} and the region near 1600 cm^{-1}, as a result of the interaction of GA with chitosan functional groups. Just like observed the FT-IR spectrum of activated carbon after immobilization, the presence of tyrosinase is confirmed by the existence of the most pronounced peak in the region of 1100 cm^{-1}, indicative of the presence of histidine, the most significant component of the tyrosinase enzyme (Kennedy et al. 2007). Immobilization is also responsible for the disappearance of the peak in the region around 2900 cm^{-1}, indicating the interaction of tyrosinase with the modified chitosan surface.

Enzyme Immobilization

Immobilization on Granular Activated Carbon

The immobilization in granular activated carbon occurs by physical adsorption, so enzyme desorption is easier to take place in case of operational changes. Immobilization assays were performed, using as support the Carbotrat AP Granular Activated Carbon, in the amount of 10.0 g and an enzyme solution with 3000.0 U of activity, up until 120 minutes of contact between the support and enzymatic crude extract. Some authors have observed that during immobilization of enzymes in GAC, the percentage of immobilized enzymes stabilized in less than 120 minutes of contact time between enzyme and support (Kennedy et al. 2007; Kumar et al. 2010; Silva et al. 2005).

Figure 5 presents the results of the immobilization rate obtained, being observed that the longer the contact time, the higher the immobilization rate of the enzyme in the support.

It can be observed that the immobilization rate increases with time. According to the test, the best immobilization time is 90 minutes, up to 55.0 % immobilization rate and enzyme activity of immobilized enzyme up to 1604.0 U. Kennedy et al. (2007) managed to immobilize tyrosinase extracted from a potato with enzymatic activity up to 30.0 x 10^4 U/L on a modified GAC, Kumar et al. (2007) and Silva et al. (2005) amanaged to immobilize almost 100.0 % of enzymes on different types of GAC, however, these authors worked with other types of enzymes.

Best stirring for physical adsorption of enzyme present in extract enzyme in Carbotrat AP granular activated carbon was investigated in immobilization using 10.0 g of support and crude enzyme extract with an activity of tyrosinase of ± 3000.0 U, with a contact time of 90 minutes and constant stirring of 10.5, 15.7, 20.9 and 31.4 rad/s. Table 1 presents the immobilization rates found in different stirring speeds after the contact time between the enzymatic solution and support.

Table 1
Variation of the immobilization rate and enzyme activity of the immobilized enzyme about to with concerning system stirring

Stirring (rad/s)	Enzymatic Activity (U)	Immobilization rate (%)
10.5	900.0	30.0
15.7	1650.0	55.0
20.9	1590.0	53.0
31.4	1200.0	40.0

The worst immobilization rate was obtained using a constant stirring of 10.5 rad/s; using 15.7 and 20.9 rad/s, it was possible to obtain an immobilization rate above 50.0 %; while stirring at 31.4 rad/s, the immobilization rate dropped to 40.0 %. The low immobilization at low stirring speed is due to insufficient tyrosinase mixture with the support. For higher stirring values, the mechanical inactivation of the enzyme occurs due to the increase of the contact area of the enzyme with air and with the surfaces of the erlenmeyer. This phenomenon was reported by Colombiè et al. (2001), Gikanga et al. (2017); Menoncin et al. (2009), and Wiesbauer et al. (2013).

Immobilization on Activated Chitosan Beads

The immobilization using activated chitosan bead support occurs by covalent bonding. Immobilization Assays were performed using 5.0 g of chitosan beads activated with 1.5 and 3.0% of GA and an enzyme solution with 2000.0 U activity, made from the crude extract during the contact time of 120 minutes and with stirring of 15.7 rad/s.

Fig. 6 presents the results of the percentage of immobilized enzymes. It is observed that the longer the contact time, the higher the immobilization rate of the enzyme in the support.

From these results, the best contact time was 120 minutes and using chitosan beads activated with 3.0 % of GA as support, resulting in immobilization of approximately 100.0 %, higher than that obtained with immobilization by adsorption on granular activated carbon and similar to that found by Chavita (2010) and Miyaguti (2011), but higher than that found by Santos et al. (2013).

Enzymatic Oxidation Assays

Influence of Initial Phenol Concentration

Phenol enzymatic oxidation assays were performed using the enzyme support system in solutions of 10.0, 20.0, 40.0, 60.0, and 100.0 mg/L for both the immobilized enzyme in the activated carbon support and the immobilized enzyme on activated chitosan beads. All assays were performed under the same stirring conditions, pH, contact time, and temperature, respectively 15.7 rad/s, pH 7.0, 120 minutes, and 298K.

The enzymatic activity of the immobilized enzymes on 10.0 g Carbotrat AP support was approximately 1500.0 U, while the enzymatic activity of the immobilized enzymes on 5.0 g activated chitosan beads was approximately 2000.0 U. The results, in terms of the final average concentration of phenol in the aqueous phenol solution and phenol removal are shown in Table 2.

Table 2
Final average phenol concentration and phenol removal from aqueous solutions containing different phenol concentrations after enzymatic oxidation

Granular Activated Carbon			
Enzymatic Activity (U)	Inicial Phenol Concentration (mg/L)	Final Phenol Concentration (mg/L)	Phenol Removal (%)
~1500.0	10.0	~0	100.0
~1500.0	20.0	2.5	87.5
~1500.0	40.0	6.7	83.3
~1500.0	60.0	19.5	67.5
~1500.0	100.0	45.6	54.4
Activated Chitosan Beads			
Enzymatic Activity (U)	Inicial Phenol Concentration (mg/L)	Final Phenol Concentration (mg/L)	Phenol Removal (%)
~2000.0	10.0	~0	100.0
~2000.0	20.0	~0	100.0
~2000.0	40.0	0.40	99.0
~2000.0	60.0	1.30	97.8
~2000.0	100.0	28.46	71.5

Using immobilized enzyme in GAC with an enzymatic activity of 1500.0 U, the phenol limit required by CONAMA 430/2011 (MMA 2011) is only reached by enzymatic oxidation on solutions with 10.0 mg phenol/L. However, despite not attending the limit for disposal, using the immobilized enzyme, it was possible to obtain phenol removal above 50.0 %, even for solutions containing 100.0 mg phenol/L.

By using tyrosinase immobilized on activated chitosan beads with the average enzymatic activity of 2000.0 U, a final phenol concentration below the limit required by Brazilian legislation for phenol solutions with an initial concentration of 10.0 to 40.0 mg/L was obtained. However, in solutions of 60.0 mg/L, it was possible to remove up to 97.0 % of phenol, and even in solutions containing 100.0 mg/L of phenol, it was possible to achieve a removal of more than 70.0 % of this compound. This was greater than those found with the immobilized enzyme on GAC and using a lower amount of support.

The decrease in removal efficiency with the increase in the initial concentration of phenol present in the solution can be explained not only by the amount of immobilized enzyme on the support but also by the fact that o-quinone, formed by the degradation of phenol through tyrosinase, inactivates the enzyme

when in higher concentrations (Bevilaqua et al. 2002; Wada et al. 1993). Another reason for solutions with high initial phenol concentration not attending the limit established by CONAMA 430/2011 (MMA 2011) is the contact time of the immobilized enzyme in the solutions, which in this work was set at 120 minutes. Some authors needed 8 hours (480 minutes) to remove practically 100.0 % phenol from solutions initially containing 100.0 mg/L (Chavita 2010; Kameda et al. 2006).

Reuse of Immobilized Enzyme

Immobilized enzymes with the initial enzymatic activity of 1500.0 U on granular activated carbon support and initial enzymatic activity of 2000.0 U on activated chitosan beads were used for the assays. After each assay, the immobilized enzyme was washed with distilled water and phosphate buffer pH 7.0 and subjected to vacuum filtration. Assays were performed using the immobilized enzyme for phenol removal from phenol-containing solutions at concentrations of 10.0 and 60.0 mg/L.

For phenol removal on 10.0 mg phenol/L solutions using the immobilized enzyme in activated carbon support Carbotrat AP, it was observed that, in the second cycle of use, there was a 4.5 % drop in yield over the 100.0 % removal yield of the first cycle, and yield drop greater than 50.0 % in its third use (Fig. 7 (a)). Removal using the immobilized enzyme on activated chitosan bead was able to remove up to 100.0 % of phenol in the first three uses. From the fourth use onwards, there was a 15.0 % drop in yield. The enzyme immobilized on activated S24 beads showed lower reductions than the enzyme immobilized on activated carbon, maintaining the phenol removal rate close to 70.0 % until the sixth use (Fig. 7 (b)).

Considering the removal of phenol in 60.0 mg/L solutions, it could be seen again that the use of the immobilized enzyme in Carbotrat AP activated carbon made a yield drop from the second cycle onwards, with an 18.9 % decrease compared to 66.2 % during the first cycle, and at the third use, the removal yield drop was 63.8 % (Fig. 8 (a)). Immobilized enzymes on activated chitosan beads were able to remove ~ 98.0 % of phenol in its first use, and from the second use, there was a yield drop of 1.2 % and, differently from removal using GAC as support, only in the fourth cycle of use phenol removal yield drop was greater than 50.0 % (Fig. 8 (b)).

The drop in enzymatic oxidation yield may cause prolonged exposure of the enzyme to the phenol degradation product (which explains a higher yield drop in more concentrated phenol solutions), the adsorption of this degradation product to the support (which causes inactivation of the tyrosinase enzyme) and also by the loss of support that occurs between uses either during enzymatic oxidation or during the washing and filtration process after testing. In addition, the sharpest drop in yield during reuse of the immobilized enzyme in GAC is caused by the enzyme desorption due to the stirring of the system during enzymatic oxidation.

Support Reuse

The reuse of the support in future immobilizations is very interesting from the economic point of view, but it was only possible to reuse the support for immobilizations in GAC, due to the type of immobilization - physical adsorption - in which it is possible to remove the immobilized enzyme after its loss of activity.

Table 3 shows the reuse of the support as a function of the immobilized enzyme rate, when the initial enzymatic activity refers to the enzymatic solution and the final enzymatic activity refers to the estimated activity of the immobilized enzyme on the support.

Table 3
Enzyme immobilization rate on the same support

Support Usage	Initial Enzymatic Activity (U)	Final Enzymatic Activity (U)	Immobilization Rate (%)
1	3000.0	1440.0	52.0
2	3015.0	1581.7	47.5
3	3002.0	1701.2	43.3

The rate of immobilization declined with each use of the same support, but this decrease was less than 10.0 %, which can be considered a very positive result, as the reuse of the support would help lower the cost of using the immobilized enzyme, giving higher economic viability for it.

Enzyme oxidation assays were performed using the immobilized enzyme on reused support (second immobilization with the support), also reusing the immobilized enzyme on this support to remove phenol from an aqueous solution containing 10 mg/L of phenol.

The results of enzymatic oxidation as a function of phenol removal rate are expressed in Fig. 9.

It was possible to reach the limit established by CONAMA 430 (MMA 2011) for phenol-containing effluent disposal in the first two uses of the immobilized enzyme. There was also a sharp drop in phenol removal from the first use to the third. Just the same as with the immobilized enzymes in new support, it was also verified, but in a smaller proportion. The drop in removal can still be explained by the loss of the enzyme during the test and its inactivation in the activated carbon.

Since it is not possible to reuse the activated chitosan beads support because adsorption occurs by covalent bonding (chemically joining the enzyme to the support surface), it has been studied a way of decreasing the yield drop of the immobilized enzyme in this support type. As a cause for the drop in phenol removal yield is the adsorption of o-quinone on the bead surface, inactivating the tyrosinase enzyme, successive washing steps with pH 6.7 and 8.0 buffers, and distilled water after each cycle were added to the enzymatic oxidation test. To verify the efficiency of the use of these new steps, enzymatic oxidation assays were carried out using the enzyme-support system for 5 cycles in 10.0 and 100.0 mg phenol/L solutions.

Fig. 10 and 11 show five cycles of enzymatic oxidation reusing the immobilized tyrosinase enzyme in aqueous solutions containing 10.0 and 100.0 mg phenol/L, respectively, with and without the addition of wash steps.

In the 10.0 mg phenol/L removal, the new cleaning methodology after each assay allowed the phenol removal yield to fall only 0.9 % after 5 cycles, unlike the enzymatic oxidation cycles using the old method. From the fourth cycle onwards, it dropped by 15.0 %, reaching 28.8 % on the fifth cycle. Yield to enzymatic oxidation to phenol is shown in Fig. 10.

For the removal of 100.0 mg/L (Fig. 11), although the use of successive washing steps after each cycle was not sufficient for the maintenance of enzymatic activity, its application still caused the decrease of phenol removal yield in the initial cycles, allowing the immobilized enzyme to be used five times with a phenol removal above 50.0 %. There has been a 33.7 % yield drop after 5 uses of the immobilized enzyme, whereas, with the old cleaning methodology, this drop was almost 50.0 %.

Immobilized enzyme storage

One of the most important parameters to consider in enzymatic immobilization is storage stability. In general, storing enzymes in solution makes them unstable and leads to a decline on activity.

In order to verify the behavior of the enzymatic activity concerning the storage time, 5 immobilizations were performed under the same conditions for each support used, using immobilized enzymes with activities of approximately 1500.0 U for immobilization in GAC and 2000.0 U for immobilization in beads. Activated chitosan was stored under refrigeration for later use in enzymatic oxidation assays at different times: 1 day, 1 week, 2 weeks, 3 weeks, and 4 weeks after immobilization.

Fig. 12 and 13 show the phenol concentration decay concerning the contact time of the aqueous solution initially containing 10.0 mg phenol/L with the immobilized enzyme on GAC (Fig. 12) and ACB (Fig. 13) after different storage times.

Enzymatic oxidation using immobilized tyrosinase 1 day after immobilization reached 100.0 % of phenol removal within 60 minutes for immobilization on both support types and similar to that found by some authors such as Chavita (2010) and Pigatto (2013). Analyzing the graphs, it is shown that phenol removal slows down as the time between immobilization and enzymatic oxidation increases and that after the fourth week of storage, the drop in activity is more pronounced in both cases.

The graph in Fig. 14 shows the yield of the enzyme oxidation reaction related to the storage time of the immobilized enzyme in the GAC (a) and ACB (b) carrier before its first use.

According to the results, the immobilized enzymes on both supports removed 100.0 % of phenol from a 10.0 mg/L solution within 120 minutes, up to 2 weeks after immobilization and storage under refrigeration. After 3 and 4 weeks of storage, the enzyme showed a rate drop of 9.5 and 16.0 % when immobilized on GAC and 7.3 % and 12.6 % when immobilized on ACB about to with concerning the first 3 weeks of storage.

The stability of the immobilized enzyme in GAC and ACB concerning the storage time between its first and second use in enzymatic oxidation assays was also investigated. Again, 5 immobilizations were

performed for each support type, under the same operating conditions, generating 5 support enzyme systems containing an initial enzyme activity of 1500.0 U and 2000.0 U, respectively. Each enzyme support system was then used in enzymatic oxidation of an aqueous 10.0 mg phenol/L solution and stored under refrigeration after the assay. Once reserved, each enzyme support system was reused with time between two different uses: 1 day, 1 week, 2 weeks, 3 weeks, and 4 weeks after their first use.

The graphs show in Fig. 15 and 16 illustrate the phenol concentration decay concerning the contact time of the aqueous phenol solution, with the initial concentration of 10.0 mg/L, with the immobilized enzyme on GAC (Fig. 15) and ACB (Fig. 16) on its second use. All assays were performed under the same conditions, stirring of 15.7 rad/s, temperature 298K, contact time 120 minutes.

The second enzymatic oxidation with the same enzyme immobilized 1 day after the first use was able to remove 94.0 % and 100.0 % of the phenol from the solution shortly after 60 minutes of contact on GAC and ACB supports, respectively, similar to what happened with the first use of the immobilized enzyme with one day of storage; this was also the assay with the fastest removal. Analyzing the graphs, it is noted that the enzymatic oxidation assay curves for the second use of the immobilized enzyme in ACB 2, 3, and 4 weeks after the first use are very similar, indicating a stabilization of the immobilized enzyme while the assay curves of the enzymatic oxidation rate related to the second use of the immobilized enzyme in GAC showed similar decays when compared to the first use of the immobilized enzyme.

Figure 17 shows the yield of the enzyme oxidation reaction related to the storage time of the immobilized enzyme in GAC (a) and ACB (b) between its first and second uses.

The stability of the immobilized enzyme in ACB can be caused by two reasons: covalent immobilization (it keeps the enzyme in a stable position compared to the free enzyme) and a stabilizing effect provided by the support (it minimizes possible distortion effects imposed by the aqueous solution on the active site of the immobilized enzyme, which did not occur in the immobilized enzyme in GAC).

Conclusions

The methodology used in this work allowed immobilizations of up to 70.0 % of enzymes present in the enzymatic solution in granular activated carbon support. The best immobilization conditions were achieved using 10.0 g of support, constant stirring of 15.7 rad/s, and 90 minutes of contact time. All assays were performed keeping the temperature at 298K and pH at 7.0.

Oxidation Assays on phenol solutions with initial concentrations ranging from 10.0 to 100.0 mg/L using GAC immobilized tyrosinase with an average enzymatic activity of 1500.0 U for 120 minutes only achieved a final phenol concentration below the limit required by Brazilian law for a solution with an initial concentration of 10.0 mg/L. However, even in solutions containing 100.0 mg phenol/L, it was possible to remove this compound greater than 50.0 %.

The immobilized enzyme could be used up to three times before phenol removal decreased by 50.0 %. It was also possible to reuse the GAC support, and in its third use, the immobilization rate fell less than 10.0 %. The immobilization of the enzyme in used support was more resistant than the immobilization of the enzyme on new support.

The methodology applied allowed immobilization of up to 100.0 % of enzymes present in the enzymatic solution, in a support of activated chitosan beads. The immobilization conditions were: 5.0 g of support, constant stirring of 15.7 rad/s, and contact time of 120 minutes. All assays were performed keeping the temperature at 298K and pH at 7.0.

Oxidation Assays on phenol solutions with initial concentrations ranging from 10.0 to 100.0 mg/L using immobilized tyrosinase on activated chitosan beads with an average enzymatic activity of 2000.0 U for 120 minutes obtained a final phenol concentration below the limit required by Brazilian law for phenol solutions with an initial concentration of 10.0 to 40.0 mg/L. However, in solutions of 60.0 mg/L, it was possible to remove up to 97.0 % of phenol, and even in solutions containing 100.0 mg/L of phenol, it was possible to achieve a removal of more than 70.0 % of this compound.

It was possible to use the immobilized enzyme on activated chitosan beads up to six times before phenol removal decreased by more than 50.0 %. This decay can be considered by prolonged exposure of the enzyme to the phenol degradation product and by adsorption of this product to the support, leading to inactivation of the enzyme and also by the loss of support between uses. The addition of cleaning steps with different buffers improved the yield drop that occurs with repeated use of the immobilized enzyme from 28.8 % to 1.3 % after 5 uses in solutions with concentrations up to 10 mg/L of phenol. In solutions containing 100 mg/L of phenol, the yield drop in 5 uses of the immobilized enzyme was 16.1 % lower compared to the methodology without the addition of steps.

Phenol removal yield remains unchanged using immobilized enzymes on GAC and ACB for up to 2 weeks. After 4 weeks, a 15.0 % yield drop occurs using GAC immobilized enzymes and 12.6 % using ACB immobilized enzymes. After its first use, the immobilized enzyme on ACB is more stable, with only a 4.4 % drop in phenol removal yield after 4 weeks.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and materials

The raw data of experimental results reported, as well as the materials used in this work, are available upon reasonable request.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

Ana Carina Cruz de Mello: Conceptualization; Data curation; Formal analysis; Investigation; Methodology; Validation; Visualization; Writing – original draft; Writing – review & editing

Felipe Pereira da Silva: Visualization; Writing – review & editing

Andrea Medeiros Salgado: Conceptualization; Methodology; Resources; Supervision; Writing – review & editing

Fabiana Valéria da Fonseca: Conceptualization; Methodology; Resources; Supervision; Writing – review & editing

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Figures

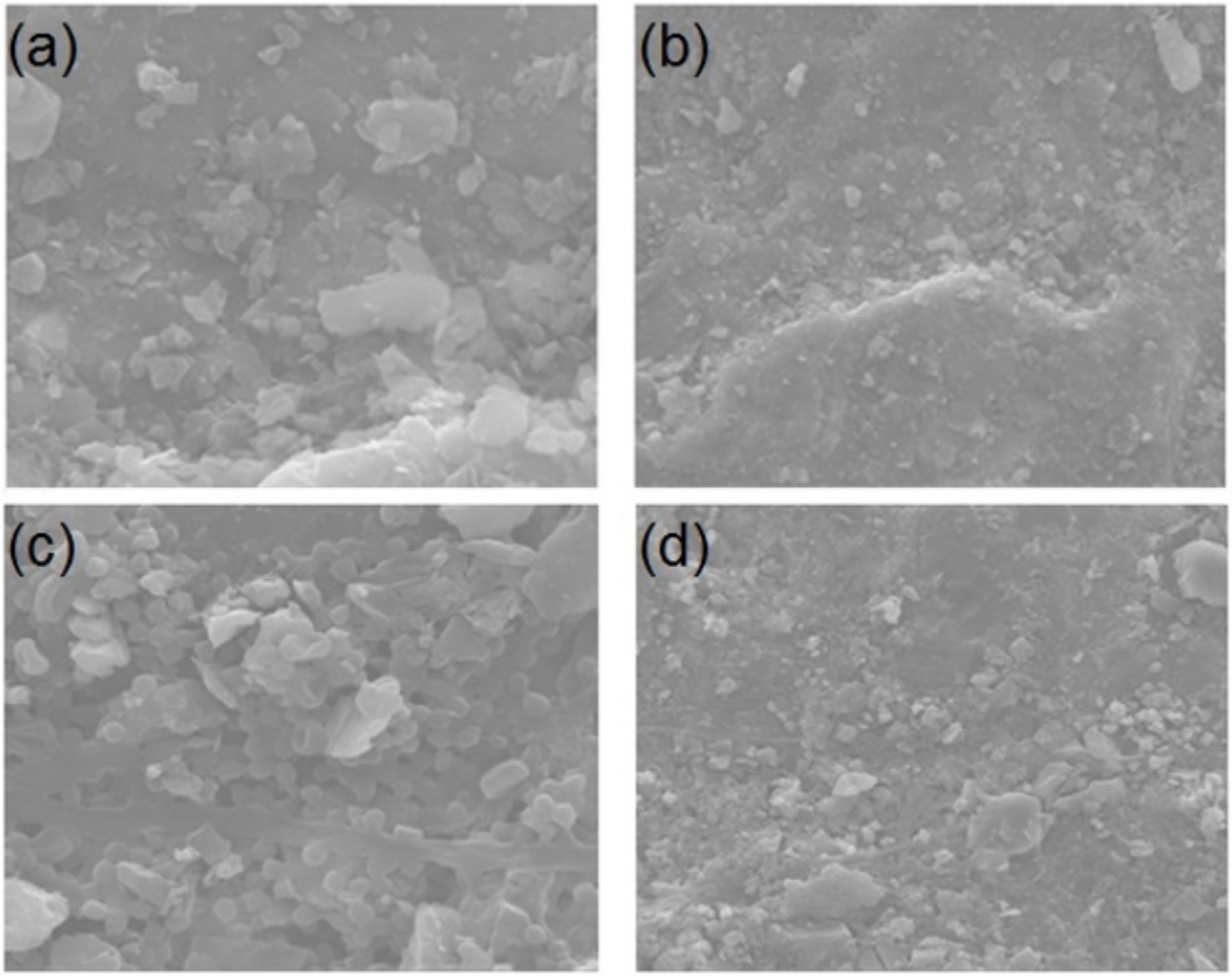

Figure 1

Image analysis of activated carbon before adsorption immobilization using SEM, (a) 10000-fold magnification, (b) 2500-fold magnification and image analysis of activated carbon after adsorption immobilization using SEM, (c) 10000-fold magnification, (d) 2500-fold magnification

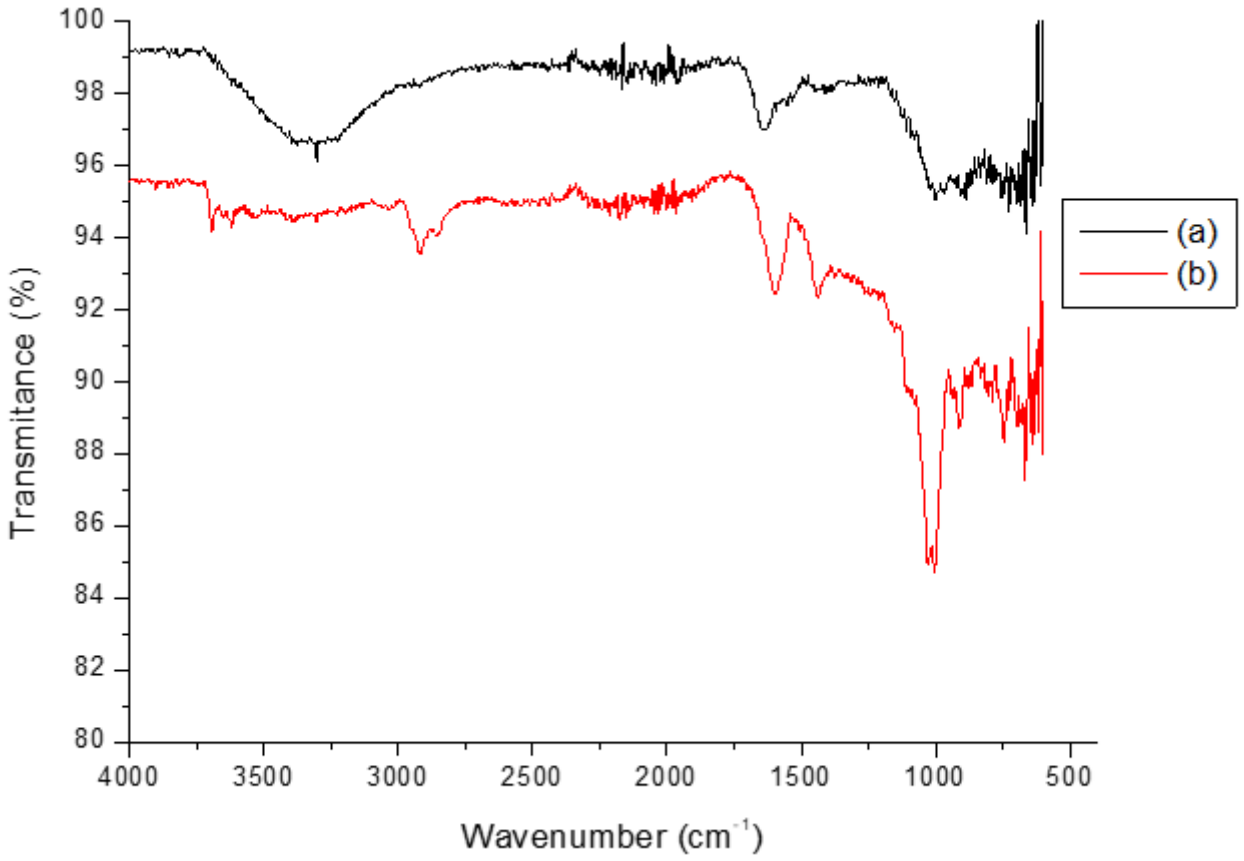

Figure 2

Carbotrat AP granular activated carbon FT-IR spectrum (a) before immobilization and (b) after the immobilization

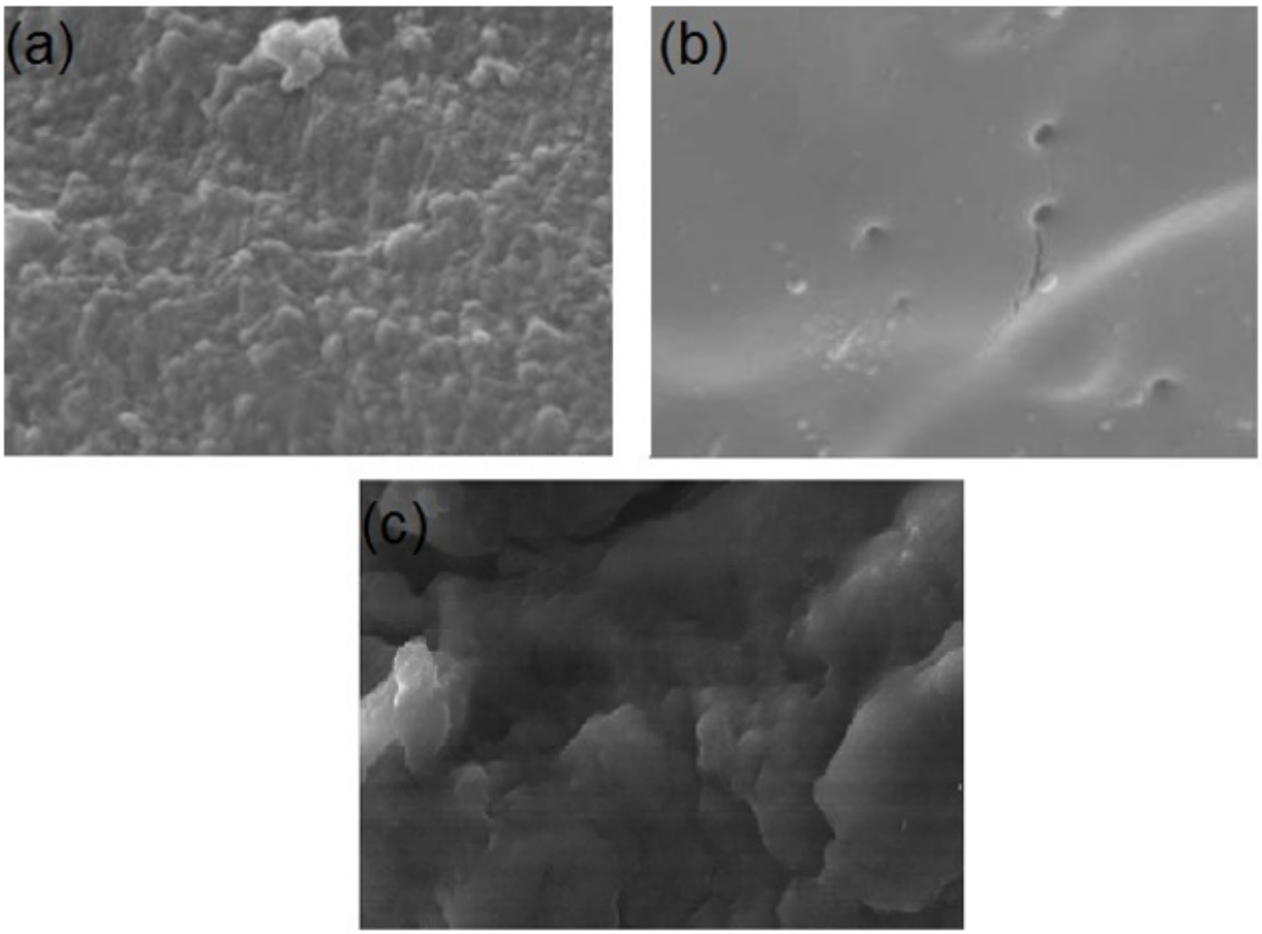

Figure 3

Chitosan bead image analysis before activation (a), after activation (b), and after immobilization of the tyrosinase enzyme (c) with 10000 magnification times and 2500 times

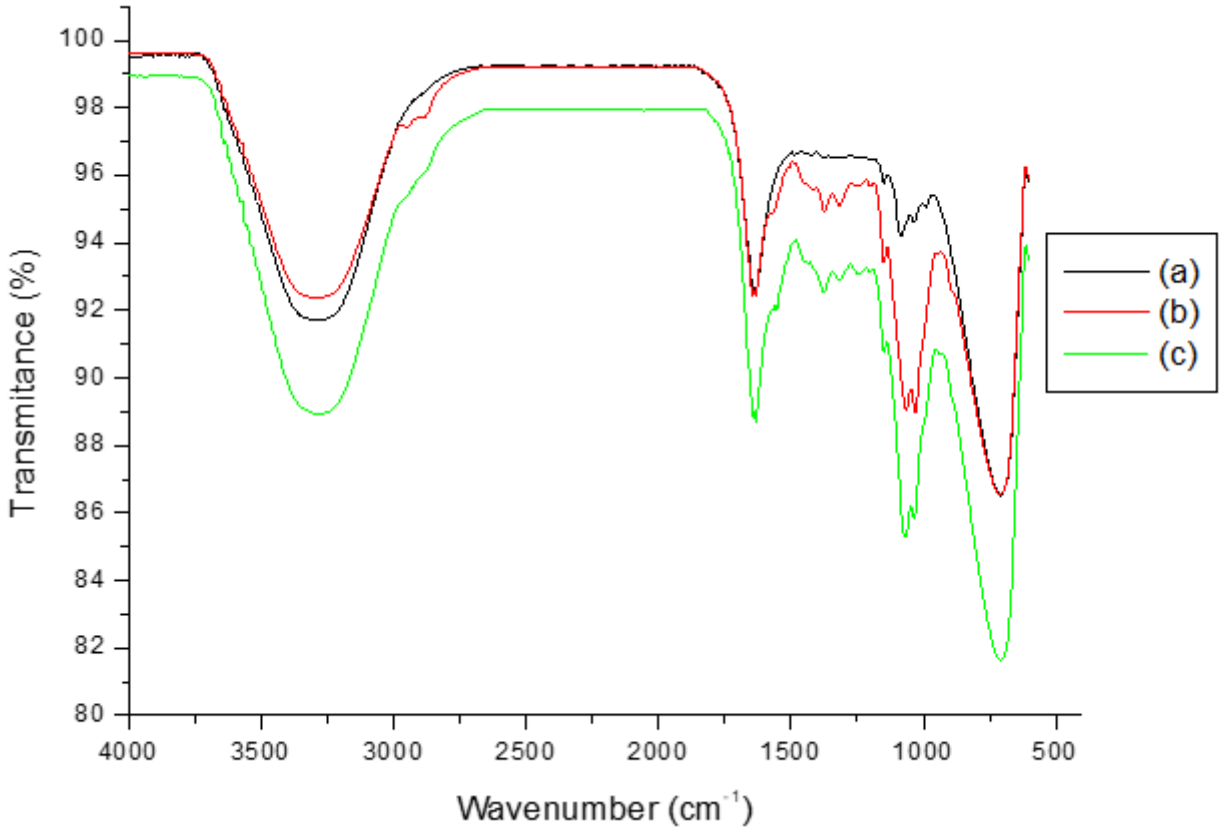

Figure 4

FT-IR spectrum of the chitosan beads (a) before activation, (b) after activation, and (c) after immobilization of the enzyme tyrosinase

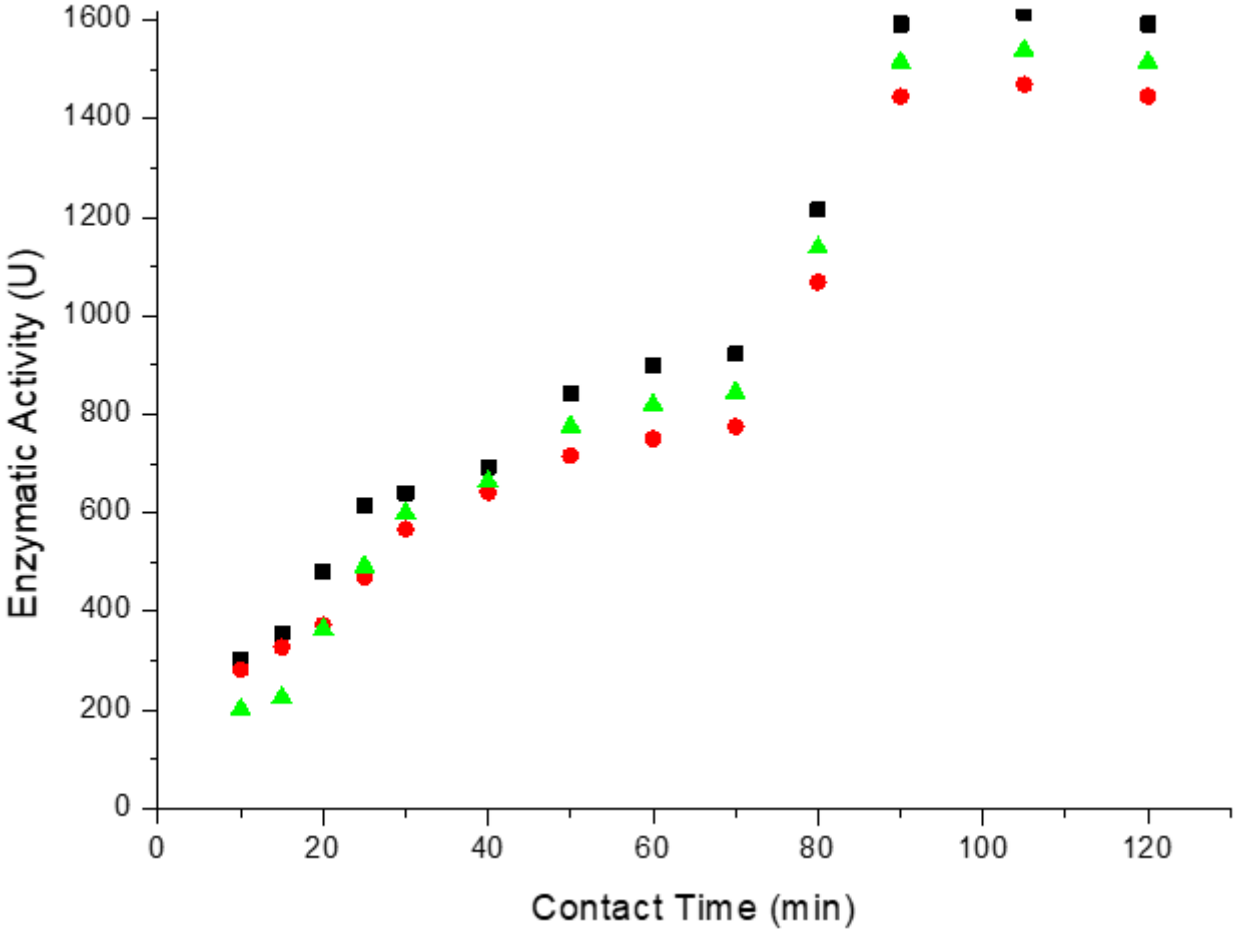

Figure 5

Variation of the enzyme activity during contact time. Experimental conditions: Initial enzymatic activity ~ 3000.0 U, 10.0 g support, constant stirring of 15.7 rad/s, and 298 K

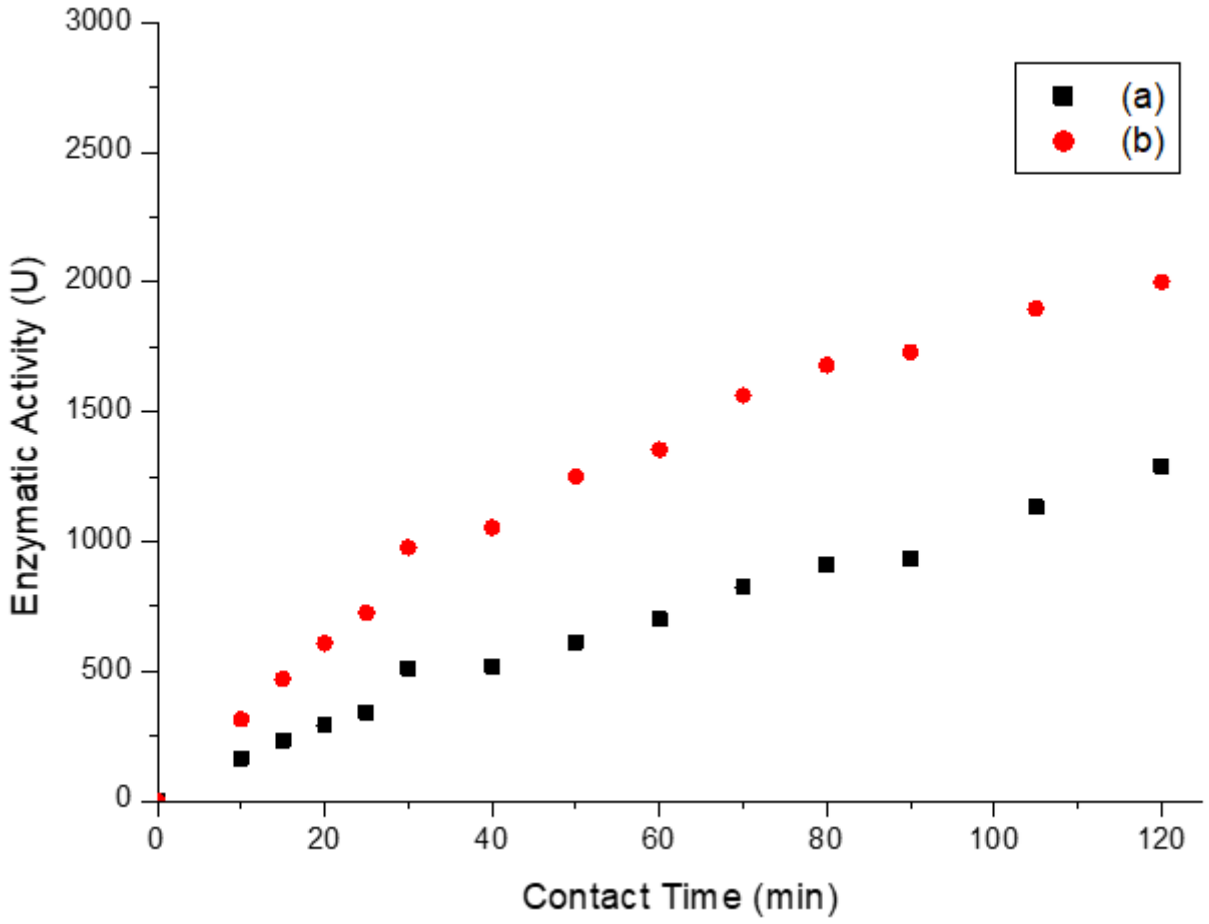

Figure 6

Enzymatic immobilization (a) 24 h drying beads activated with 1.5 % of GA and (b) 24 h drying beads activated with 3.0 % GA. Experimental conditions: Initial enzymatic activity ~ 2000.0 U, 5.0 g support, stirring of 15.7 rad/s, 298 K

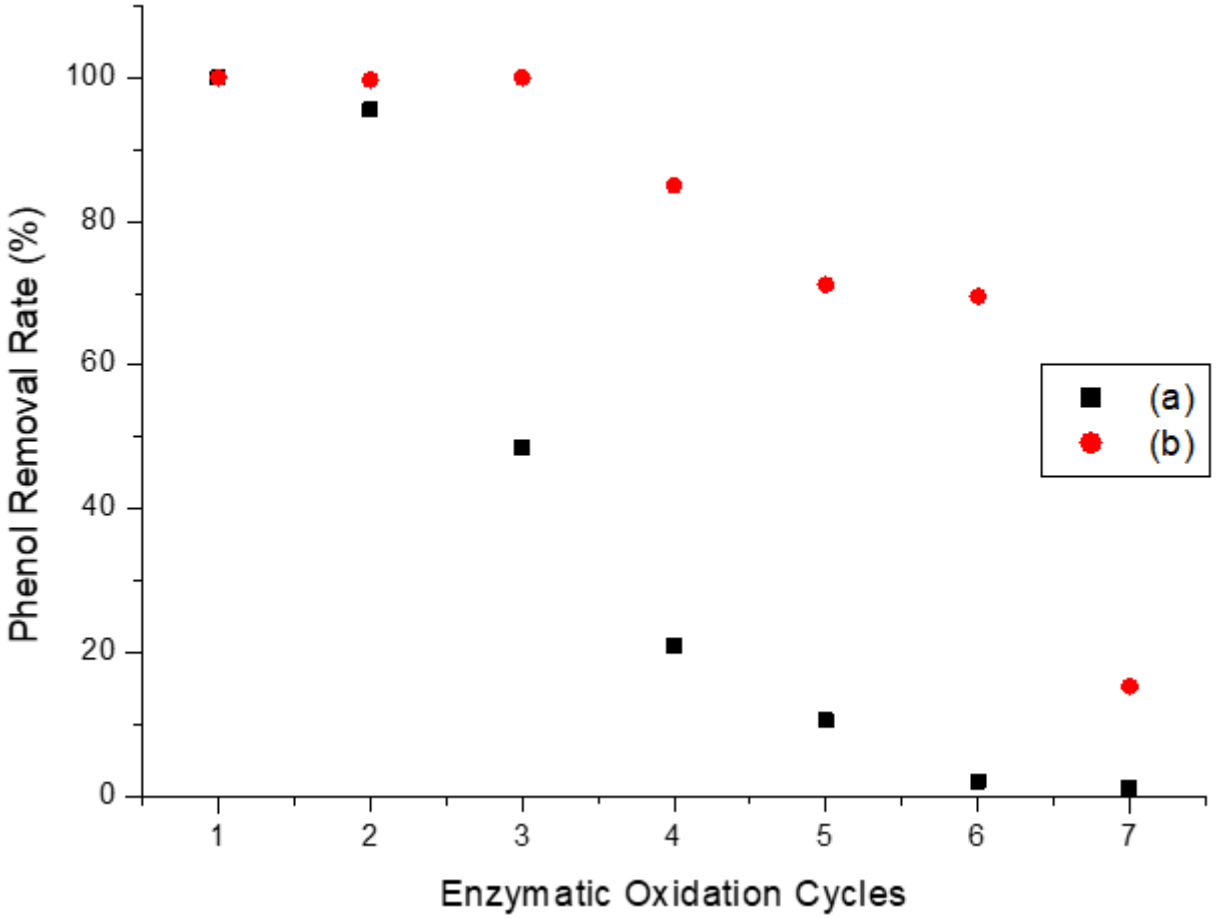

Figure 7

Removal of phenol from 10.0 mg/L solution using (a) tyrosinase with an initial activity of 1500.0 U on Carbotrat AP granular activated carbon support and (b) tyrosinase with an initial activity of 2000.0 U on 5.0 g of activated chitosan beads support.Experimental conditions: constant stirring of 15.7 rad/s and temperature of ~ 298K, in contact time of 120 minutes

Figure 8

Phenol removal from 60.0 mg/L solutions using (a) tyrosinase with an initial activity of 1500.0 U on Carbotrat AP Granular Activated Carbon support and (b) tyrosinase with an initial activity of 2000.0 U on 5.0 g of activated chitosan beads support. Experimental conditions: constant stirring of 15.7 rad/s and temperature of ~ 298K, in contact time of 120 minutes

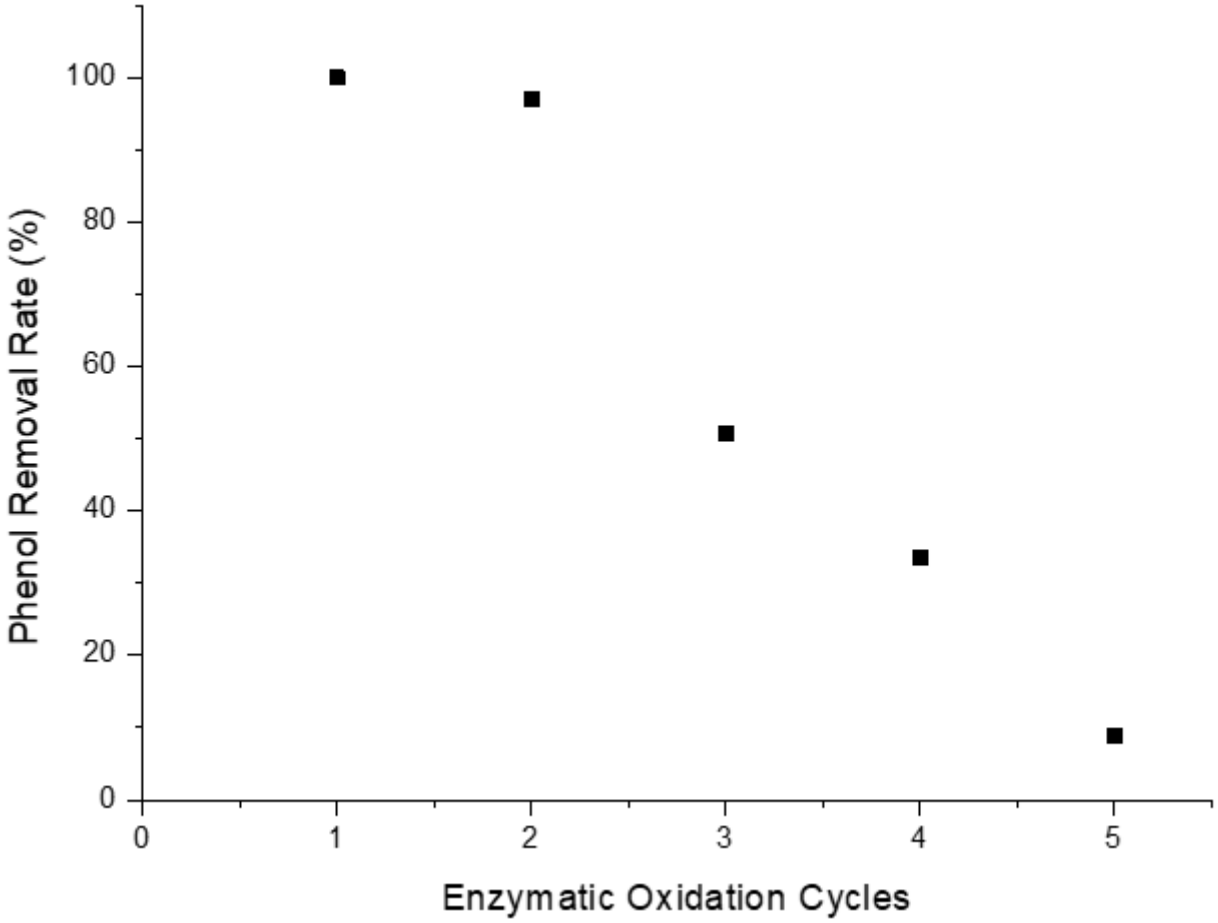

Figure 9

Phenol removal rate from 10.0 mg/L aqueous phenol solution using tyrosinase on granular activated carbon reused support. Experimental conditions: initial activity of 1500.0 U, constant stirring of 15.7 rad/s g and temperature of ~ 298 K, and contact time of 120 minutes

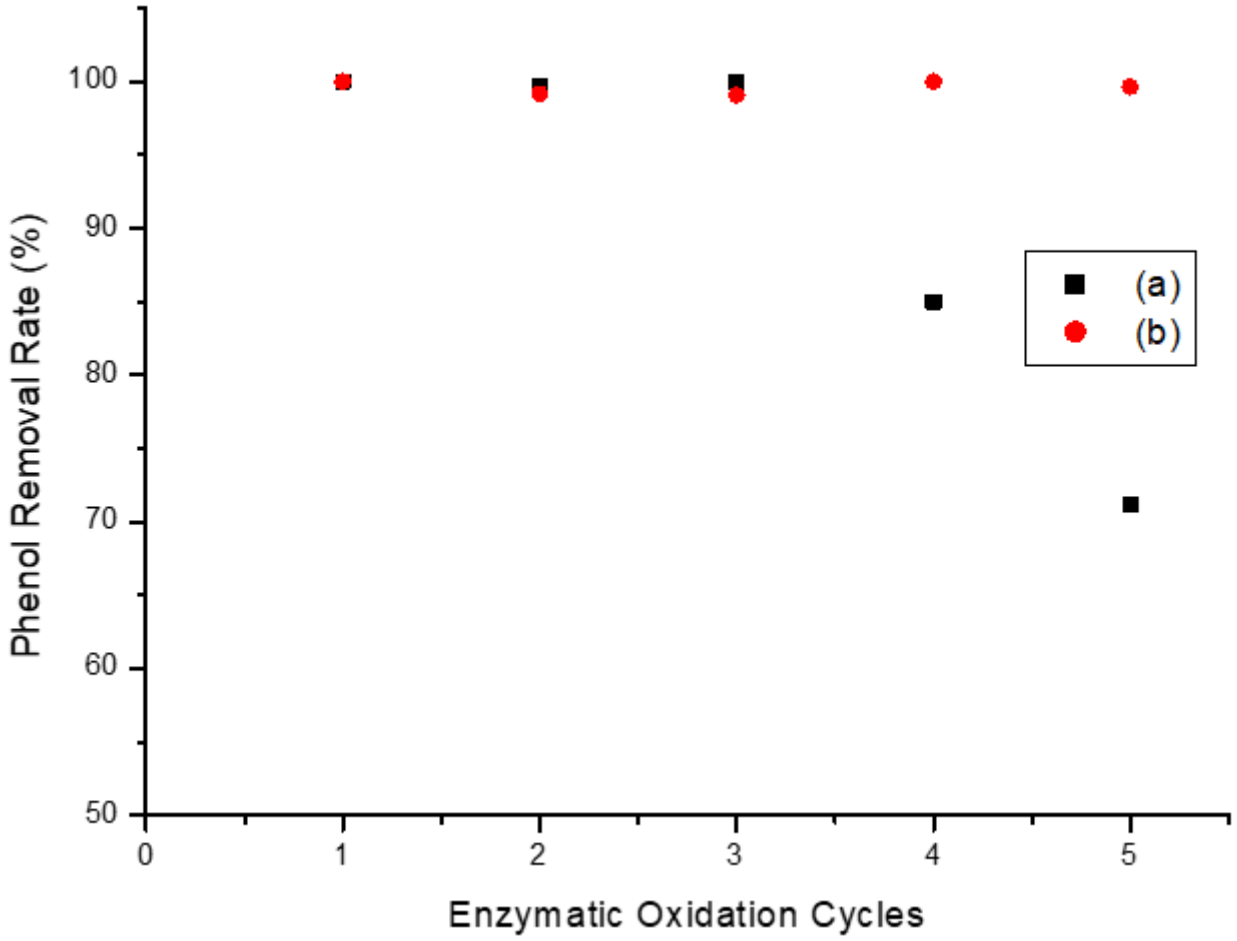

Figure 10

Removal phenol rate on enzymatic oxidation cycles in 10.0 mg/L aqueous phenol solution using tyrosinase on 5.0 g of activated chitosan beads (a) without washing steps and (b) with washing steps. Experimental conditions: initial activity of 2000.0 U, 15.7 rad/s orbital stirring, 298 K, and 120 minutes contact time

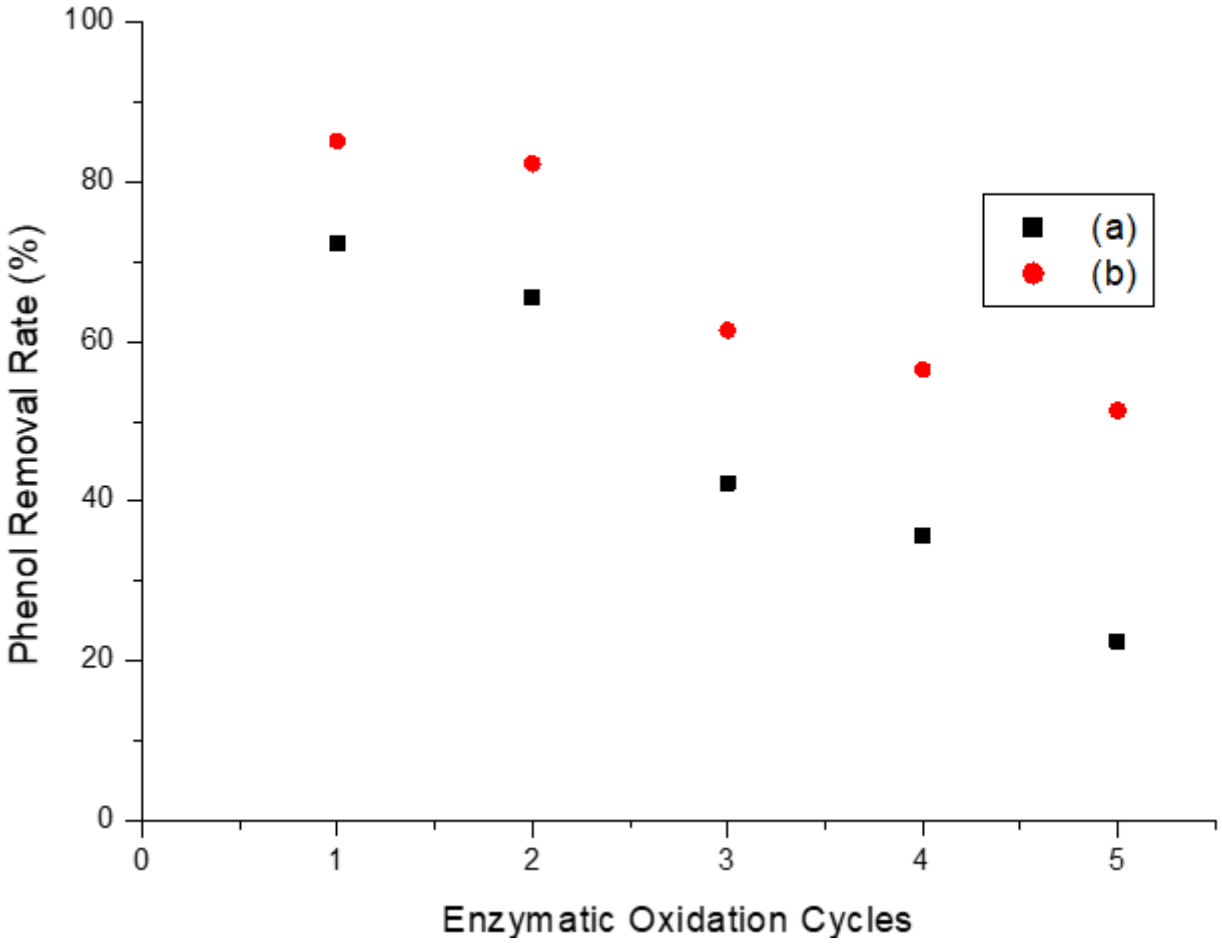

Figure 11

Removal of 100.0 mg/L phenol using tyrosinase on 5.0 g of support ACB (a) without washing steps and (b) with washing steps. Experimental conditions: initial activity of 2000.0 U, 15.7 rad/s orbital stirring, 298 K, and 120 minutes contact time

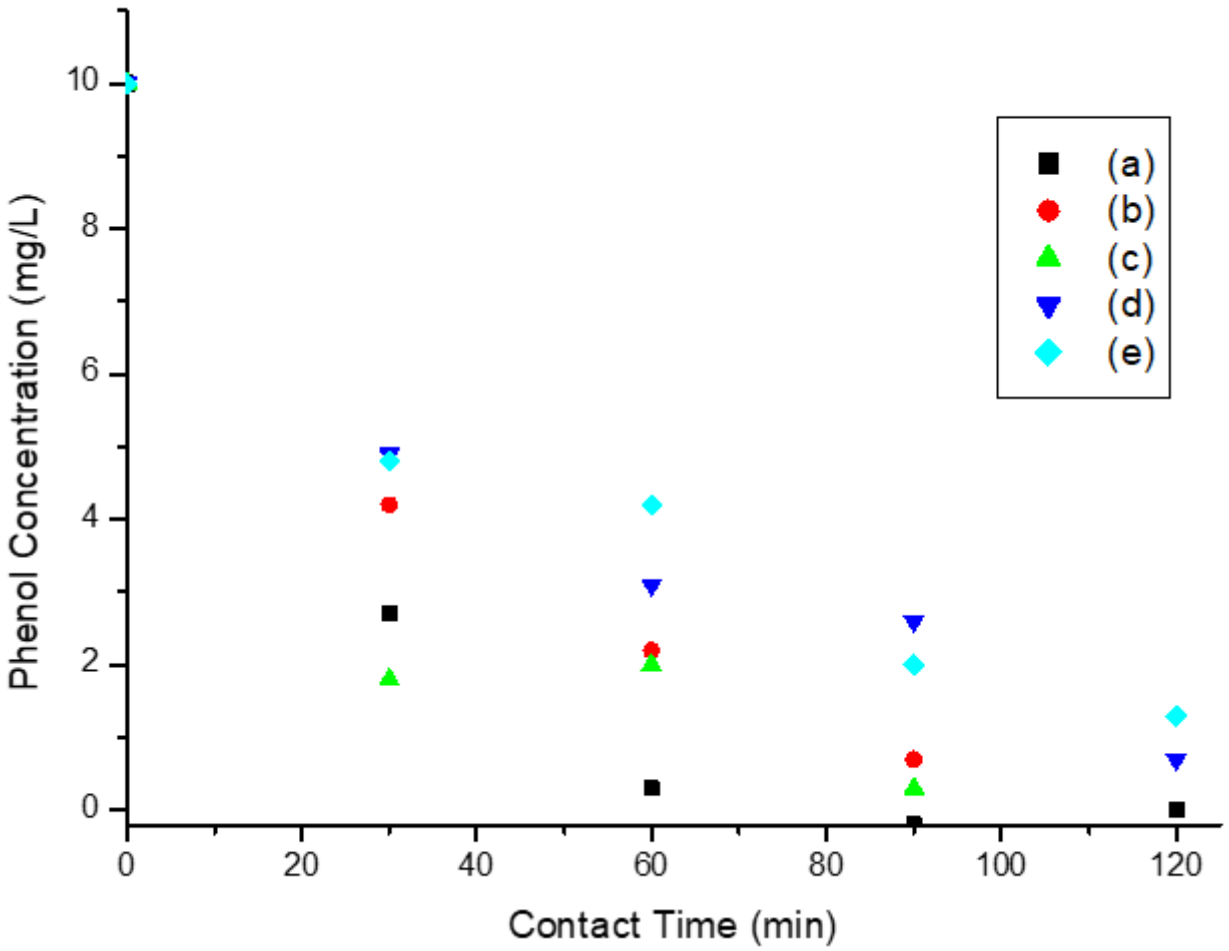

Figure 12

Enzymatic oxidation using (a) immobilized tyrosinase on GAC 1 day before of its use, (b) immobilized tyrosinase 1 week before use, (c) immobilized tyrosinase 2 weeks before use, (d) immobilized tyrosinase 3 weeks before use, and (e) immobilized tyrosinase 4 weeks before use. Experimental conditions: initial activity of 1500.0 U, constant stirring of 15.7 rad/s, 298 K, and 120 minutes contact time

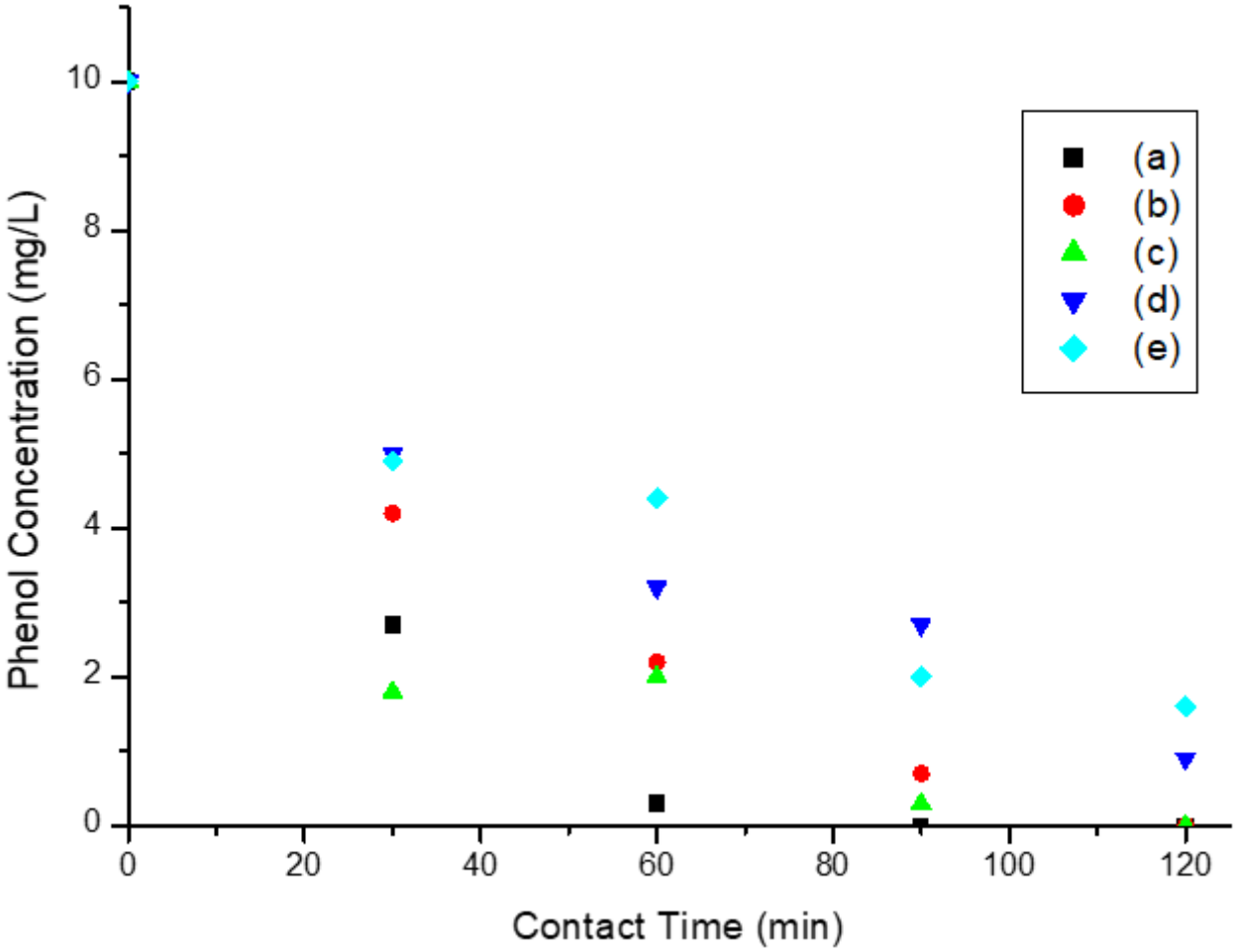

Figure 13

Enzymatic oxidation using (a) immobilized tyrosinase on ACB 1 day before of its use, (b) immobilized tyrosinase 1 week before use, (c) immobilized tyrosinase 2 weeks before use, (d) immobilized tyrosinase 3 weeks before use, and (e) immobilized tyrosinase 4 weeks before use. Experimental conditions: initial activity of 2000.0 U, constant stirring of 15.7 rad/s, 298 K, and 120 minutes contact time

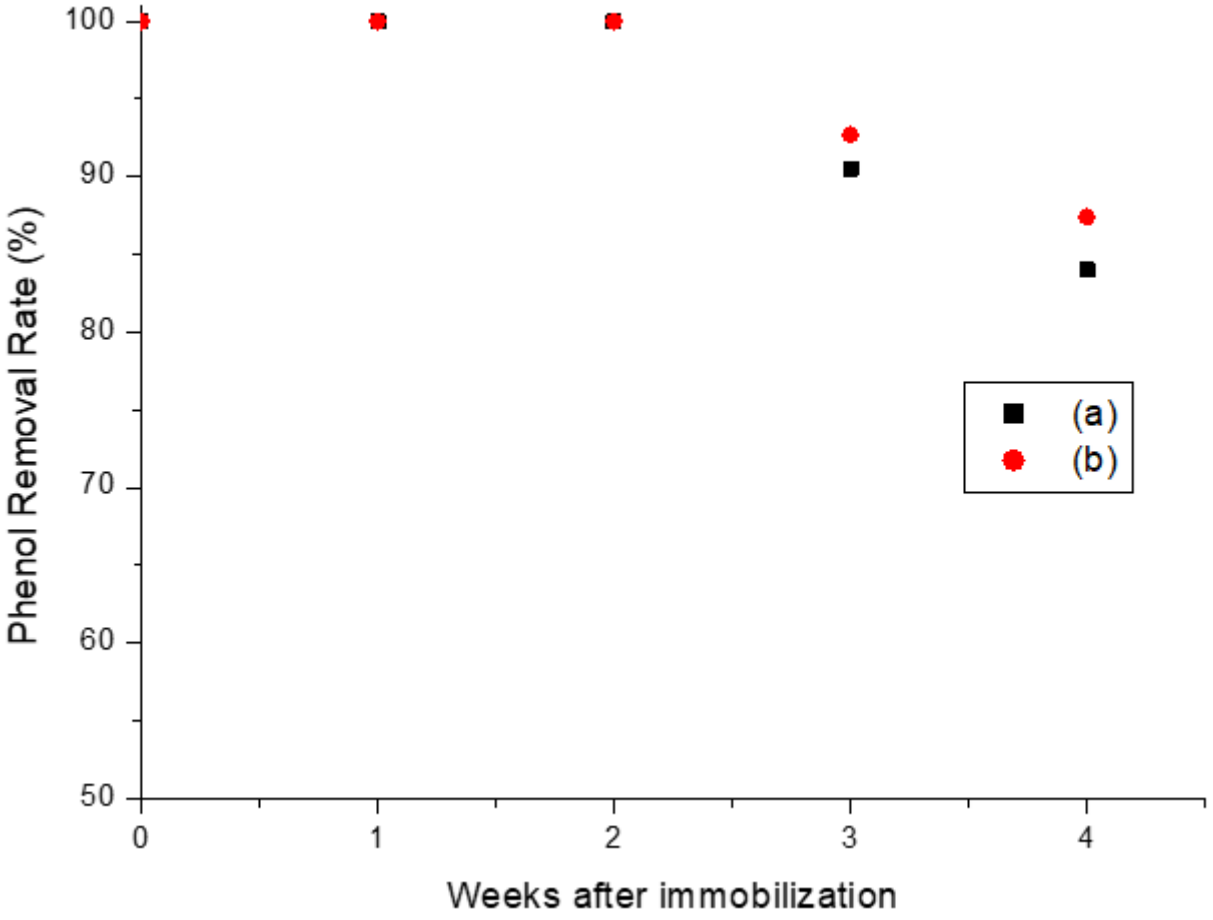

Figure 14

Phenol removal rate after enzyme oxidation assay concerning the time between immobilization and its first use (a) in GAC and (b) ACB

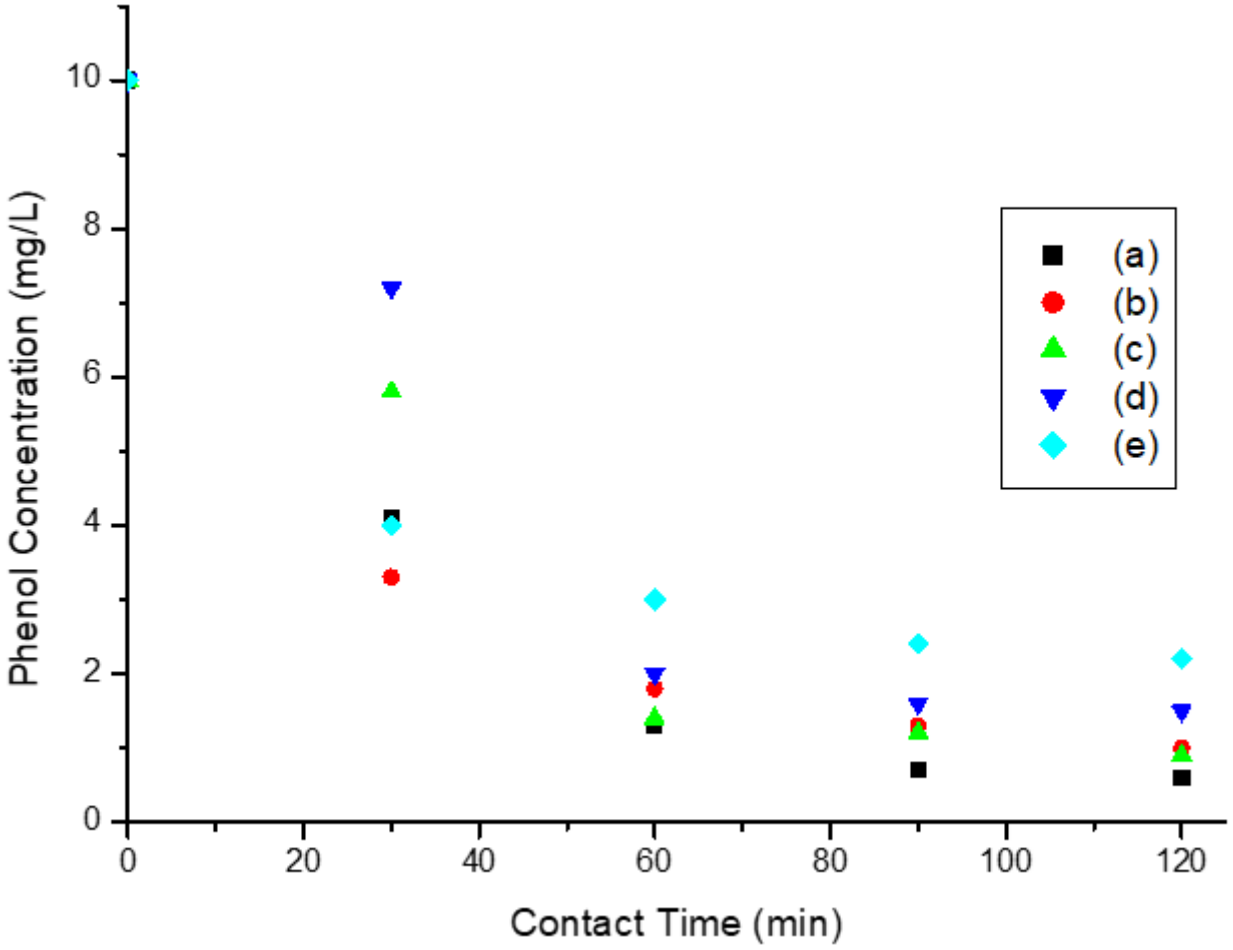

Figure 15

Enzymatic oxidation reusing GAC immobilized tyrosinase (a) 1 day after its first use, (b) 1 week after its first use, (c) 2 weeks after its first use, (d) 3 weeks after first use, and (e) 4 weeks after first use. Experimental conditions: initial activity of ~ 1500.0 U, constant stirring of 15.7 rad/s, ~ 298 K, and 120 minutes contact time

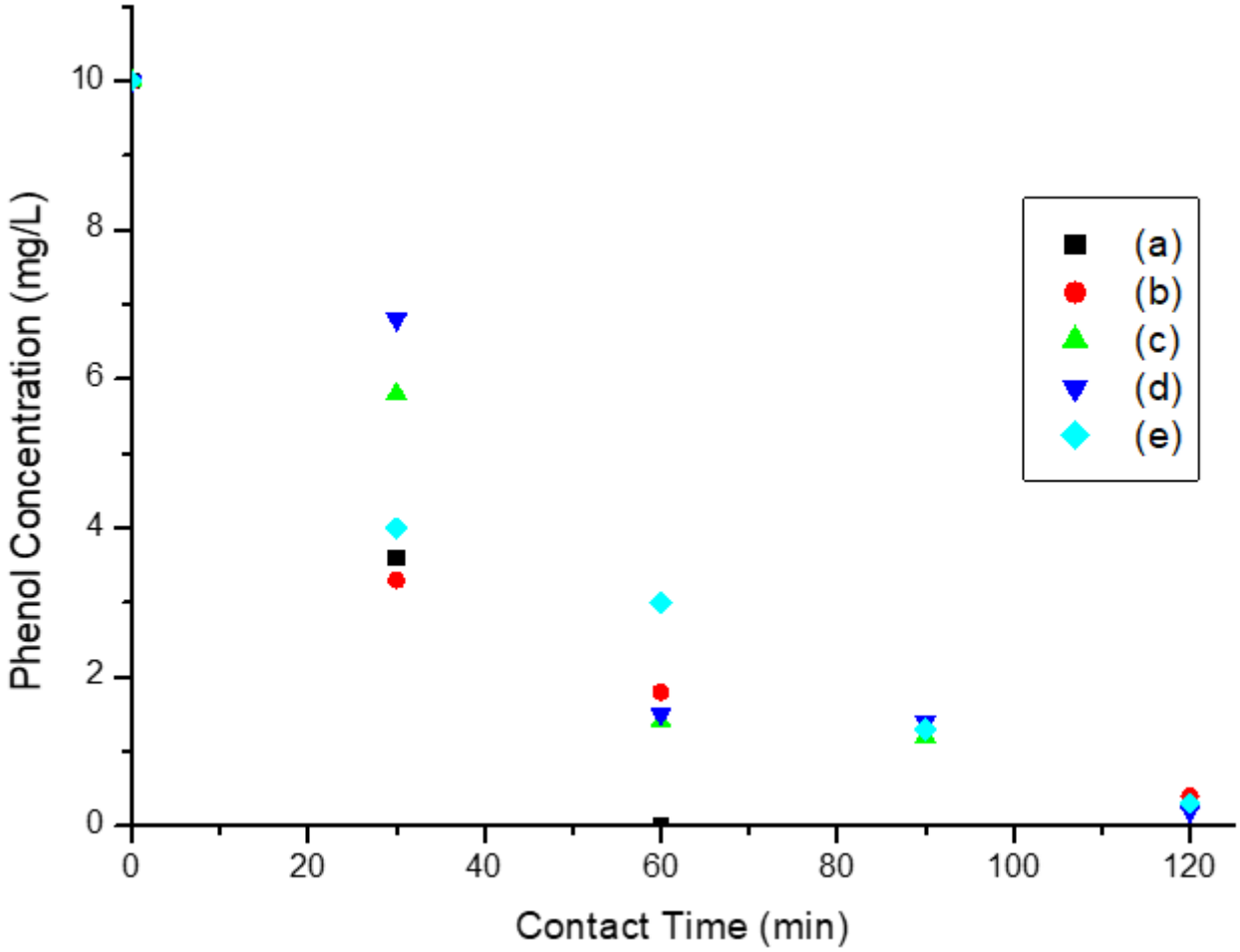

Figure 16

Enzymatic oxidation reusing ACB immobilized tyrosinase (a) 1 day after its first use, (b) 1 week after its first use, (c) 2 weeks after its first use, (d) 3 weeks after first use, and (e) 4 weeks after first use. Experimental conditions: initial activity of 2000.0 U, constant stirring of 15.7 rad/s, ~ 298 K, and 120 minutes contact time

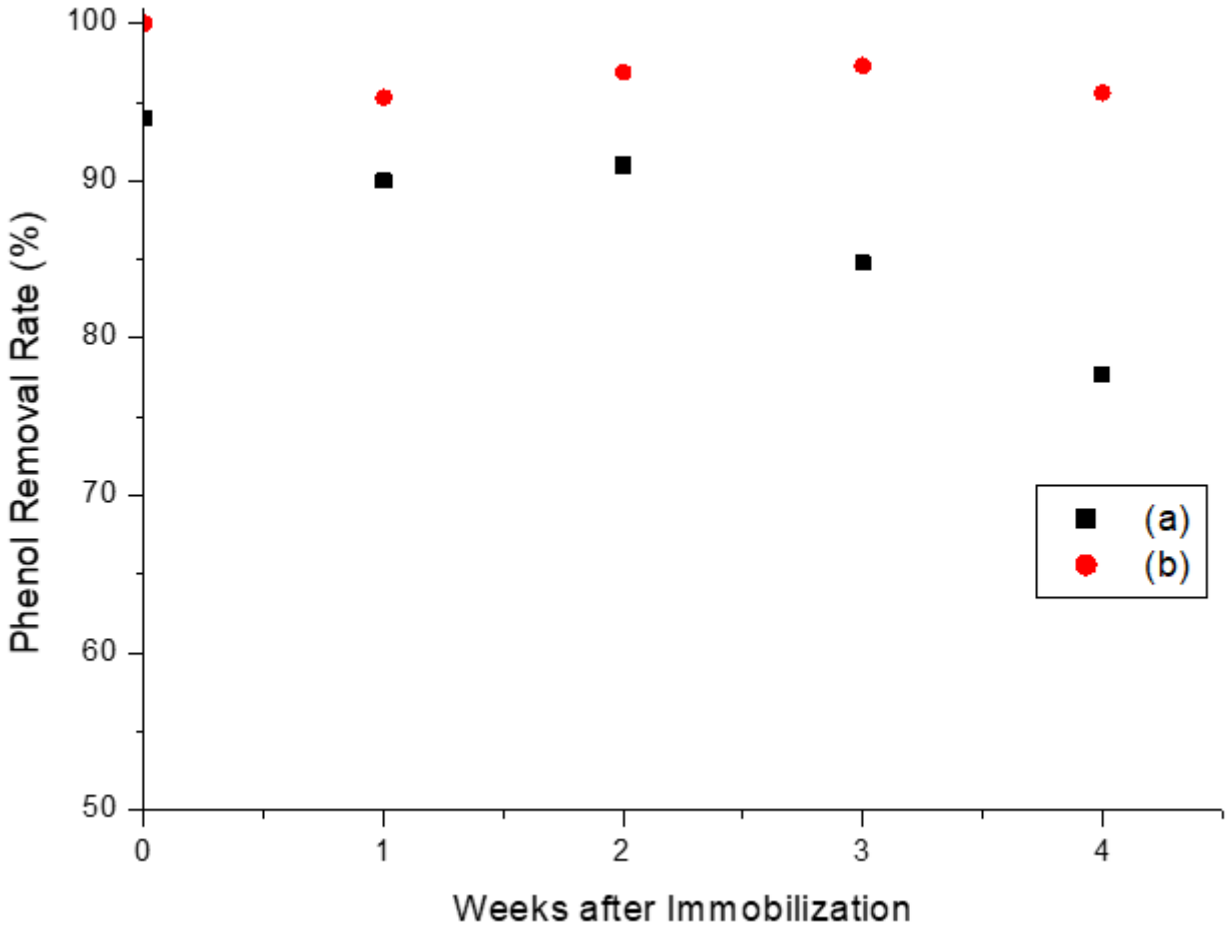

Figure 17

Rate of phenol removal with enzyme oxidation about the time between its first and second uses of the immobilized enzyme in (a) GAC and (b) ACB