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EVALUATION OF MAGNETIC CARRIERS EMPLOYED FOR IMMOBILIZATION OF LIPASE FROM *CANDIDA RUGOSA*

AVALIAÇÃO DE SUPORTES MAGNÉTICOS UTILIZADOS PARA IMOBILIZAÇÃO DA LIPASE DE CANDIDA RUGOSA

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ABSTRACT

Currently, the use of magnetic nanoparticles has aroused interest in industrial processes, and the combination of their properties with the immobilization of lipases has been developed in order to produce carriers of easy separation of the reaction medium. In this context, the objective of the present study was to immobilize Candida rugosa *lipase in magnetic nanoparticles, such as magnetite and maghemite, by physical adsorption and covalent bonding. The biocatalysts were evaluated by infrared spectroscopy (FTIR) and hydrolytic activity analysis. Thus, from the analyses performed, the best biocatalyst obtained was the immobilized by covalent bond in maghemite, presenting a hydrolytic activity of 174.67 U/g.*

RESUMO

Atualmente, o uso de nanopartículas magnéticas tem despertado interesse em processos industriais, e a combinação de suas propriedades com a imobilização de lipases tem sido desenvolvida a fim de produzir suportes de fácil separação do meio reacional. Nesse contexto, o objetivo do presente estudo foi imobilizar a lipase de Candida rugosa em nanopartículas magnéticas, como magnetita e maghemita, por adsorção física e ligação covalente. Os biocatalisadores foram avaliados por espectroscopia de infravermelho (FTIR) e análise de atividade hidrolítica. Dessa forma, a partir das análises realizadas, o melhor biocatalisador obtido foi o imobilizado por ligação covalente em maghemita, apresentando atividade hidrolítica de 174,67 U/g.

NOMENCLATURE

In the study were used: *Candida rugosa* lipase (CRL); maghemite (MH), magnetite synthesized using sodium hydroxide (MN1) and ammonium hydroxide (MN2), in addition to immobilized biocatalysts by physical adsorption (A): lipase immobilized in maghemite (MH-A-CRL) and magnetite (MN1-A-CRL and MN2-A-CRL); and covalent bonding (C): immobilized in maghemite (MH-C-CRL) and magnetite (MN1-C-CRL and MN2-C-CRL).

1. INTRODUCTION

In the current industrial context, the search for ways of reusing biocatalyst has gained ample space among research to improve and reduce costs in biotechnological processes, such as the use of enzymes. These are protein polymers formed by peptide bonds between two adjacent amino acids, presenting a three-dimensional region formed by amino acid residues (CAMPESTRINI et al., 2005). Among the enzymes, lipases stand out for its ability to perform hydrolysis of long-chain triglycerides, whose industrial applications range from use in the food industry to obtaining bioactive molecules (REZANKA et al., 2017). In addition, the use of this biocatalyst is more ecological due to the lower production of toxic agents and by-products (ZHENG et al., 2017). Among the lipases from several microorganisms, the biocatalyst used in the study was *Candida rugosa* lipase (CRL).

Considering the high added value and the advantages of the use of lipases, immobilization is an alternative for its reuse, influencing and modifying parameters such as stability after immobilized, and can be performed by different methodologies (CARVALHO et al., 2015). Adherence by physical adsorption is based on the interaction of the enzyme with the carrier used. This interaction occurs through weak forces such as hydrophobic, ionic, hydrogen bonds and Van der Waals forces. However, this method is advantageous due to its easy execution, having as a disadvantage the high chance of desorption due to the weak interaction of the derivative with the support used. (SHELDON; PELT, 2013).

Immobilization by covalent bonding occurs due to the strong chemical bond between enzyme and support. In this methodology, the carrier should be modified with some functionalizing agent in order to facilitate that regions of the enzyme bind covalently. A positive outlook for this immobilization methodology is the irreversible characteristic of the binding formed by the enzyme and the support, avoiding the desorption that occurred when immobilized by physical adsorption (TAN et al., 2010). However, because it is a chemical bond, there may be a decrease in the biological activity of the enzyme due to a possible impediment of the active site with immobilization (BRÍGIDA, 2010). For the use of a material as a carrier for immobilization, the contact surface, ions, solubility, mechanical strength, and biocatalyst, being classified according to its composition, inorganic and organic, and morphological structure, in non-porous, porous and gel structure (CARVALHO et al., 2015).

Therefore, due to the search for improvements in biotechnological processes and the use of new supports for immobilization of enzymes, magnetic supports draw attention due to advantages such as resistance to high temperatures, mass transfer and the available surface for immobilization (PINTO, 2017). In this study, the carriers used for immobilization were maghemite (γFe_2O_3) and magnetite (Fe_3O_4) and the crystalline structure of each of the nanoparticles is shown in Figures 1 and 2.

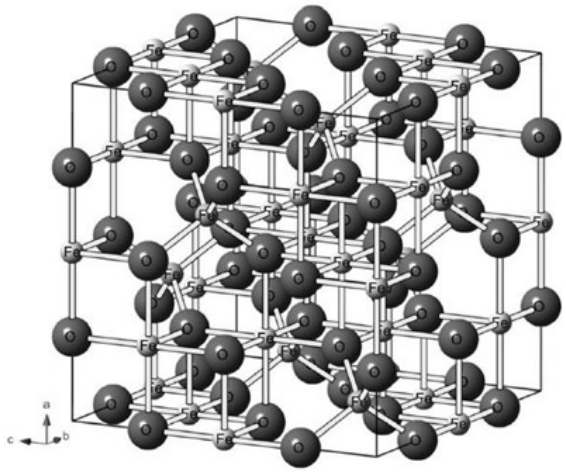

Figure 1. Maghemite crystalline structure. Source: Oliveira et al. (2013).

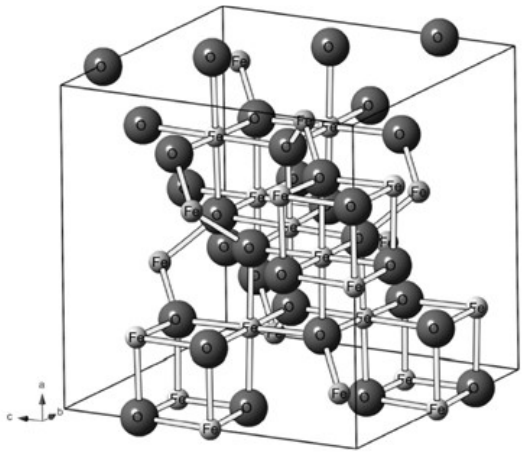

Figure 2. Magnetite crystalline structure. Source: Oliveira et al. (2013).

Magnetite nanoparticles are iron oxides formed by a structure composed of oxygen ions, whose interstitial spaces are filled with ferrous iron ions (Fe^{2+}), ferric iron ions (Fe^{3+}), differing by the absence of ferrous iron for maghemite synthesis (DUNLOP and ÖZDEMIR, 1997). Moreover, the main justification for the use of magnetic materials is the easy removal of the immobilized derivative with a magnetic field after the process (PINTO, 2017).

2. MATERIAL E METHODS

2.1 Maghemite Synthesis

The synthesis followed modified methodology of Junior et al. (2010), adding 30 mL of ultra-pure water and 20 mL of Na_2SO_3 solution (1 M), in this order, in 30 mL of $FeCl_3.6H_2O$ solution (2 M, prepared in HCl 2 M), under agitation until the solution turned yellow. 51 mL ammonium hydroxide (25%) were added in 870 mL of ultra-pure water, followed by the addition of the previously obtained yellow solution, maintaining at 3000 rpm for 1 hour. At the end, the dark mass obtained was washed with ultra-pure water and dried at 100ºC for 24h. The dried mass was taken to laboratory muffle at 250ºC for 1 h. the synthesis yield (Y) was calculated according to Equation 1.

$$Y\ (\%) = \frac{m(MH)}{M_T} * 100 \quad (1)$$

where m(MH) is the mass of maghemite obtained in the synthesis and M_T is the total mass of $FeCl_3$ used.

2.2 Magnetite Synthesis

The synthesis was carried according to the co-precipitation methodology of Fe^{2+} and Fe^{3+}, proposed by Mijone (2014), using 30 mL of $FeCl_2$ solution (0.6 M) and 300 mL of ultra-pure water under high agitation, followed by the addition of 30 mL of $FeCl_3.6H_2O$ solution (1.1 M), maintained at 80ºC. Then, was adjusted pH 11 adding solution (4 M) of precipitating agents (sodium hydroxide and ammonium hydroxide, independent and separate processes). After 30 min, the container was kept under a magnet and the recovered mass was washed with ethyl acetate and ultra-pure water (1:1), dried at 100ºC for 24 h. The yield was calculated according to Eq. 1, and the M_T value corresponding to the mass of $FeCl_2$ and $FeCl_3$ used in the synthesis

2.3 Surface Modification of Nanoparticles

For immobilization by covalent bond, a step of modification of the nanoparticles was necessary, and the activation process with APTES (3- aminopropyltrithoxysilane) proposed by Kumar et al. (2013) and the process of functionalization with glutaraldehyde described by Vescovi et al. (2016). For activation, the nanoparticles were dried at 200ºC for 2 h and then added 500 mg in a solution prepared with 20 mL heptane and 240 µL APTES (3- aminopropylthetoxysilane). The mixture was kept in an atmosphere of N_2 at 75°C for 3h. The obtained solid was washed with hexane and stored for lipase immobilization. The activated nanoparticles were functionalized by adding phosphate buffer solution pH 7.4 (0.1 M), ratio 1:18 (m/v), adding 2 mL of glutaraldehyde (25%) for each 1 g of support. The mixture was kept under agitation at room temperature for 3 h, followed by 1 h at rest at the end of the process. The mass obtained was washed with alcohol, water and buffer solution and maintained at 4ºC until immobilization.

2.4 Immobilization of CRL

The immobilization process of *Candida rugosa* lipase (L1754, EC. 3.1.1.3, type VII and protein content of 16% m/m) was carried using the unmodified nanoparticles (immobilization by physical adsorption) and modified nanoparticles (covalent bonding), according to modified methodology by Paula (2012). The nanoparticles were immersed in phosphate buffer solution pH 5 (0.1 M), in a ratio 1:10 (m/v), maintained at room temperature for 2 h. Then, for each 1 g of support, 200 µL of PEG 1500 solution (0.5 mg/mL) and 0.25 g of lipase were added, remaining under agitation at 8ºC for 24 h. The biocatalyst was washed with buffer solution and kept in desseccator until use.

2.5 Physical Characterization

In order to analyze the processes of modification and immobilization, infrared spectroscopy (FTIR) was performed, using the PLATINUM-ATR infrared spectrometer (Bruker). The graphs were plotted using ORIGIN 8.0 software.

2.6 Determination of Hydrolytic Activity (A)

The activity was determined using Spanish Extra Virgin Olive Oil CARBONELL and phosphate buffer pH 7 (0.1 M), according to the modified methodology described by Paula (2012). Thus, 4.5 mL of olive oil emulsion, olive oil:water: phosphate buffer (5:5:8) were added in an Erlenmeyer and left at 37ºC in thermostatized bath (Dubnoff) for 10 min. Then, 50 mg of biocatalyst was added and the same temperature in constant agitation for 5 min. At the end, 10 mL of ethanol:acetone:water (1:1:1) and 10 mL of potassium hydroxide solution (0.05 M) were added. Excess potassium hydroxide was titrated with HCI solution (0.05 M) with blue bromothymol indicator (1% m/v). The determination was calculated using the Equation 2, where the unit of activity (U) was defined as the necessary amount of enzyme for the release of 1 µmol of fatty acid per minute, under the study conditions.

$$A\ (U/g) = \frac{(V_S - V_E) * [HA] * 1000}{m * t} \quad (2)$$

where V_S is the volume (mL) of acid titrated in standard Erlenmeyer, V_E is the volume (mL) in each experiment, HA the standardized concentration of the acid solution used (mol/L), m is the mass of biocatalyst used and t is the reaction time (min).

3. RESULTS AND DISCUSSION

3.1 Synthesis of Magnetic Nanoparticles

For the synthesis of maghemite, was used 250ºC instead of 200ºC to avoid the inversion of the maghemite structure in hematite, considering its minimum inversion temperature (T_{inv}) at 250ºC (DUNLOP and ÖZDEMIR, 1997). Figure 3 shows the nanoparticles obtained at the end of the synthesis.

Thus, starting from 5.52 g of $FeCl_3$ and 2.30 g of $FeCl_2$ for magnetite synthesis, a mass of 4.56 g and 4.63 g was obtained, yield of 58.31% and 59.21% for synthesis using sodium hydroxide and ammonium hydroxide. The synthesis of maghemite, starting from 10.03 g of $FeCl_3$, was obtained mass of 4.91 g, yield of 49%. During the synthesis of maghemite, the change in color of the solution from ferric chloride to dark red after the addition of sodium sulfite solution is justified by the formation of the complex $[Fe_2(SO_3)]^{4+}$, making the solution

yellow with the total reduction of dissolved iron. In addition, the formation of nanoparticles by the addition of precipitating agents in both synthesis corresponds to iron oxidation, resulting in MH, MN1 and MN2 nanoparticles (PIMENTA, 2010).

Figure 3. Magnetic nanoparticles obtained.

3.2 Modified Nanoparticles

The modification was performed in part of the nanoparticles for immobilization by covalent bond, being the activation process with APTES, a silane and amino groups carbonyls, increasing interaction between the enzyme and modified nanoparticles (LIANG et al., 2014; HAO et al., 2019; PEREIRA, 2009; NELSON and COX, 2014). The infrared spectroscopy of the supports after the modification process is shown in Figure 4 coupler reagent, and functionalization with glutaraldehyde, promoting irreversible cross reactions and resulting in a difference of carbonyl electrons in the functional group.

Analyzing the Figure 4, it is possible to observe stretching regions (v) of characteristic groups of the compounds used for the modification process, having the largest stretch in the region between the range 1200 – 900 cm^{-1}, corresponding to the stretch of the carbonyl group (C-O: 1100 cm^{-1}), in addition to the alkoxy and silane groups (Si-O-C: between 1160 – 1070 cm^{-1}). Another region identified is for the stretching of the amino groups (NH_2: between 1650 – 1475 cm^{-1}) coupled during the activation of the support, as well as the stretching of iron oxide (Fe-O: 575 cm^{-1}) (CASAGRANDE and REPETTE, 2018; PADILLA et al. 2011; PAVIA et al., 2010).

In this context, when compared to each other, it was found that the lower transmittance characteristic of the stretching of these groups was seen for the analysis of maghemite, showing the best grouping of compounds in relation to the other nanoparticles. However, as the purpose of the functionalization process is the bonding of these free groups with glutaraldehyde, the best for the efficiency in the process would be a small stretch band, showing the presence of few free groups (PAVIA et al., 2010). Thus, it is possible to conclude that the functionalization process was less efficient in the MN2 sample compared to the other samples analyzed.

3.3 Physical Characterization of Immobilized Biocatalyst

After the process of modification in part of the nanoparticles, the enzyme was immobilized by covalent bonding using the modified supports and by physical adsorption in the synthesized pure material. Then, the physical characterization of the biocatalyst immobilized by infrared spectroscopy was performed. The spectroscopies of the immobilized by physical adsorption (Figure 5) and by covalent bonding (Figure 6) are shown.

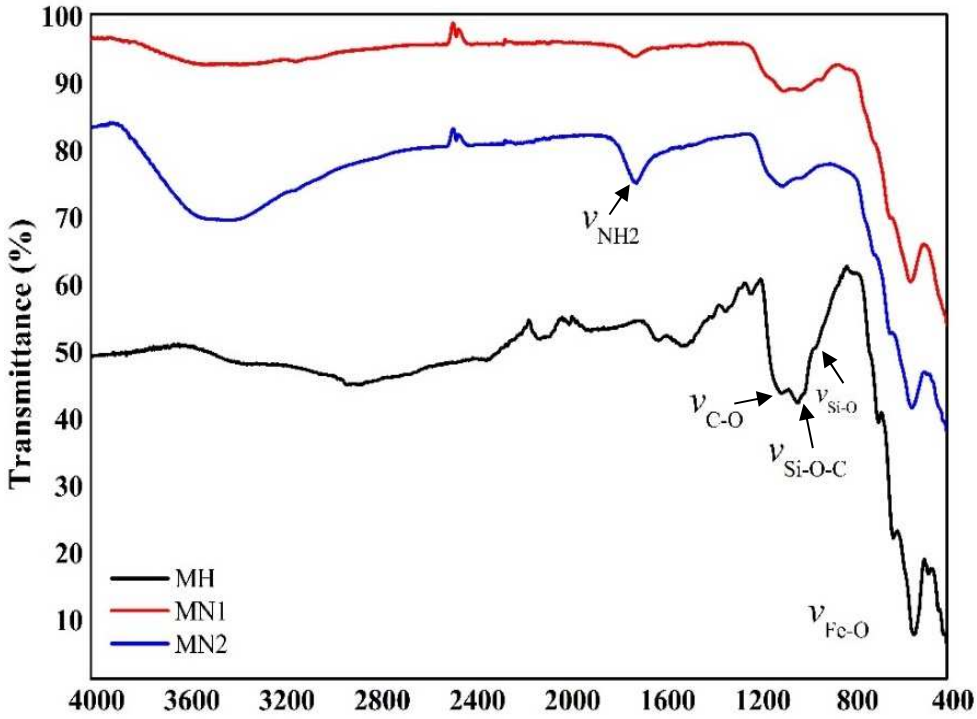

Figure 4. Infrared spectroscopy of modified samples of MN1, MN2 and MH.

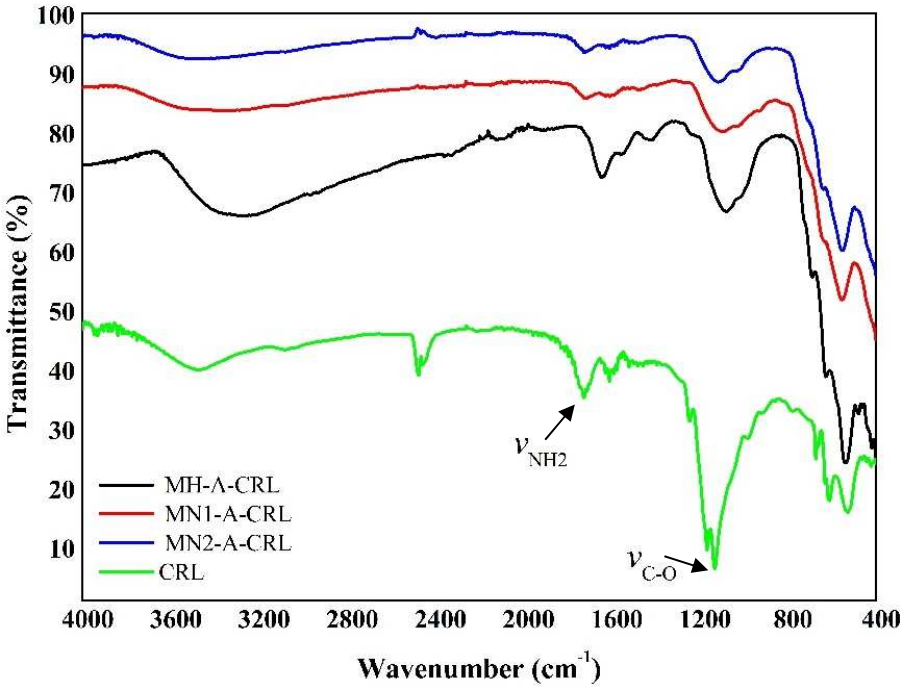

Figure 5. Infrared spectroscopy of biocatalysts immobilized by physical adsorption in samples MH-A-CRL, MN1-A-CRL and MN2-A-CRL.

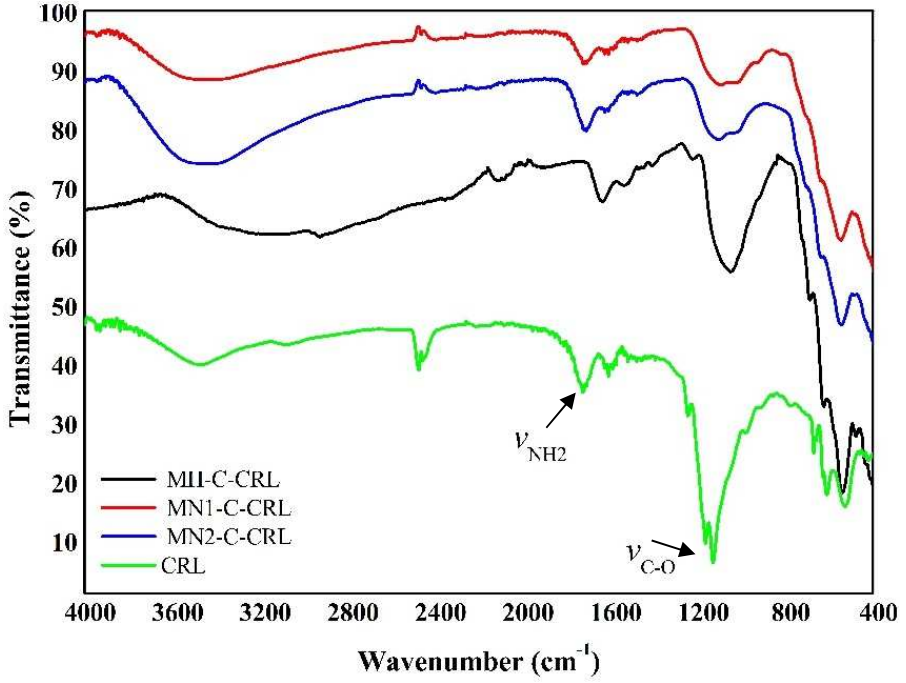

Figure 6. Infrared spectroscopy of biocatalysts immobilized by covalent bonding in samples MH-C-CRL, MN1-C-CRL and MN2-C-CRL

Based on the Figures 5 and 6, it is possible to verify that when comparing the immobilization processes, the presence of regions with characteristic stretching of groups of lipase interest was higher for immobilization by covalent bonding. Comparing the immobilization processes separately, it was found that for both immobilization processes, maghemite was better than others supports for lipase immobilization due to the lower transmittance resulting from the analysis, since the lower the percentage of transmittance higher the absorbance of the compound in the analyzed material (PADILLA *et al.* 2011). In addition, to the infrared spectroscopy analysis, it was also carried the determination of the hydrolytic activity of biocatalysts resulting from both immobilization processes, in order to verify the best immobilization efficiency between the methods studied.

3.4 Hydrolytic Activity of Immobilized Biocatalysts

The results obtained for the determination of the hydrolytic activity of biocatalysts immobilized by physical adsorption are shown in Table 1 and by covalent bonding in Table 2.

Table 1. Average Hydrolytic Activity (U/g) of biocatalysts immobilized by physical adsorption.

Samples	Hydrolytic Activity (U/g) ± STDEV
MH-A-CRL	126.37 ± 19.66
MN1-A-CRL	115.18 ± 24.02
MN2-A-CRL	121.87 ± 12.77

Table 2. Average Hydrolytic Activity (U/g) of biocatalysts immobilized by covalent bonding.

Samples	Hydrolytic Activity (U/g) ± STDEV
MH-A-CRL	167.27 ± 12.93
MN1-A-CRL	144.35 ± 12.81
MN2-A-CRL	144.89 ± 12.05

Analyzing the results presented in the previous tables, it was identified that the values of hydrolytic activity obtained for the immobilized biocatalyst by covalent bonding were better than the results obtained by physical adsorption. The highest hydrolytic activity was obtained from the immobilization of lipase in maghemite, in both methods of immobilization, showing greater efficiency in the process when using this nanoparticle as magnetic support.

Comparing with other studies, Silva and Silva (2014) presented an activity of 1.389 U/g when POS-PVA was used for covalent bond immobilization. Hou et al. (2015) showed an activity of 119.43 U/g for the immobilization in magnetic shell of polydopamine/alginate by covalent bond. Jafarian et al. (2018) points out that, when immobilized in graphene oxide nanosheets by covalent bond, the activity obtained was 167.04 U/g.

The activity values obtained in the study of immobilization in magnetite and maghemite are closer to the values presented by the studies that used polydopamine/alginate magnetic shell and graphene oxide nanosheets. The small difference presented between the authors and the results obtained can be justified by the difference in the surface area, being greater in porous materials such as magnetic nanoparticles used in this study, being a better use of the structure for enzyme immobilization (BRÍGIDA, 2010). However, the great difference seen between the other activities presented can be justified by the process and support used for immobilization, since such a process can cause problems regarding the steric impediment of the substrate to the binding site (PAULA et al., 2008).

4. CONCLUSION

Studying the synthesis of magnetic nanoparticles of maghemite (MH) and magnetite, synthesized with sodium hydroxide (MN1) and ammonium hydroxide (MN2), the highest yield obtained was 59.21% for the synthesis of MN2 nanoparticles. Analyzing the infrared spectroscopies, there was a greater stretch of lipase characteristic regions when immobilized in maghemite, in both methods of physical adsorption and covalent bonding. In addition, in relation to hydrolytic activity, better results were obtained when MH nanoparticles were used as immobilization support in both immobilization processes, with the highest activity of 174.67 U/g obtained for the immobilization of CRL in MH by covalent bond. Therefore, when the use of magnetic nanoparticles for the immobilization of *Candida rugosa* lipase was evaluated, a better performance was verified for the use of maghemite as an immobilization support.

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