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Candida rugosa lipase immobilized on hydrophobic support Accurel MP 1000 in the synthesis of emollient esters

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Abstract

In the present work, *Candida rugosa* lipase (CRL) was immobilized by physical adsorption in organic medium on Accurel MP 1000 (AMP) with a protein load of 6.5 mg g^{-1} (mg protein/g support). CRL-AMP was applied with 5 and 10% of catalyst/volume of medium (m v^{-1}) in esterification reactions of stearic acid with lauryl and cetyl alcohols producing the wax esters such as dodecanoyl octadecanoate **1** and hexadecanoyl octadecanoate **2** in a heptane medium. Six reaction cycles were studied to evaluate the stability and recyclability of the prepared biocatalyst. The specific activity (A_{sp}) for CRL-AMP was 200 ± 20 U mg^{-1}. Its catalytic activity was 1300 ± 100 U g^{-1}. CRL-AMP was used in the synthesis of esters in heptane medium with a 1:1 acid:alcohol molar ratio at 45°C and 200 rpm. In synthesis **1**, conversion was 62.5 ± 3.9% in 30 min at 10% m v^{-1} and 56.9 ± 2.8% in 54 min at 5% m v^{-1}, while in synthesis **2**, conversion was 79.0 ± 3.9% in 24 min at 10% m v^{-1}, and 46.0 ± 2.4% in 54 min at 5% m v^{-1}. Reuse tests after 6 consecutive cycles of reaction showed that the biocatalyst retained approximately 50% of its original activity for both reaction systems. CRL-AMP showed a high potential in the production of wax esters, since it started from low enzymatic load and high specific activities and conversions were obtained, in addition to allowing an increase in stability and recyclability of the prepared biocatalyst.

Keywords: Accurel MP 1000; Biocatalysis*;* Esterification; Immobilization.

1. Introduction

Emollient esters are long chain organic compounds (from 12 carbon atoms) with high molecular weight, formed by long chain alcohols and carboxylic acids (Serrano-Arnaldos et al., 2016). These esters are neither toxic nor greasy (Khan et al., 2015), they are classified as fine chemical products (Rani et al., 2015) and are commonly used in the cosmetic, pharmaceutical and lubricant industries (Lima et al., 2018; Miguez et al., 2018). Naturally, wax esters can be obtained from animal and vegetable sources and have distinct compositions (Ungcharoenwiwat et al., 2015). However, natural wax esters are not readily available, which increases their commercial value. Thus, synthetic esters are considered promising substitutes to natural esters (Kuo et al., 2012).

Wax ester synthesis reactions are usually catalyzed by toxic and corrosive chemical compounds such as hydrofluoric acid and sulfuric acid (Esfandmaz et al., 2018). The disadvantages of processes catalyzed by strong acids are high reaction temperatures, difficult separation of reaction media, low yields and environmental concerns (Cui et al., 2020). However, the enzymatic route has proved attractive for the production of these esters because it is a process with low energy consumption, high productivity, and good stability compared to organic solvents (Bandikari et al., 2018), in addition to having broad substrate specificity and exhibiting high enantioselectivity (Benamia et al., 2016).

Among the enzymes used in the synthesis of esters with emollient properties, lipases (triacylglycerol acyl-hydrolases, EC 3.1.1.3) stand out (Novaes et al., 2018; Cea et al., 2019) for their application in the hydrolysis of triacylglycerols into glycerol and free fatty acids in aqueous media. In organic medium, they catalyze transesterification, interesterification and esterification reactions for producing valuable esters such as wax esters (De Menezes et al., 2021). Lipases present two different configurations, closed configuration, where contact between the active site and the reaction medium is blocked, and open configuration, where the active site is exposed to the reaction medium (Manoel et al., 2015). In order to prolong the applicability of lipases in syntheses of emollient esters and other industrial processes, the study of new techniques that allow its reuse is necessary, among these techniques, immobilization stands out (Cea et al., 2019).

Enzymatic immobilization is the confinement of protein on an insoluble solid support in aqueous and in organic solvents media and is applied to improve catalytic efficiency and allow its reuse (Manoel et al., 2015; Hadadi et al., 2019). Physical adsorption is a technique of enzymatic immobilization that

occurs through non-covalent bonds such as hydrophobic interactions, Van der Waals forces, hydrogen bonds and ionic bonds (Bolina et al., 2018). In this type of immobilization, lipases are adsorbed on relatively porous supports and this process involves the open form of lipases, which makes it adequate (Francolini et al., 2020).

Accurel MP 1000 (AMP) is a hydrophobic and macroporous polypropylene polymer (Cesarini et al., 2014) widely used as a support in the immobilization of lipases (Baron et al., 2011; Scherer et al., 2011; Cunha et al., 2013; Alnoch et al., 2015; Madalozzo et al., 2015; Manoel et al., 2015). Lipases immobilized on AMP are used for several purposes, such as catalysis in synthesis of biodiesel esters (Madalozzo et al., 2015; De Menezes et al., 2021), pharmacological derivatives, such as myo-inositol, a supplement that helps in hormonal disorders and fertility (Manoel et al., 2016), and catalysis of biolubricating esters from vegetable oil by-products (Fernandes et al., 2020).

Thus, this work aimed to immobilize the *Candida rugosa* lipase (CRL) on Accurel MP 1000 (AMP) by adsorption in organic medium and apply it in the synthesis of stearic acid with lauryl and cetyl alcohol to obtain esters with emollient properties. CRL was selected for having high hydrolytic potential, besides its efficiency in synthesis under limited water conditions (Cavalcanti et al., 2018). Stearic acid was used as a donor because it is one of the main fatty acids present in beef tallow (Aransiola et al., 2014), a low-cost raw material that is easy to obtain in Brazil, which may lead to new studies aiming at the use of bovine tallow as a substrate for the enzymatic synthesis of wax esters.

2. Materials and Methods

Candida rugosa lipase, an enzymatic powder preparation with protein concentration of 27.42 ± 2.70 mg g^{-1}, hydrolytic activity 37591.20 ± 75.18 U g^{-1} and specific activity 1370.90 ± 1.40 U mg^{-1}, was acquired from Sigma-Aldrich Co. (St. Louis, MO, USA) and it was used without prior treatment. Lauryl and cetyl alcohols were also acquired from Sigma Aldrich Co. Accurel® MP 1000 beads which is a polypropylene carrier, with particle diameter <1.5 mm and mean pore diameter of 25 nm (Sabbani et al., 2006), was obtained from 3M (Germany). Ethyl alcohol, acetone, hexane, heptane, stearic acid and acacia gum were obtained from Synth® (São Paulo, SP, Brazil). Olive oil (Carbonell, Córdoba, Spain) was purchased at the local trade market (Itabuna, Bahia, Brazil). All other reactants and organic solvents were of analytical grade supplied by Synth®.

2.1 Immobilization of *Candida rugosa* lipase on Accurel MP 1000

2.1.1 Scanning electron microscopy (SEM)

The morphology of the Accurel MP 1000 support was investigated by fixing the samples on a "Stub" holder and then metallized, about 20 to 30 nm thick by a sputtering evaporation system using the Sputter Coater, BAL-TEC SCD050 (Fig. 1). In Fig. 1a, with a 20 µm zoom, the pores of the support are shown, while in Fig. 1b, with a 100 µm zoom, besides the pores, its conformation is shown.

2.1.2 Immobilization of *Candida rugosa* lipase

Accurel MP 1000 (1 g) was immersed in hexane (15 mL) at a ratio of 1:10 (m/v) in a becker (100 mL) and kept at -6 ºC for 4 h in a vertical freezer. Then, 237 mg of commercial CRL powder extract (that corresponds to an initial protein loading of 6.5 mg g^{-1}) were added to this suspension. The resulting suspension consisting of CRL powder extract, support and solvent was kept at -6 ºC overnight in a vertical freezer to obtain the immobilized lipase. After, the biocatalyst prepared (CRL-AMP) was recovered via filtration in a Buchner funnel under vacuum and repeatedly washed with hexane to remove unbounded enzyme molecules. The immobilized lipase was then stored under refrigeration at 4 °C in a BOD incubator (TE-371, Tecnal, Piracicaba, Brazil). The immobilized protein concentration (IP – mg g^{-1}) was determined according to method of the Bradford et al. (1976), using bovine serum albumin (BSA) as standard (Ferreira et al., 2017), as shown in Eq. (1).

$$IP = \frac{V_{enz} x (C_0 - C_e)}{m} \quad (1)$$

where *IP* is the immobilized lipase concentration (mg g^{-1}); V_{enz} is the volume of solution (mL); C_0 and C_e are respectively the initial and residual (at equilibrium) protein concentration in the immobilization supernatant (mg/mL); and *m* is the mass of support (g).

Immobilized yield (IY – %) was defined as the ratio between the immobilized protein concentration at equilibrium (IP – mg g^{-1}) and initial protein concentration used to prepare the heterogeneous biocatalyst (6.5 mg g^{-1}).

2.1.3 Determination of hydrolytic activity

The hydrolytic activity (HA) of crude free CRL and immobilized lipase (CRL-AMP) was determined on the hydrolysis of olive oil emulsion (Carvalho et al., 2017; Dos Santos et al., 2020). The emulsion was prepared by mixing 2.5 g of olive oil with 2.5 g of Arabic Gum solution at 7% m/v and 5 mL of 100 mmol L^{-1} buffer sodium phosphate pH 7.0. This mixture was placed in a 125 mL conical flask and incubated in an incubator with orbital agitation (Tecnal, Piracicaba, São Paulo, Brazil) at 200 rpm and 37 ºC and added 0.1 g of immobilized lipase. Following this, 10 mL of ethanol solution at 95% m/m was added to the reaction mixture and titrated with a 0.0313 M NaOH solution using phenolphthalein as indicator. Hydrolytic activity was determined as shown in Eq. (2). One international unit (U) of activity was defined as being the mass of enzyme required to release 1 µmol of free fatty acid per minute of reaction. These assays were performed with three replications.

$$HA\ (U\ g^{-1}) = \frac{(V_s - V_c)x\ M\ x\ 10^3}{t\ x\ m} \quad (2)$$

where *HA* is the hydrolytic activity of the immobilized lipase (U g^{-1}), V_S is the volume of NaOH solution used to titrate the sample (mL), V_C is the volume of NaOH solution used to titrate control sample (mL), *M* is the concentration of NaOH solution (mol L^{-1}), *t* is the reaction time (min) and *m* is the mass of immobilized lipase (g).

Specific Activity (A_{sp} – U mg^{-1}) was calculated according to Eq. (3) (Teodoro et al., 2019):

$$A_{sp}\ (U\ mg^{-1}) = \frac{HA}{IP} \quad (3)$$

where *HA* is the hydrolytic activity of the prepared biocatalysts (U g^{-1}) and *IP* is the immobilized protein concentration at equilibrium (mg g^{-1}).

2.2 Application of CRL-AMP

2.2.1 Esterification reaction

The esterification reactions between stearic acid and alcohols (lauryl and cetyl) were performed in closed Duran flasks (25 mL), containing 6 mL of reaction medium and 500 mmol L^{-1} of each reagent (acid:alcohol, 1:1) in heptane medium. The reaction mixtures were immersed in an orbital shaker (Tecnal, Piracicaba, São Paulo, Brazil) at 200 rpm and 45 °C by 15 min to dissolve starting materials (alcohols and fatty acids) before adding the immobilized lipase (CRL-AMP). The schematic synthesis reactions of the two esters are shown in Fig. 2. In each flask, the CRL-AMP was added in different concentrations (5 and 10% of catalyst/volume of medium) (m v^{-1}). Periodically, aliquots (100 μL) from the reaction mixture were removed, diluted in 10 mL of an ethanol/acetone 1:1 (v/v) mixture and titrated with NaOH solution (0.0313 M) using phenolphthalein as indicated (Alves et al., 2016; Lage et al., 2016). The conversion (%), based on acid consumption in the reaction, was calculated according to Eq. (4). Control assays were conducted by adding AMP beads to the reaction mixtures and no acid consumption was detected under such experimental conditions.

$$Conversion\ (\%) = \left(\frac{A_{in} - A_{fin}}{A_{in}}\right) \text{x} 100 \quad (4)$$

Where A_{in} and A_{fin} are the initial and final concentrations of fatty acid in the reaction medium (mmol L^{-1}).

2.2.2 Reuse of CRL-AMP

Two different reactional media were employed, both consisting of 6 mL and in the presence of heptane. The first containing lauryl alcohol and stearic acid and the second containing cetyl alcohol and stearic acid. The media were placed in Duran flasks (25 mL) at 45 ºC and 200 rpm in an incubator with orbital agitation (Tecnal, Piracicaba, São Paulo, Brazil). The reaction was initiated by the addition of CRL-AMP in concentrations 5 and 10% of catalyst/volume of medium (m v^{-1}) and the duration of each reaction is described in Table 1. The conversion was determined by titulometric method (Silva et al.,

2017). After a reaction cycle, the CRL-AMP was filtered and washed with excess refrigerated hexane (50 mL), to remove molecules from reagents or products retained in the microenvironment of the catalyst. Finally, the CRL-AMP was stored at 4 ºC in a BOD incubator (TE-371, Tecnal, Piracicaba, Brazil) for 24 hours. This process was performed 6 times, totaling 6 reaction cycles.

3. Results and Discussion

3.1 Obtaining the CRL-AMP

The catalytic properties of the prepared CRL-AMP were determined after quantification of the HA (determining property in catalytic efficiency) in free and immobilized lipase to determine the catalytic activity of the immobilized enzyme, whose observed HA values were 8917.57 ± 249.29 U g^{-1} and 1300 ± 100 U g^{-1}, respectively. The immobilization yield was 100%, since the immobilization procedure was performed in organic solvent medium without free water due to the high hydrophobicity of AMP (Cesarini et al., 2014). This reduces the interaction of the aqueous phase containing the enzyme with the support, thus, immobilization in organic medium was chosen, a method that has been used by several other researchers (Bento et al., 2017; Silva et al., 2018; Da Silva et al., 2020). Complete immobilization was confirmed by method of the Bradford et al. (1976).

The specific activity (A_{sp}) of the free and immobilized enzyme was determined, obtaining 324 ± 32 and 200 ± 20 U mg^{-1}, respectively. After being immobilized, the lipase reduced its A_{sp} (324 ± 32 U mg^{-1}) to 200 ± 20 U mg^{-1}. This reduction indicates that the lipase molecules are not accessible to the drops of olive oil and the immobilization of CRL occurred in the internal part of the AMP, because CRL is a globular protein with molecular size of 50Å × 42Å × 33Å (Gao et al., 2010) and molecular diameter of near 0.5 nm (much smaller than the average diameter of the AMP pores, 25 nm (Sabbani et al., 2006)). The molecular diameter of CRL was determined by equations 5 and 6:

$$R_{enz} = \sqrt[3]{\frac{3V_{enz}}{4\pi}} \quad (5)$$

$$D_{enz} = 2R_{enz} \quad (6)$$

Where R_{enz} is the molecular radius, V_{enz} is the molecular volume and D_{enz} is the molecular diameter.

3.2 Application of CRL-AMP in synthesis of emollient esters

CRL-AMP was applied in the synthesis of dodecanoyl octadecanoate **1** and hexadecanoyl octadecanoate **2** via direct esterification reactions of octadecanoic acid (stearic acid) and alcohols in heptane medium. In order to obtain larger conversions, the influence of time on the esterification reaction was evaluated by removing aliquots at fixed-time intervals (6 min) in the respective concentrations of CRL-AMP (Fig. 3). The free enzyme (powder extract) contains in its composition stabilizing agents such as proteins, sugars and salts, and when using it as a catalyst in the esterification reaction, the water produced in the reaction is adsorbed in its microenvironment causing strong aggregation, thus decreasing its catalytic activity. On the other hand, by using the immobilized enzyme on hydrophobic support, this adsorption in water is lower, thus, the dispersion of the catalyst in the medium improves significantly and favors contact of the enzyme with the raw material, besides increasing the rate of the reaction (Lage et al., 2016).

In synthesis **1** (Fig. 2) (Table 1), using CRL-AMP 10% in mass of biocatalyst by volume of medium (m v^{-1}), conversion 62.5 ± 3.9% (Fig. 3) was obtained in 30 min of reaction, after this time the conversion rate remained stable. The conversion was adequate, since the protein load of the CRL-AMP used was only 6.5 mg g^{-1}. Meanwhile, CRL-AMP 5% m v^{-1} obtained 56.9 ± 2.8% (Fig. 3) in 54 min, and in the first 30 min the reaction produced less than 20% of ester and after 54 min it remained constant. In synthesis **2** (Fig. 2) (Table 1), conversion was 46.0 ± 2.4% in 54 min and 79.0 ± 3.9% (Fig. 3) in 24 min were obtained with CRL-AMP 5 and 10% m v^{-1}, respectively, and after the indicated times, the conversions remained constant, indicating that the reaction reached chemical equilibrium. When the enzymatic concentration of catalyst was doubled (5 to 10% m v^{-1}), there was an increase in the conversion and reduction in time, thus, the synthesis reaction was controlled by kinetics (not mass tranfer) (Yadav et al., 2012).

The productivity (μmol min^{-1} $mg_{protein}^{-1}$) was calculated as indicated by Da Silva et al. (2020):

$$P\left(\mu mol\ min^{-1}\ mg_{protein^{-1}}\right) = \frac{N_a x\ 10^3}{t_{eq} x\ m} \quad (7)$$

Where N_a is the concentration of consumed carboxylic acid (mmol); t is the reaction time in equilibrium; and m is the concentration of immobilized protein.

After the calculations, the productivities 2700 and 2670 µmol min^{-1} $mg_{protein}^{-1}$ were found for 5 and 10% m v^{-1}, respectively (synthesis **1**) and 2180 and 4220 µmol min^{-1} $mg_{protein}^{-1}$ for 5 and 10% m v^{-1}, respectively (synthesis **2**), noting that the reaction in synthesis **2** using CRL-AMP 10% m v^{-1} showed higher productivity (4220 µmol min^{-1} $mg_{protein}^{-1}$) and, consequently, better efficiency. It was expected that this reaction would be slower, because the alcohol used (cetyl alcohol) presents a greater number of carbon atoms in its structure, which would make the contact of the enzyme with the substrate more difficult. However, the system presented better productivity, that is, the enzyme presented a greater affinity to the system in the presence of cetyl alcohol. This result probably indicates that in synthesis **2** the diffusive and barrier effect were smaller, in other words, there was an accelerated mass transfer between the substrate and the enzyme, thus, the orientation of the enzymatic active site directed to the reaction medium improved the contact of the molecules of the substrate with the enzyme. The results obtained corroborate with Machado et al. (2019), in which the authors obtained faster reactions in the presence of cetyl alcohol.

3.3 Reuse of CRL-AMP

Immobilization has relevant industrial importance, since it improves the operational performance and, consequently, the cost-benefit ratio of enzymes in sustainable biocatalytic processes (Sheldon et al., 2013). Thus, the reuse of CRL-AMP at 5 and 10% m v^{-1} was performed in the synthesis of esters **1** and **2**.

In synthesis **1**, CRL-AMP 5% m v^{-1} retained 37.5% of the catalytic activity after the fourth cycle, while with 10% m v^{-1} concentration, it retained 46.5% after the fifth cycle and after the sixth cycle the activity decreased 20% (Fig. 4a). In synthesis **2**, it was observed that after the fifth reaction cycle with CRL-AMP 5% m v^{-1}, 69.4% of the catalytic activity was still present, while with CRL-AMP 10%, it retained 33.5% after the fifth reaction cycle (Fig. 4b). For all reuse calculations, 100% of the reaction activity that preceded the cycles was considered.

Among the six cycles performed, the results obtained show that immobilization increases the ability to reuse lipase after five reaction cycles. A progressive decrease of activity after consecutive cycles of reaction could be due to thermal inactivation of some CRL molecules and/or possible accumulation of water or alcohol molecules on the biocatalyst surface that lead to formation of a hydrophilic layer that restricts the partition of carboxylic acids to its microenvironment (Nasef et al., 2014; Lage et al., 2016).

When comparing the activity of the lipase used in this work after reuse with data from the literature, it is observed that the immobilization procedure was efficient, since, Zare et al. (2018) immobilized *Candia rugosa* lipase on MIL-101 chromium terephthalate and obtained a residual activity of 20 to 30% after the first cycle of reuse, a value lower than that found in the present work in the fourth cycle, whose value obtained was 46.5% in synthesis **1** with 10% m v^{-1} (Fig. 4a), and 69.4 and 33.5% in synthesis **2** with 5% m v^{-1} (Fig. 4b).

Halin et al. (2019) reported the immobilization of CRL on a support of nylon microfibers and its application as a catalyst. An enzymatic load of 1000 mg g^{-1} was used, a content of immobilized enzyme that is approximately 10^3 higher than the one used in the present study and approximately 50% residual activity was obtained after 4 cycles. The results obtained in the present study, with activity retention close to 50% after 5 cycles, suggests that AMP support can be used in the immobilization of CRL, since results similar to the consolidated data in the literature were obtained (Halin et al., 2019). Thus, the use of the AMP support in CRL immobilization is a promising alternative to preserve catalytic activity in esters synthesis using small amounts of protein, as well as enriching data from the literature in terms of enzymatic loading, specific activity, stability and recyclability.

4. Conclusion

The immobilization of *Candida rugosa* lipase on Accurel MP 1000 evaluated in this study showed high values of hydrolytic activity and specific esterified activity in organic medium, whose values demonstrate the efficiency of the immobilization process. The use of CRL-AMP in wax ester synthesis showed good conversions, since the reactions were performed in 60 min to 5 and 10% support and 6.5 mg g^{-1} protein load. Through the CRL-AMP reusability tests, it was possible to verify that the immobilization

made the CRL more stable and consequently, made reuse possible, since it showed activity retention after six cycles. Therefore, the application of this procedure at industrial levels becomes interesting.

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Availability of data and material Not applicable

Code availability Not applicable

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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Figure captions

Fig. 1 Scanning electron microscopy of the adsorbent resin Accurel MP 1000 (a) and (b) porosity with 20 and 100 µm zoom, respectively

Fig. 2 Synthesis of dodecanoyl octadecanoate **1** and hexadecanoyl octadecanoate **2** catalyzed by CRL-AMP

Fig. 3 Reactions catalyzed by CRL-AMP in the synthesis of (a) dodecanoyl octadecanoate **1** and (b) hexadecanoyl octadecanoate **2** to 5 and 10% m v^{-1} to 40 ºC, 200 rpm and 500 mmol L^{-1} of each reagent (acid:alcohol) in heptane medium. The values are represented as mean ± standard deviation of three repetitions

Fig. 4 Studies of CRL-AMP reuse after 6 successive cycles of the synthesis of (a) dodecanoyl octadecanoate **1** and (b) hexadecanoyl octadecanoate **2** to 5 and 10% m v^{-1} in heptane medium

Table captions

Table 1 Conversion of esters synthesized by CRL-AMP (5 and 10 % m v^{-1}) at 40 ºC and 200 rpm

Figures

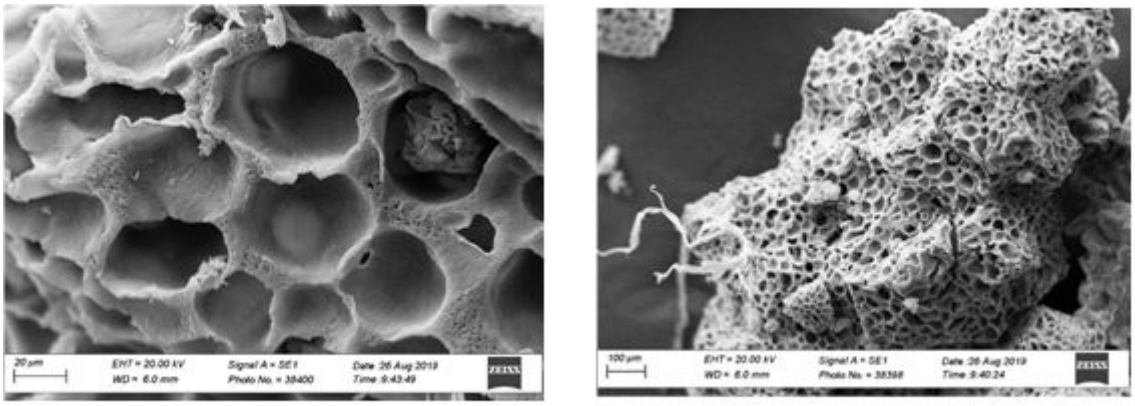

Figure 1

Scanning electron microscopy of the adsorbent resin Accurel MP 1000 (a) and (b) porosity with 20 and 100 µm zoom, respectively

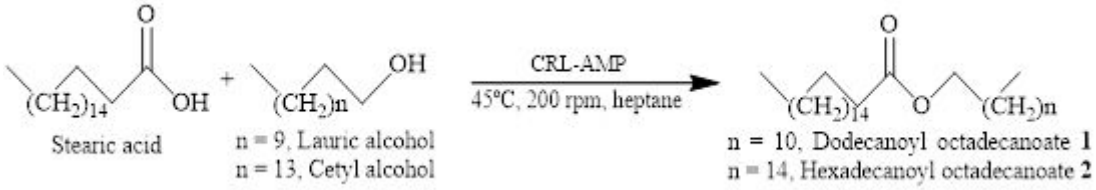

Figure 2

Synthesis of dodecanoyl octadecanoate 1 and hexadecanoyl octadecanoate 2 catalyzed by CRL-AMP

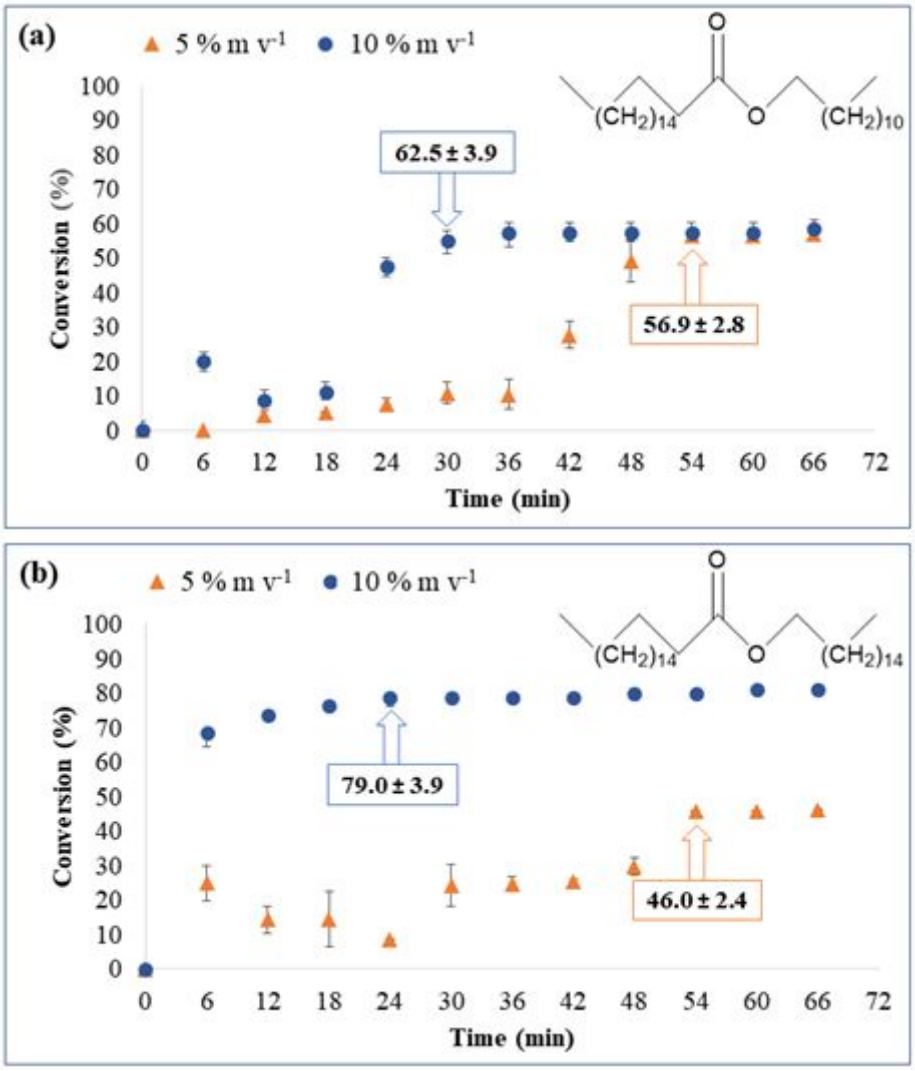

Figure 3

Reactions catalyzed by CRL-AMP in the synthesis of (a) dodecanoyl octadecanoate 1 and (b) hexadecanoyl octadecanoate 2 to 5 and 10% m v-1 to 40 ºC, 200 rpm and 500 mmol L-1 of each reagent (acid:alcohol) in heptane medium. The values are represented as mean ± standard deviation of three repetitions

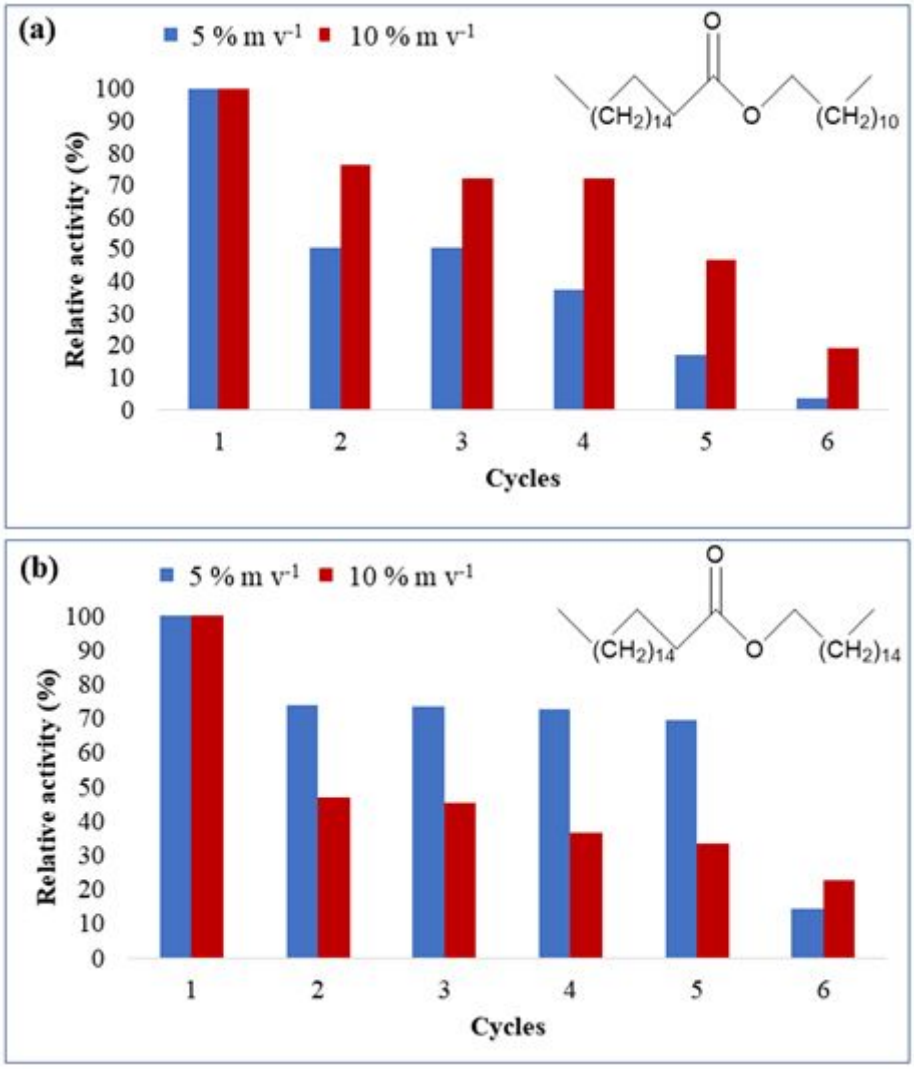

Figure 4

Studies of CRL-AMP reuse after 6 successive cycles of the synthesis of (a) dodecanoyl octadecanoate 1 and (b) hexadecanoyl octadecanoate 2 to 5 and 10% m v-1 in heptane medium