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

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**Abstract**

**Objectives** To immobilize *Candida rugosa* lipase in Accurel MP 1000 (CRL-AMP) by physical adsorption in organic medium and apply in the synthesis of wax esters dodecanoyl octadecanoate 1 and hexadecanoyl octadecanoate 2 in a heptane medium, as well as evaluating the stability and recyclability of CRL-AMP in six reaction cycles.

**Results** 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 six consecutive cycles of reaction showed that the biocatalyst retained approximately 50% of its original activity for both reaction systems.

**Conclusions** 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

**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 and Kittikun 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 catalysed by toxic and corrosive chemical compounds such as hydrofluoric acid and sulfuric acid (Esfandmaz et al. 2018). The disadvantages of processes catalysed 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 catalyse 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 organic solvent media and is applied to improve catalytic efficiency and allow its reuse (Manoel et al. 2015; Hadadi and Habibi 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 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.

Materials and methods

Candida rugosa lipase, an enzymatic powder preparation with protein concentration of 27.42 ± 2.70 mg g⁻¹, hydrolytic activity of 37,591.20 ± 75.18 U g⁻¹ and specific activity of 1370.90 ± 1.40 U mg⁻¹, was acquired from Sigma-Aldrich Co. (St. Louis, MO, USA) and was used without prior treatment. Lauryl and cetyl alcohols were also acquired from Sigma Aldrich Co. Accurel® MP 1000 beads, a polypropylene carrier with a particle diameter less than 1.5 mm and a mean pore diameter of 25 nm (Sabbani et al. 2006), were obtained from 3 M (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®.

**Immobilization of *C. rugosa* lipase on Accurel MP 1000**

**Scanning electron microscopy (SEM)**

The morphology of the Accurel MP 1000 support was investigated by fixing the samples on a ‘Stub’ holder then metallized, about 20–30 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.

**Immobilization of *C. rugosa* lipase**

The immobilization in an organic medium was adapted from Simões et al. (2011). Accurel MP 1000 (1 g) was immersed in hexane (15 mL) at a ratio of 1:10 (m/v) in a beaker (100 mL) and kept at −6 °C for 4 h in a vertical freezer. After freezing, 237 mg of commercial CRL powder extract (which 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 °C 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 unbound 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 (1976), using bovine serum albumin (BSA) as a standard (Ferreira et al. 2017), as shown in Eq. (1).

\[
IP = \frac{V_{enz} \times (C_0 - C_e)}{m}
\]

where *IP* is the immobilized lipase concentration (mg g⁻¹), *V_{enz}* is the volume of solution (mL), *C₀* 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).

**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 °C and 0.1 g of immobilized lipase was added. Following this, 10 mL of ethanol solution at 95% m/m

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![Fig. 1](https://example.com/fig1.png)

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

$$HA (U \cdot g^{-1}) = \frac{(V_S - V_c) \times M \times 10^3}{t \times m}$$  \hspace{1cm} (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 the 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\(_{sp}\) – Um g\(^{-1}\)) was calculated according to Eq. (3) (Teodoro et al. 2019):

$$A_{sp} (U \cdot mg^{-1}) = \frac{HA}{IP}$$  \hspace{1cm} (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\(^{-1}\)).

Application of CRL-AMP

**Esterification reaction**

During the selection process of the best synthesis conditions, tests were carried out under fixed experimental conditions: 45 °C e 200 rpm, according to Barbosa et al. (2021), to determine the acid/alcohol ratio. Three different conditions (1:2, 1:1 e 2:1) were analyzed using 5% CRL-AMP at 45 min. After the reaction period, the molar ratio (1:1) showed an increase of 35% in the ester conversion compared to the others, this condition was selected to continue the work, followed of the analysis of the concentration of CRL-AMP and reaction time. The esterification reactions between stearic acid and alcohols (lauryl and cetyl) were performed in closed Duran flasks (25 mL) at 45 °C 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 titrimetric 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 °C in a BOD incubator (TE-371, Tecnal, Piracicaba, Brazil) for 24 h. This process was performed six times, totalling six reaction cycles.

$$Conversion(\%) = \left(\frac{A_{fin} - A_{in}}{A_{in}}\right) \times 100$$  \hspace{1cm} (4)

where A\(_{in}\) and A\(_{fin}\) are the initial and final concentrations of fatty acid in the reaction medium (mmol L\(^{-1}\)).
Results and discussion

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. 2020a, b). Complete immobilization was confirmed by method of Bradford (1976).

The specific activity (A_sp) of the free and immobilized enzyme was determined, obtaining values of 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 50 Å x 42 Å x 33 Å (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 using Eqs. 5 and 6:

\[ R_{enz} = \sqrt[3]{\frac{3V_{enz}}{4\pi}} \]  
\[ D_{enz} = 2R_{enz} \]

where \( R_{enz} \) is the molecular radius, \( V_{enz} \) is the molecular volume and \( D_{enz} \) is the molecular diameter.

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 stabilizing agents, such as proteins, sugars, and salts, and when used 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 favours contact of the enzyme with the raw material, as well as increasing the rate of the reaction (Lage et al. 2016).

In synthesis 1 (Fig. 2; Table 1), using (10% CRL-AMP in mass of biocatalyst by volume of medium; m v⁻¹), a conversion of 62.5 ± 3.9% (Fig. 3) was obtained in 30 min of reaction, after which the

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**Table 1** Conversion of esters synthesized by CRL-AMP (5% and 10% m v⁻¹) at 40 °C and 200 rpm

| Reaction | C (%) | Time (min) | c (%) |
|----------|-------|------------|-------|
| 1        | 5     | 54         | 56.9 ± 2.8 |
| 10       | 30    | 62.5 ± 3.9 |
| 2        | 5     | 54         | 46.0 ± 2.4 |
| 10       | 24    | 79.0 ± 3.9 |

C (%) support concentration (m v⁻¹); c (%) conversion to ester.
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\(^{-1}\) obtained a conversion of 56.9 ± 2.8% (Fig. 3) in 54 min. 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, obtained with CRL-AMP 5% and 10% m\(^{-1}\), respectively. After the indicated times, the conversions remained constant, indicating that the reaction had reached chemical equilibrium. When the enzymatic concentration of catalyst was doubled (5–10% m\(^{-1}\)), there was an increase in the conversion and reduction in time, indicating the synthesis reaction was controlled by kinetics (not mass transfer) (Yadav and Devendran 2012).

The productivity (\(\mu\text{mol min}^{-1} \text{ mg}_{\text{protein}}^{-1}\)) was calculated as indicated by Da Silva et al. (2020a, b):

\[
P(\mu\text{mol min}^{-1} \text{ mg}_{\text{protein}}^{-1}) = \frac{N_a \times 10^3}{t_{\text{eq}} \times m}\]  

where \(N_a\) is the concentration of consumed carboxylic acid (mmol), \(t_{\text{eq}}\) is the reaction time in equilibrium, and \(m\) is the concentration of immobilized protein.
After the calculations, the productivities 2700 and 2670 \( \text{mol min}^{-1} \text{mg protein}^{-1} \) were found for 5% and 10% \( \text{m v}^{-1} \), respectively (synthesis 1) and 2180 and 4220 \( \text{mol min}^{-1} \text{mg protein}^{-1} \) for 5% and 10% \( \text{m v}^{-1} \), respectively (synthesis 2), noting that the reaction in synthesis 2 using CRL-AMP 10% \( \text{m v}^{-1} \) showed higher productivity (4220 \( \text{mol min}^{-1} \text{mg protein}^{-1} \)) 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 likely 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.

**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 and Van-Pelt 2013). Thus, the reuse of CRL-AMP at 5% and 10% \( \text{m v}^{-1} \) was performed in the synthesis of esters 1 and 2.

In synthesis 1, CRL-AMP 5% \( \text{m v}^{-1} \) retained 37.5% of the catalytic activity after the fourth cycle; while with 10% \( \text{m v}^{-1} \) 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% \( \text{m v}^{-1} \), 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.

It is possible to observe in synthesis 2 (Fig. 4b) that 10% of the enzyme proved to be less reusable than 5%. It is likely that successive washings with hexane between one cycle and another caused an increase in diffusive and barrier effects, decreasing the contact between substrate molecules and the enzyme, thereby slowing down the reaction.

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 C. rugosa lipase on MIL-101 chromium terephthalate and obtained a residual activity of 20–30% after the first cycle of reuse. This value was lower than that found in the present work in the fourth cycle, 46.5% residual activity in synthesis 1 with 10% \( \text{m v}^{-1} \) (Fig. 4a), and 69.4% and 33.5% in synthesis 2 with 5% \( \text{m v}^{-1} \) and 10% \( \text{m v}^{-1} \), respectively (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 four cycles. The results obtained in the present study, with activity retention close to 50% after five 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.
Conclusion

The immobilization of *C. rugosa* lipase on Accurel MP 1000 evaluated in this study showed high values of hydrolytic activity (1300 ± 100 U g⁻¹) and specific activity (200 ± 20 U mg⁻¹) 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. The stability of the CRL-AMP was verified by reuse tests, which were evaluated by six reaction cycles, obtaining better responses when using 10% m v⁻¹ of CRL-AMP for the dodecanoyl octadecanoate 1 and 5% m v⁻¹ for hexadecanoyl octadecanoate 2.

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Declarations

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