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In situ immobilization of Cal B enzyme in mesoporous materials of the MCM-48 type using different ionic solids: synthesis and application in esterification reactions

Imobilização *in situ* da enzima Cal B em materiais mesoporosos do tipo MCM-48 utilizando diferentes sólidos iônicos: síntese e aplicação em reações de esterificação

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Abstract
The ionic solids [C_{16}MI]Cl and [C_{14}MI]Cl were used as structure directors on MCM-48 mesoporous support with immobilized CALB lipase for the synthesis of esters from geraniol. Significant results were obtained in the synthesis of geranyl acetate and geranyl butyrate. Enzyme concentration, temperature and molar ratio of substrates were parameters that affect the synthesis yield. The highest temperature (60°C) and highest enzyme concentration (13%) conditions showed the best ester conversion results. For both esters, the ionic liquid [C_{16}MI]Cl showed better conversion results and reached a value above 38% of conversion. Furthermore, the support tested in the present study for application in ester synthesis is cost-effective. In addition, the enzymatic route to obtain the esters

presents mild reaction conditions and the results show potential for application in industrial applications.
Keywords: Ionic Solids. Synthesis of geranyl. Enzymatic route catalyst. Industrial applications.

Resumo
Os sólidos iônicos [C_{16}MI]Cl e [C_{14}MI]Cl foram utilizados como diretores de estrutura em suporte mesoporoso MCM-48 com lipase CALB imobilizada para a síntese de ésteres de geraniol. Resultados significativos foram obtidos na síntese de acetato de geranila e butirato de geranil. A concentração da enzima, a temperatura e a razão molar dos substratos foram parâmetros que afetaram o rendimento da síntese. As condições de maior temperatura (60°C) e maior concentração de enzima (13%) apresentaram os melhores resultados de conversão de ésteres. Para ambos os ésteres, o líquido iônico [C_{16}MI]Cl apresentou melhores resultados de conversão e atingiu valor acima de 38% de conversão. Além disso, o suporte testado no presente estudo para aplicação na síntese de ésteres é custo-efetivo. Além disso, a rota enzimática para obtenção dos ésteres apresenta condições reacionais brandas e os resultados mostram potencial para aplicação em aplicações industriais.
Palavras-chave: Sólidos Iônicos. Síntese de geranil. Catalisador de rota enzimática. Aplicações industriais.

1. Introduction

Mesoporous materials have gained prominence in research due to their diverse applicability, among them, the immobilization of enzymes is one of the most researched. Finding supports to fulfill this function that provide the best enzyme/support interaction is essential to achieve better results in reactions of any type. MCM-type materials have shown promise for enzyme immobilization, considering that the large pores of these materials allow bulky enzyme molecules to diffuse into them. Furthermore, the silanol groups present on the surface of MCMs facilitate the binding of the enzyme to the support through hydrogen bonds. Furthermore, the possibility of inserting the enzyme into a support with a well-defined design prevents its denaturation and can provide greater stability (Reato *et al*., 2023).

Esters find several industrial uses, being used as fuels, plasticizers, solvents, flavor and aroma compounds, and pharmaceuticals. Ester synthesis is traditionally based on the esterification of carboxylic acids with alcohol. The reaction takes place in the presence of inorganic catalysts at high temperatures (>100 °C), requires a large amount of energy and can be slow. In addition, acid catalysis becomes inefficient when the substrates used for the production of esters present unsaturations requiring the additional steps of protection and deprotection of the double bonds during the esterification reaction, increasing the cost and time of the process (Sbardelotto *et al*., 2018). Because of these drawbacks, the study of new and more efficient methods for flavor esters synthesis processes is necessary. The use of lipases as biocatalysts to obtain esters has several advantages, such as milder temperature conditions, high productivity, selectivity, in addition to being able to characterize the products obtained as natural flavors (Basso & Serban, 2019; Filho *et al*., 2019; Quayson *et al*., 2020).

Esters produced from monoterpene alcohols have a wide range of applications due to their flavoring properties. Geraniol is a low-toxicity monoterpene alcohol found naturally in essential oils of several aromatic plants used as a natural agent in pest control and this property limits its use in the food and cosmetics industry. However, the esterification of geraniol can overcome this deficiency, altering olfactory characteristics and biological properties (Bahadori *et al*., 2020; Zeferino *et al*., 2021). Among the geraniol esters, geraniol acetate, propionate and butyrate are the most sought after. Geranyl butyrate ($C_{14}H_{24}O_2$, 224.34 g/mol) is found in nature has a fruity, cherry-like odor, and is a cosmetic product used in lipsticks and soaps. Geranyl acetate ($C_{12}H_{20}O_2$, 196.29 g/mol) is the most common and valuable ester of geraniol. It has a fruity flavor of sweet lemon and a fragrance of rose and lavender. These esters can be obtained by extraction from plants or chemical

synthesis. However, enzymatic routes have become attractive and the use of enzymes immobilized on/in different supports has drawn attention. For this purpose, lipases are used (Bourkaib *et al*., 2020; Murcia *et al*., 2018; Zeferino *et al*., 2021).

Regarding the choice of lipase for the present study, Candida antarctica type B (CALB) stands out in terms of the efficiency of its applications and is well established for biocatalysis purposes. Our research group successfully immobilized CALB on the MCM-48 structure using the ionic solids [C_{16}MI]Cl (1-hexadecyl-3-methylimidazolium chloride) (Battiston *et al*., 2017) and [C_{14}MI]Cl (1-tetradecyl-3-methylimidazolium chloride) (Battiston *et al*., 2022) structure-directing agents. However, no studies were found in the literature regarding the use of CALB immobilized on MCM-type supports in the synthesis of geranyl acetate and geranyl butyrate. Furthermore, the synthesis of esters from geraniol using CALB immobilized *in situ* in a mesoporous support of the MCM-48 type using the ionic solids [C_{16}MI]Cl and [C_{14}MI]Cl as structure directors is unprecedented.

2. Material and methods

For the synthesis of ionic solids, the following were used: 1-methylimidazolium (99%, Aldrich), 1-chlorotetradecane (98%, Aldrich), 1-chlorohexadecane (95%, Aldrich), acetonitrile (99.5%, Vetec) and ethyl (99.5%, Vetec). For the synthesis of the supports, the following were used: tetraethylorthosilicate (TEOS) (98%, Aldrich), ammonium hydroxide P.A (Quimex), ethyl alcohol P.A (99.5%, Merck) and deionized water.

The enzyme used in immobilization was the commercial lipase from Candida antarctica type B (Novozyme).

To determine the esterification activity, the following were used: ethyl alcohol P.A (99.5%, Merck), acetone (99.5%, Merck), oleic acid (65-88%, Synth), sodium hydroxide (micro bead P.A, CRQ) and distilled water.

For the synthesis of esters, the following were used: geraniol (97%, SAFC), acetic acid (99.8%, Aldrich), butyric acid (99%, Vetec), 4 Å molecular sieves (Sigma-Aldrich) and distilled water.

2.1. Synthesis of MCM-48 Mesoporous Material and in situ Immobilization

The synthesis of the ionic solids [C_{16}MI]Cl (1-hexadecyl-3-methylimidazolium chloride) and [C_{14}MI]Cl (1-tetradecyl-3-methylimidazolium chloride) and use in the immobilization of CALB in MCM-48 was performed as previously described by (Battiston *et al*., 2022).

One of the main advantages of using an immobilized enzyme is its reuse. Thus, it is necessary to prevent its leaching as much as possible. With this in mind, the *in situ* immobilization method was used. In this way, the enzyme is retained within the pore of the support, as the immobilization occurs simultaneously with the formation of the crystalline network of the material.

The synthesis of the MCM-48 material was performed according to the methodology described in literature, with adaptations (Kumar *et al*., 2001). The ionic solid was diluted in 25 ml of deionized water under magnetic stirring, according to the concentrations defined in the experimental design. After dilution, also according to the experimental design, the mass defined of the enzyme was added into the system followed by more 25 mL of deionized water. 50 ml of absolute ethanol and 12 ml of ammonium hydroxide (NH_4OH) were added to the reaction medium and the system was stirred for 10 min. Then, 3.4 g of tetraethylorthosilicate (TEOS) were added and the system was stirred for 2 h at room temperature (22 °C). Finally, the support was washed by centrifugation with aliquots of deionized water until neutral pH, and kept at rest at room temperature for 24 h.

Specific area

The analysis of specific area was carried out according to the BET method (Brunauer *et al*., 1938). To characterize the porosity of the support without enzyme, approximately 200 mg of sample was subjected to the calcination process at 700 °C for 420 min. Prior to adsorption, 20 to 50 mg of

each calcined sample were degassed for 12 h at 350 ºC under vacuum. For the support with enzyme, 8 h at 80 ºC was used as a pretreatment. The nitrogen adsorption isotherms were obtained at 77 K (Nova 2200e, Quantachrome).

X-ray diffraction analysis

X-ray diffraction analysis (XRD) was used to confirm the formation of the ordered structures of MCM-48 materials obtained through characteristic peaks. The X-ray diffraction patterns were collected in a diffractometer with a Siemens D500 type goniometer, with a copper emitting tube that emits K alpha particles (λ = 1.54 Å). The generator operates at a voltage of 40 kV and a current of 17.5 mA. The scan was performed at a rate of 0.05º/2s.

2.2. Synthesis of esters from geraniol

To analyze the influence of the independent variables of the process on the synthesis of esters using geraniol alcohol and acetic/butyric acids, a central composite design (DCC 2^3) was used. The variables studied were molar ratio (alcohol/acid), concentration of the immobilized enzyme (% m/m in relation to the substrate), and temperature (ºC). Table 1 shows the variables and levels studied for the synthesis of geranyl acetate and geranyl butyrate.

Enzymatic esterification reactions were carried out with the preparation of a solution of geraniol and acetic acid (geranyl acetate) or geraniol and butyric acid (geranyl butyrate), according to molar ratios defined in the experimental designs (Table 1). To start the reaction, the enzyme immobilized on the supports MCM-48[C_{16}MI]Cl or MCM-48[C_{14}MI]Cl was added to 5 mL of the solution with the previously established molar ratio. 15% (w/w) of immobilized enzyme was added in relation to the substrate.

Table 1 – Independent variables and levels of variation (real and coded values) for the synthesis of geranyl acetate and butyrate

Independent variables	Levels		
	-1	0	+1
Molar ratio (alcohol/acid) - X1	1:1	3:1	5:1
Immobilized enzyme concentration (%) - X2	7	10	13
Temperature (ºC) - X3	40	50	60

The experiments were carried out in closed glass flasks, with constant orbital agitation at 180 rpm for 6 h at the temperature established in the experimental design. Afterwards, 500 µL were removed from the reaction medium and 15 mL of acetone-ethanol solution (1:1) (v/v) were added. The reaction yield was determined by titration with 0.05 M sodium hydroxide (NaOH) to pH 9.3 for geranyl acetate and up to pH 9.4 for geranyl butyrate. Assays of sample controls were performed using 500 µL of the geraniol and acetic/butyric acid solution and 15 mL of the acetone-ethanol solution. All assays were performed in triplicate.

3. Results and discussion

3.1. Isotherms and XRD

The N_2 adsorption-desorption isotherms, specific surface area, specific pore volume (Vp) and diameter (dp) of the support with MCM-48[C_{14}MI]Cl or MCM-48[C_{16}MI]Cl were determined from nitrogen adsorption-desorption measurements, and are shown in Figure 1 and Table 2.

The support MCM-48[C_{14}MI]Cl (Figure 1) showed a type II isotherm, in which the relative pressure varies little and the volume increases, indicating the formation of adsorbent materials with capillary(Teixeira *et al*., 2001). The support MCM-48[C_{16}MI]Cl presented the same form in relation to the adsorption-desorption isotherm.

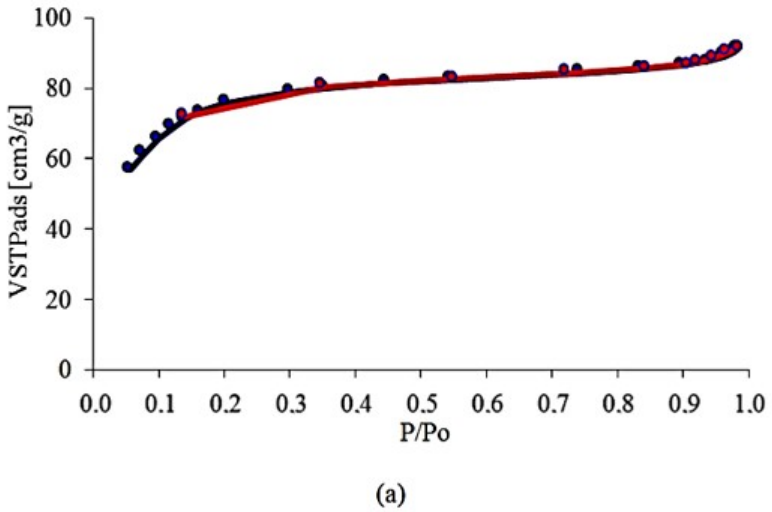


Figure 1 – Textural analysis of N_2 adsorption/desorption for MCM-48 with MCM-48[C_{14}MI]Cl.

Table 2 – Characteristics of the MCM-48 [C_{14}MI]Cl or [C_{16}MI]Cl support.

Support	Specific area ($m^2.g^{-1}$)	Pore volume ($cm^3.g^{-1}$)	Pore size (Å)
MCM-48[C_{14}MI]Cl	312	0.14	20.30
MCM-48[C_{16}MI]Cl	286	0.13	23.19

According to Table 2, all samples analyzed are classified as mesoporous materials according to IUPAC (IUPAC, 1985). Figure 2 shows the resulting diffractogram, indicating the peaks corresponding to both MCM-48[C_{14}MI]Cl or with MCM-48[C_{16}MI]Cl.

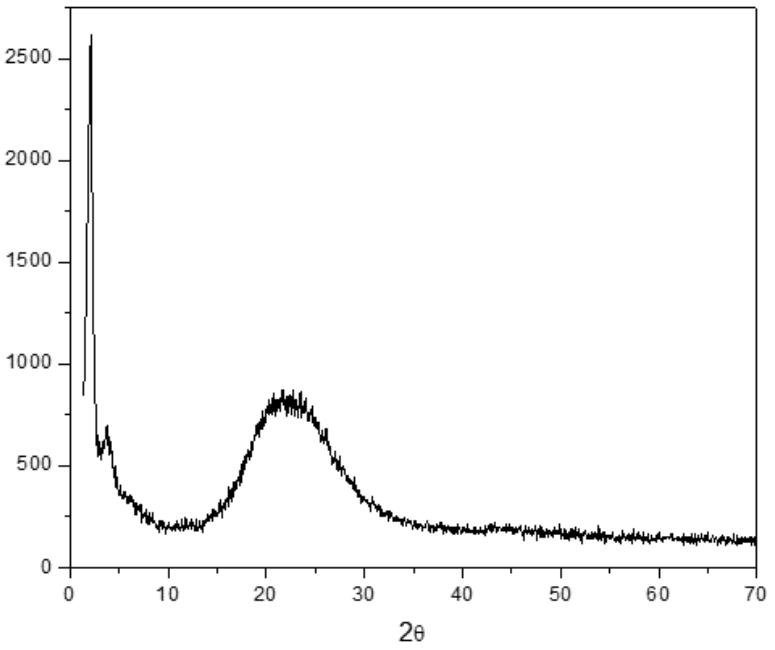


Figure 2 – X-ray diffraction analysis of MCM-48 with [C_{14}MI]Cl.

In the diffractogram (Figure 2), there are peaks that characterize the material as crystalline. In comparison with the literature, the position of all peaks identifies the phase obtained as being characteristic of the pure and crystalline MCM-48 mesoporous material (Bordin *et al*., 2021; Kim & Ryoo, 1998). For the sample MCM-48[C_{16}MI]Cl and also when in the presence of the immobilized enzyme, both with [C_{14}MI]Cl and[C_{16}MI]Cl, the X-ray diffractograms obtained showed the same X-ray diffraction pattern.

3.2. Synthesis of Geranyl Acetate

Table 3 presents the matrix of the design of experiments with the real and coded values of the independent variables, as well as the conversions to geranyl acetate and geranyl butyrate using the enzyme immobilized on the two supports studied.

As can be seen (Table 3), the enzyme immobilized on MCM-48 [C_{16}MI]Cl support showed greater responses in terms of yields to catalyze the production of the two geraniol esters, obtaining better responses for the production of geranyl acetate. This behavior can be attributed to the structure of the CALB binding site, which has a tapered elliptical shape, with a narrow slit. Thus, as the number of carbons of the acylating agent increases, there begins to be a certain steric hindrance, resulting in low efficiency of the enzymatic reaction(Pleiss *et al*., 1998). A similar trend was observed when evaluated the influence of the fatty acid chain on the formation of rutin esters and obtained a gradual decrease in conversion rates as the carbon chain of the acids studied increased (Viskupicova *et al*., 2010).

Table 3 – Matrix of DCC 2^3 (coded and real values) and responses in term of conversion of geranyl acetate and geranyl butyrate.

Run	X_1	X_2	X_3	Conversion (%)			
				IE MCM-48 [C_{14}MI]Cl		IE MCM-48 [C_{16}MI]Cl	
				Geranyl acetate	Geranyl butyrate	Geranyl acetate	Geranyl butyrate
1	-1 (1:1)	-1 (7)	-1 (40)	10,0	5,9	19,8	9,2
2	1 (5:1)	-1 (7)	-1 (40)	22,7	10,5	17,8	9,5
3	-1 (1:1)	1 (13)	-1 (40)	15,9	13,5	26,9	12,0
4	1 (5:1)	1 (13)	-1 (40)	24,3	18,7	22,0	19,6
5	-1 (1:1)	-1 (7)	1 (60)	18,5	9,4	25,9	12,0
6	1 (5:1)	-1 (7)	1 (60)	26,3	17,9	20,1	15,4
7	-1 (1:1)	1 (13)	1 (60)	32,5	12,4	42,5	13,8
8	1 (5:1)	1 (13)	1 (60)	29,4	18,0	38,6	22,9
9	0 (3:1)	0 (10)	0 (50)	19,3	13,2	30,2	17,5
10	0 (3:1)	0 (10)	0 (50)	19,0	13,2	30,2	17,1
11	0 (3:1)	0 (10)	0 (50)	19,1	12,9	30,2	17,1

X1: molar ratio (alcohol/acid); X2: immobilized enzyme concentration (wt.%); X3: temperature (ºC) IE: immobilized enzyme

It can be seen (Table 3) that the immobilized enzyme on the different supports showed similar behavior depending on the levels studied, with the highest conversion rates shown in assay 7, where the enzyme concentration and temperature are at their highest levels.

Independently analyzing each variable, it is observed, for both supports, that the increase in temperature (assays 1 and 5, 2 and 6, 3 and 7, 4 and 8), regardless of the molar ratio and enzyme concentration, provided an increase of conversion to geranyl acetate. This tendency can be explained by the endothermic nature of the esterification reactions. Positive enthalpies (ΔH) were observed by different authors for the enzymatic synthesis of esters, such as isoamyl oleate(Lage *et al*., 2016),

hexyl levulinate (Badgujar & Bhanage, 2014), geranil butanoate (Sbardelotto *et al*., 2018) and in the acylation of the flavonoid rutin with lauric acid (Razak & Annuar, 2015), using the lipases *Candida antarctica B* and *Thermomyces lanuginosus* immobilized on different supports. The endothermic character of the esterification reactions indicates that it occurs with heat absorption and that the increase in temperature will provide a shift in the equilibrium reaction system for the formation of products, thus increasing its yield. Temperature may also be contributing to mass transfer, mainly due to the decrease in density and viscosity of the reaction medium. The increase in temperature provides an increase in intermolecular distances, thus decreasing the attractive forces between the molecules and, consequently, the viscosity, which varies proportionally with the force of attraction between the molecules.

Regarding the molar ratio variable, some studies show that excess alcohol positively interferes with the conversion processes (Azudin *et al*., 2013; Chiaradia *et al*., 2012), similar to that observed in this study, when this variable is analyzed independently. The improvement in the conversion results is related to the decrease in acidity in the medium and the balance shift due to excess alcohol. As it is a reversible reaction (esterification), excess alcohol alone can positively affect the conversion process, by shifting the equilibrium towards the products, that is, towards the production of the ester. On the other hand, there are also studies that report that there is a limit for the addition of alcohol, as excess above the critical level results in lower conversion rates due to the formation of bonds between the alcohol and the active site of the enzyme (Güvenç *et al*., 2002).

Another extremely important variable is the mass of catalyst used. For this variable, analyzed independently (assays 1 and 3, 2 and 4, 5 and 7, 6 and 8), an increase in the conversion of geranyl acetate was observed as the enzyme concentration increased. This increase in conversion was expected and is related to the increase in the number of active sites present in the reaction medium provided by the increase in the enzyme concentration. A study also observed that increasing the mass of catalyst used in the reaction directly and positively affects the conversion process in esterification reactions (Salum *et al*., 2008).

Considering the conditions of the central point, there is a better performance in the production of geranyl acetate, with a conversion of 30%, for the immobilized derivative enzyme/MCM-48[C_{16}MI]Cl. In the same tests, for the immobilized derivative enzyme/MCM-48[C_{14}MI]Cl the conversion was 17%. The results presented in this study follow the same trend of the results obtained for the esterification activity from the optimal conditions described by the mathematical models, when the conditions of the studied supports were optimized. Thus, it is possible to relate the best results presented for the enzyme immobilized on the MCM-48[C_{16}MI]Cl support with the largest size of the carbon chain of the ionic solid, which increases its hydrophobic character and improves the efficiency of the enzymatic activity, allowing the formation of a hydration layer around the enzyme and consequently favoring the lipolytic activity, inducing the water closer of the enzyme.

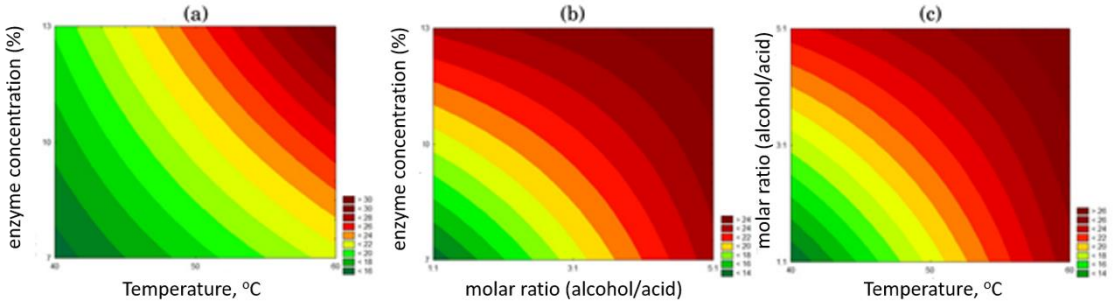
From the results obtained in the analysis of variance (Table 4), it is possible to observe the validation of both plans, as they presented values of $F_{calculated}$ (8,47 for enzyme immobilized in MCM-48[C_{14}MI]Cl and 13,18 for the enzyme immobilized in MCM-48[C_{16}MI]Cl) higher than the $F_{tabulated}$ (6,16), with coefficients of determination (R^2) greater than 0,93.

All factors evaluated were statistically significant ($p \leq 0,05$) with regard to the conversion of geranyl acetate. Analyzing the regression coefficients (Table 4) for the sample where the enzyme was immobilized on the MCM-48[C_{14}MI]Cl support a positive effect is observed for all variables. As for the interaction between the variables, there is a negative effect for the molar ratio x enzyme concentration and for the molar ratio x temperature. For the interaction between enzyme concentration x temperature, the effect is positive. For the MCM-48[C_{16}MI]Cl support, there is a positive effect for the variables immobilized enzyme concentration and temperature and a negative effect for the molar ratio. The interaction between the variables showed the same tendency of the enzyme immobilized on the MCM-48[C_{14}MI]Cl support, with positive effect only for interaction between enzyme concentration x temperature.

The data obtained allow the construction of contour curves (Figure 3) referring to the interaction of the variables for the enzyme immobilized in the two studied supports, thus enabling

the analysis of the trend of the most appropriate conditions and that maximize the response, using the additive C_{14}MI.Cl.

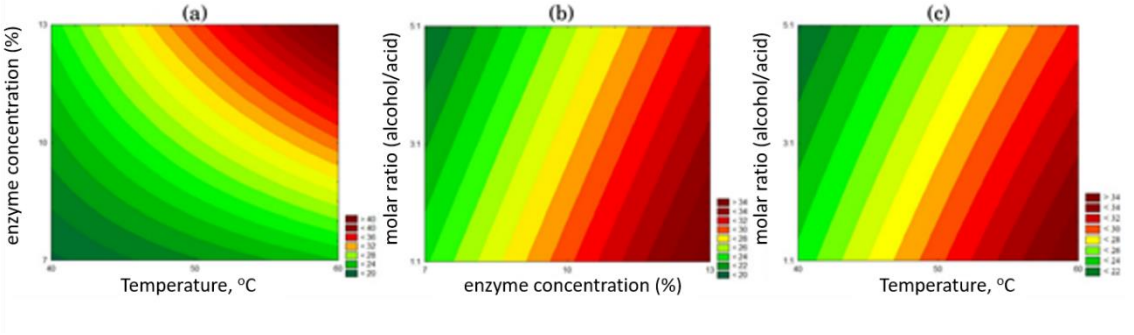
Figure 3 – Contour curve for the conversion of geranyl acetate from immobilized enzyme in MCM-48[C_{14}MI]Cl



Source: Authors (2023)
Subtitle: (a) Geranyl acetate conversion rates as a function of enzyme concentration (%) and temperature (ºC); (b) molar ratio (alcohol/acid) and enzyme concentration (%); (c) molar ratio (alcohol/acid) and temperature (ºC).

Analyzing Figure 2a, which represents the effect of the interaction between the enzyme concentration x temperature variables, an increase in the values of both variables leads to higher values of the conversion of geranyl acetate. The same occurs in Figure 2, 2b and 2c, which indicate the effect of the enzyme concentration x molar ratio variables and molar ratio x temperature, respectively. Figure 3 shows the effects of interactions using the additive C_{16}MI.

Figure 3 – Contour curve for the conversion of geranyl acetate from immobilized enzyme in MCM-48[C_{16}MI]Cl



Source: Authors (2023)
Subtitle: (a) Geranyl acetate conversion rates as a function of enzyme concentration (%) and temperature (ºC); (b) molar ratio (alcohol/acid) and enzyme concentration (%); (c) molar ratio (alcohol/acid) and temperature (ºC).

Analyzing Figure 3a, which represents the effect of the interaction between the enzyme concentration x temperature variables, an increase in the values of both variables leads to higher values of the conversion of geranyl acetate. For Figures 3b and 3c, which indicate the effect of the enzyme concentration x molar ratio and molar ratio x temperature variables, respectively, it can be seen that the highest conversion rates are presented at the highest levels of enzyme concentration and temperature, however at the lowest level of molar ratio.

Some studies presented in the literature regarding the production of geranyl acetate ester will be exposed next: using polymer-immobilized lipase from *Pseudomonas cepacia*, it was obtained

99% of conversion after 3 h of reaction at 55 ºC, in the study a high catalyst load was used (Badgujar & Bhanage, 2014). Geranyl acetate was synthesized catalyzed by lipase *Thermomyces lanuginosus* immobilized on nanofiber membranes, by adsorption and covalent bonding, and obtained conversion rates of 90 and 66%, respectively (Gupta *et al*., 2013). *Candida rugosa* lipase was immobilized on zinc oxide nanoparticles and used as a catalyst in the synthesis reaction of geranyl acetate and it was obtained 94% of conversion after 6 h of reaction(Patel *et al*., 2016). Although the results presented for the conversion of geranyl acetate are lower than those reported in the literature, they are significant and promising, as to date there are no published works that report the use of immobilized lipase in supports similar to those studied. However, it can be seen that different conversion rates in the ester synthesis are mainly related to the characteristics of the enzyme, the support used and the immobilization technique, in addition, the parameters used in the process also exert a great influence, namely, temperature, molar ratio of reagents, enzyme concentration and reaction time.

3.3. Synthesis of Geranyl Butyrate

In Table 4 presents the planning matrix with the real and coded values of the independent variables, as well as the conversions of geranyl butyrate using the enzyme immobilized on the two supports studied.

Table 4 – DCC 2^3 matrix (values and coding) with geranyl butyrate conversion responses.

Experiment	X_1	X_2	X_3	Conversion (%)	
				IE MCM-48 [C_{14}MI]Cl	IE MCM-48 [C_{16}MI]Cl
1	-1 (1:1)	-1 (7)	-1 (40)	5,9	9,2
2	1 (5:1)	-1 (7)	-1 (40)	10,5	9,5
3	-1 (1:1)	1 (13)	-1 (40)	13,5	12,0
4	1 (5:1)	1 (13)	-1 (40)	18,7	19,6
5	-1 (1:1)	-1 (7)	1 (60)	9,4	12,0
6	1 (5:1)	-1 (7)	1 (60)	17,9	15,4
7	-1 (1:1)	1 (13)	1 (60)	12,4	13,8
8	1 (5:1)	1 (13)	1 (60)	18,0	22,9
9	0 (3:1)	0 (10)	0 (50)	13,2	17,5
10	0 (3:1)	0 (10)	0 (50)	13,2	17,1
11	0 (3:1)	0 (10)	0 (50)	12,9	17,1

X1: molar ratio (alcohol/acid); X2: immobilized enzyme concentration (%); X3: temperature (ºC)
IE: immobilized enzyme

From Table 4, it is possible to observe that in both supports the highest conversion rates were presented in assays 4 (18.7% for IE MCM-48[C_{14}MI]Cl and 19.6% for IE MCM-48[C_{16}MI]Cl) and 8 (18.0% for IE MCM-48[C_{14}MI]Cl and 22.9% for IE MCM-48[C_{16}MI]Cl), where the enzyme concentration is at its highest levels.

Analyzing each variable independently, a similar behavior is observed for the molar ratio for both immobilized derivatives (assays 1 and 2, 3 and 4, 5 and 6, 7 and 8), showing an increase in conversion with increasing molar ratio (alcohol/acid). This behavior is similar to that observed in the synthesis of geranyl acetate and was linked to the positive effect of excess alcohol, which acts by decreasing the acidity of the medium and shifting the balance towards the products.

Regarding the concentration of the enzyme for both immobilized derivatives, as occurred for the synthesis of geranyl acetate, there is an increase in the ester conversion with the addition of the values of this variable (assays 1 and 3, 2 and 4, 5 and 7, 6 and 8). This trend is associated with the increase in active sites, provided by the increase in the concentration of the enzyme.

The temperature variable, analyzed independently, showed a different behavior among the immobilized derivatives. While for the enzyme immobilized on the MCM-48[C_{16}MI]Cl support, the temperature had a positive effect for all conditions analyzed (assays 1 and 5, 2 and 6, 3 and 7, 4 and 8), for the enzyme immobilized on the MCM-48[C_{14}MI]Cl support, the temperature only provided a positive effect for the lowest enzyme concentrations, that is, for the samples with 7% (assays 1 and 5, 2 and 6), regardless of the molar ratio. For the enzyme concentration equivalent to 13%, there was practically no temperature effect.

Considering the conditions of the central point, a better performance was observed in the production of geranyl butyrate, with a conversion of 17% to the immobilized derivative enzyme/MCM-48[C_{16}MI]Cl. In the same assays, for the immobilized enzyme/MCM-48[C_{14}MI]Cl derivative the conversion was 13%. This tendency was also observed in the synthesis of geranyl acetate and in the esterification activities when the conditions of the studied supports were optimized. This behavior is attributed to the increase in the size of the carbonic chain of the ionic solid.

The data thus allow the construction of the contour curves presented in Figure 4 referring to the interaction of the variables for the enzyme immobilized in the two studied supports, thus enabling the analysis of the trend of the most appropriate conditions that maximize the response, using the additive C_{14}MI.Cl.

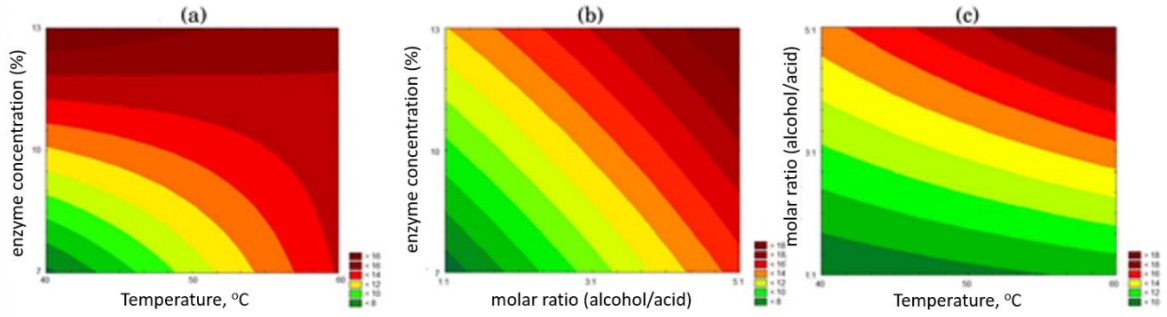


Figure 4 – Contour curve for the conversion of geranyl butyrate from the enzyme immobilized in MCM-48[C_{14}MI]Cl

Source: Authors (2023)
Subtitle: (a) Geranyl butyrate conversion rates as a function of enzyme concentration (%) and temperature (ºC); (b) molar ratio (alcohol/acid) and enzyme concentration (%); (c) molar ratio (alcohol/acid) and temperature.

Analyzing Figure 4a, which represents the effect of the interaction between the enzyme concentration x temperature variables, it can be seen that the highest conversion rates are presented at the highest levels of enzyme concentration. For temperature, the effect on conversion (%) is more pronounced at lower enzyme concentrations. As the enzyme concentration increases, the effect of temperature becomes smaller, presenting to the upper extreme, with 13%, in a region where we see practically no effect of temperature on yield.

For Figures 4b and 4c, which indicate the effect of the enzyme concentration x molar ratio and molar ratio x temperature variables, respectively, an increase in the values of both variables leads to higher values of geranyl butyrate conversion. Figure 5 shows the effects of interactions using the additive C_{16}MI.Cl.

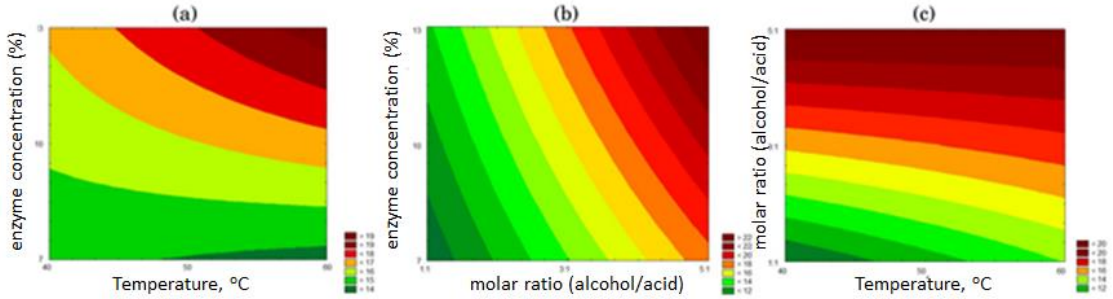


Figure 5 – Contour curve for the conversion of geranyl butyrate from the enzyme immobilized in MCM-48[C_{16}MI]Cl

Source: Authors (2023)
Subtitle: (a) Geranyl butyrate conversion rates as a function of enzyme concentration (%) and temperature (ºC); (b) molar ratio (alcohol/acid) and enzyme concentration (%); (c) molar ratio (alcohol/acid) and temperature (ºC)

Analyzing Figures 5a and 5a, which represent the effect of the interaction between the enzyme concentration x temperature and enzyme concentration x molar ratio variables, respectively, it can be seen that the highest conversion rates are presented at the highest levels of all variables. For Figure 5c, which indicates the effect of the molar ratio x temperature variables, an increase in the molar ratio values in all studied temperature ranges presents the best conversion rates.

To our knowledge, to date, there are no works published in the literature that report the synthesis of geranyl butyrate using lipase immobilized on mesoporous supports for comparison, however, some works also using lipases for synthesis of said ester will be presented. Applying Candida rugosa lipase immobilized in commercial polymers it was obtained 99% of conversion to geranyl butyrate after 48 h of reaction in a batch process and 78.9% conversion after 10 h of reaction in a fluidized bed reactor (Damnjanović *et al*., 2012). The influence of water activity on the conversion of geranyl butyrate catalyzed by lipase Mucor miehei was analyzed and it was obtained 75% yield after 75 h of reaction (Karra-Chaabouni *et al*., 2002). For the synthesis of this ester, variables such as catalyst mass, temperature and molar ratio will also influence the results obtained. The conversion values obtained in this study are important and promising for the synthesis of geranyl butyrate.

Regarding the effect of the carbon number of the acid chains (acetic acid – $C_2H_4O_2$ and butyric acid - $C_4H_8O_2$) in the formation of the studied esters, it is observed that the highest conversions were achieved for the shorter chain acid (acetic acid), regardless of the immobilized derivative used. This behavior can be attributed to the structure of the CALB binding site, which has a tapered elliptical shape, with a narrow slit. Thus, as the number of carbons of the acylating agent increases (from 2 to 4), there begins to be a certain steric hindrance, resulting in low efficiency of the enzymatic reaction (Pleiss *et al*., 1998). A similar trend was observed when evaluating the influence of the fatty acid chain on the formation of rutin esters and obtained a gradual decrease in conversion rates as the carbon chain of the acids studied increased (Viskupicova *et al*., 2010).

4. Conclusions

In this study, the application process of Candida antarctica B lipase immobilized on a MCM-48 type mesoporous support synthesized with two ionic solids, 1-hexadecyl-3methylimidazolium chloride ([C_{16}MI]Cl) and 1-tetradecyl-3methylimidazolium chloride ([C_{14}MI]Cl) was studied. The results obtained in this work showed that the immobilization by the *in situ* technique was effective,

allowing the entrapment of the enzyme inside the crystalline lattice of the material and consequently hindering its leaching into the medium. In addition, the MCM-48 type mesoporous material is efficient and promising for the immobilization of CALB due to its large surface area, thermal stability and easy recovery of the reaction medium.

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