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Enzymatic Synthesis of Thymol Octanoate, a Promising Hybrid Molecule

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Abstract: Interest in the synthesis and application of thymol esters has increased in recent years due to the numerous applications associated with its biological activities. The enzymatic synthesis of thymol octanoate by esterification of thymol and octanoic acid was explored using soluble lipases and immobilized lipase biocatalysts in solvent-free systems. *Candida antarctica* lipase B in its soluble form was the most active biocatalyst for this reaction. Different thymol and lipase feeding strategies were evaluated to maximize thymol octanoate production. The results suggest that there could be lipase inhibition by the ester product of the reaction. In this way, the optimal reaction condition was given using a thymol/acid molar ratio of 1:4 mol/mol. Under these conditions the conversion of thymol was close to 94% and the lipase maintained more than 90% of its initial activity after the reaction, showing the potential of the enzyme to be used in successive reaction cycles.

Keywords: thymol octanoate; enzymatic synthesis; biocatalysts; *Candida antarctica* lipase B

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1. Introduction

Natural products are widely demanded due to their content of bioactive molecules [1] and play an important role in drug development, there being a significant number of drugs obtained from them that are currently marketed [2]. Recently, thymol and its derivatives have gained a lot of attention due to their various biological functions, their phyto-pharmaceutical applications, and as a food preservative [1].

Thymol (2-isopropyl-5-methylphenol) is the main monoterpene phenol found in essential oils extracted from plants belonging to the Lamiaceae family, such as those of the genera Thymus, Ocimum, Origanum, Satureja, Thymbra, and Monarda [3–7]. Thymol has antioxidant [8–10], anti-inflammatory [11–13], antibacterial [14–16], antifungal [17], antiparasitic [18,19], mosquito repellent [20], and larvicide [21] properties, among many others [22–24].

Occasionally, the use of thymol is limited due to low palatability (unpleasant taste and odor) [25], moderate cytotoxicity for human and animal cells [26,27], reduced passive transport through membranes due to its hydrophobicity [28], high volatility [29], and problems of dissolution in water, emulsification, and chemical stability [30], among others.

The esterification of terpenic alcohols, and in particular thymol, has solved several of these problems and, at the same time, enhanced the biological activity [1,31–35]. Thymol esters with antifungal [36,37], antibacterial [28,38], antiparasitic [19–39], larvicidal [40,41], anti-inflammatory [42,43], antinociceptive [44,45], and anticonvulsant [46] activity have been reported. In addition to increased activity, thymol derivatives showed low toxicity [28,39,44], fewer adverse effects [42,43], higher lipophilicity [28,43], lower volatility [28], and an easier transport through membranes [33,47].

Thymol esters and carboxylic acids (linear and/or branched) have been extensively studied. Figure 1 presents some of these thymol derivatives. The esters of thymol and carboxylic acids showed greater activity and stability than the starting compounds, while their toxicity was reduced. Esterification considerably reduces the odor and taste of thymol and, on the other hand, protects the carboxyl group, reducing unwanted effects of acids.

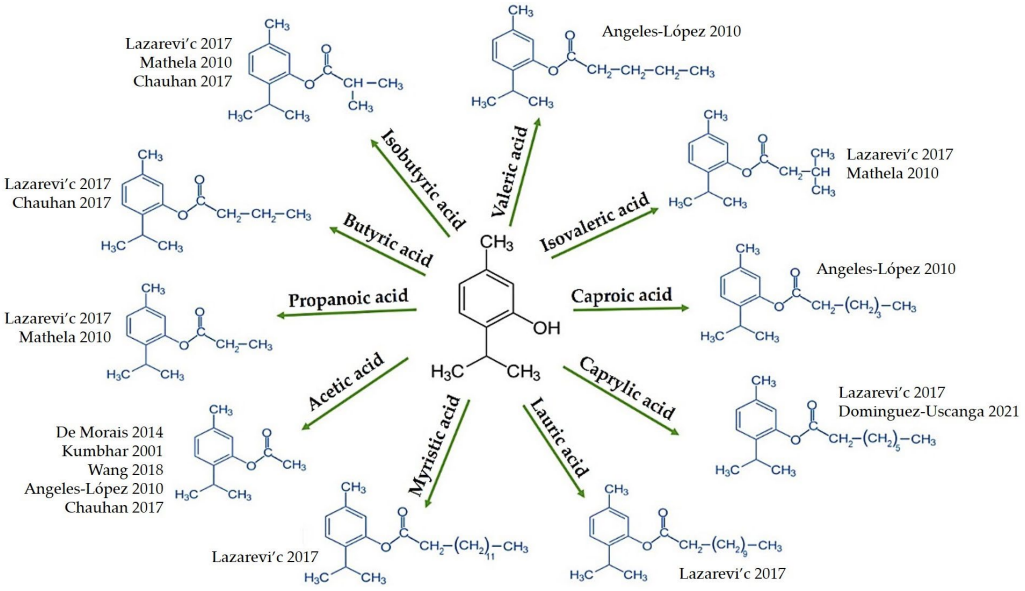

Figure 1. Main esters with biological activity obtained by esterification of thymol and linear and branched carboxylic acids [19,28,36–39,44,48].

Terpene esters have been shown to be active before undergoing enzymatic hydrolysis [46]. On the other hand, the hydrolysis of the ester bond allows the gradual release of the esterified compounds [49], and consequently a gradual release of the activity. These compounds can be considered prolonged-release prodrugs [49].

Even though the interest in the synthesis of thymol esters has grown remarkably, the reaction methodologies have not been updated; however, three basic approaches and some slight modifications of these have been reported. Thymol esters have been obtained from carboxylic acid chlorides dissolved in dichloromethane (CH_2Cl_2). This solution is slowly added to a solution of thymol and NaOH [36,44]. A modification of this methodology involves the use of triethylamine (Et_3N) as the base instead of NaOH [28,37,38]. Steglich esterification [50] has been used for the synthesis of a large number of thymol esters. In this methodology, thymol and the corresponding carboxylic acid are dissolved in CH_2Cl_2, the addition of *N*,*N*′-dicyclohexylcarbodiimide (DCC) allows the activation of the carboxylic group by increasing the electrophilicity of the carbonyl, and 4-dimethylaminopyridine (DMAP) is used as the catalyst [41,42,46,47]. A modification of this synthesis methodology involves the use of the carboxylic acid anhydride and thymol in a solution of Et_3N, DMPA as the catalyst, and CH_2Cl_2 as the reaction solvent [41]. The reaction between chlorides or anhydrides of the corresponding carboxylic acid and thymol, dissolved in pyridine, has been reported as the third form of synthesis of thymol esters. In this case, pyridine would act as the solvent and catalyst of the reaction [19,48].

As mentioned, thymol esterification has only been studied chemically, using highly corrosive, irritating, allergenic, and/or toxic compounds that are dangerous to human health and the environment [36–38,41,42,46–48]. The processes for the separation and purification of thymol esters involve the use of chromatography and/or hazardous solvents [43,45–47], which makes them impractical on other scales.

Enzymatic esterification is an extremely attractive methodology due to the mild reaction conditions and the lesser or null generation of by-products. The enzymatic

esterification of phenolic alcohols has been scarcely studied, contrary to what occurs with phenolic acids (phenolcarboxylic acids) [51–53]. However, some phenol esters have been obtained using free and/or immobilized lipases with promising results. Tyrosol and hydroxytyrosol esters were synthesized with yields ranging from 25 to more than 90% using Novozym 435 [54–57]. Vanillic alcohol has been esterified with different fatty acids with yields greater than 70% [58,59]. Capsaicin esters and capsaicin analogs were obtained with yields between 25 and 80% depending on the synthesis conditions and using lipase B from *Candida antarctica* (CALB) [60]. On the other hand, the synthesis of *m*- and *p*-cresol esters was carried out successfully using organic acids with chain lengths between C2 and C18 and lipase from porcine pancreas and *Rhizomucor miehei* lipase (RML) [61]. Esters of alcohols derived from catechol were synthesized using Novozym 435 [62,63]. Recently, the esterification of different phenolic compounds and hexanoic acid was carried out with CALB and the effect of the substituents in the aromatic ring on the biological activity of the obtained esters was evaluated [64]. In this work, the enzymatic esterification of thymol and octanoic acid was studied for the first time to obtain a hybrid molecule (or mutual prodrug) [65,66] that combines and enhances the properties of thymol and the antimicrobial activity of the octanoic acid [67,68]. Different experimental conditions were explored to minimize enzyme inhibition and optimize ester production. All reactions were carried out in solvent-free systems, which make this synthesis methodology even more attractive from economic and environmental points of view [69–72].

2. Results and Discussion

2.1. Evaluation of the Performance of Lipases in Free Form

The performances of *Candida antarctica* lipase B (CALB), *Rhizomucor miehei* lipase (RML), *Thermomyces lanuginosus* lipase (TLL), *Candida rugosa* lipase (CRL), *Burkholderia cepacia* lipase (BCL), and porcine pancreas (PPL), all in free form and in aqueous solution, were evaluated in the esterification reaction of thymol and octanoic acid in a solvent-free system. Table 1 shows the conversion of octanoic acid after 24 h of reaction at 50 °C using different lipases.

Table 1. Esterification of thymol and octanoic acid using lipases in soluble form in a solvent-free system. Reaction conditions: 2 mmol of octanoic acid, 2 mmol of thymol, 200 μL of enzymatic aqueous solution, 50 °C and 24 h of reaction.

Lipase Source	Octanoic Acid Conversion (%)
Candida antarctica	30.8 ± 2.4
Rhizomucor miehei	0.0 ± 0.0
Thermomyces lanuginosus	10.2 ± 1.2
Candida rugosa	0.0 ± 0.0
Burkholderia cepacia	1.4 ± 0.0
Porcine pancreas	0.0 ± 0.0

Although lipases have a similar architecture and reaction mechanism, the shapes and physicochemical properties of the substrate binding sites for each of them are different. In the selected lipases, the binding site is located within a pocket; the shape, depth, and physicochemical properties of the substrate-binding pocket vary for each of the lipases. The substrate-binding pocket of CALB is a steep elliptical funnel of 9.5 × 4.5 Å, the BCL-binding pocket is also an elliptical funnel with a diameter of 4.5 Å but with a length of 17 Å. The length of the binding site is 22 Å for CRL and RML, with diameters of 4 and 4.5 Å, respectively [73].

With this simplified idea of lipase binding sites, we can understand that, when faced with bulky substrates, the greatest activity will occur in enzymes with binding sites of shorter length and greater diameter. In this way, the interactions of the substrates with the different amino acid residues present in the binding site of the substrates are

minimized. Figure 2 shows a reduced model of CALB with thymol in the vicinity of the acyl enzyme formed after the coordination of octanoic acid with serine from the lipase active site. It is possible to see the proximity of the thymol to the walls of the CALB binding site; this generates numerous interactions with the present amino acid residues, limiting the approximation towards the acyl enzyme.

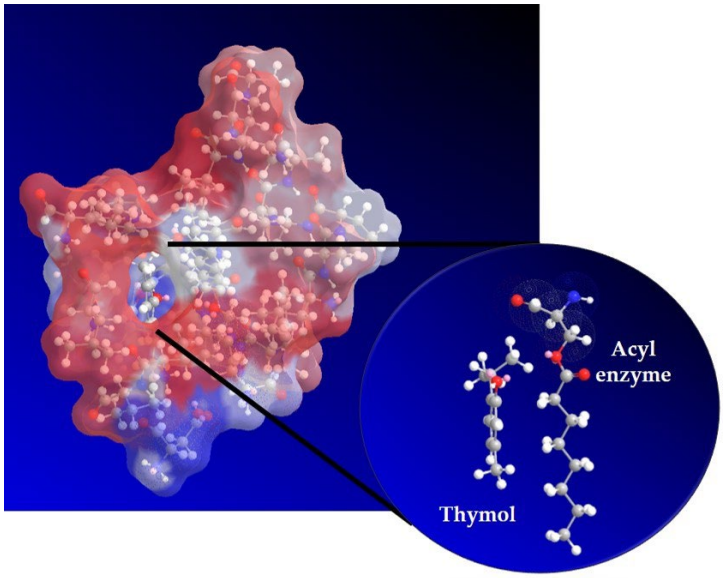

Figure 2. Reduced model of CALB with thymol in the vicinity of the acyl enzyme.

2.2. Performance Evaluation of Immobilized Lipases

The use of the immobilized enzyme, whenever possible, is extremely interesting since it provides several advantages. First, the immobilized enzyme biocatalyst can be easily recovered and reused. Secondly, immobilization usually improves the stability of the enzyme (against changes in temperature, pH, solvents, etc.). Even more, on some occasions, depending on the enzyme and the type of immobilization technique, it is possible to increase its activity.

In this context, the esterification reaction of thymol and octanoic acid was carried out using immobilized *Candida antarctica* lipase B. On the one hand, the performance of the commercial biocatalyst Novozym 435 was evaluated, and on the other hand, this lipase was immobilized on Immobead 150. Immobead 150 is a resin functionalized with epoxy groups. This solid has interesting characteristics for the immobilization of lipases. First, its hydrophobic nature allows the opening of the lipase lid due to the polar/non-polar interface that occurs between the support and the (aqueous) enzymatic solution. Secondly, the surface oxirane groups can react with the residues of some amino acids of the lipase, generating immobilization by means of covalent bonding [74]. Thus, an active biocatalyst can be obtained while minimizing the leaching of the lipase into the reaction medium. The immobilization process was also combined with the molecular imprinting or bioimprinting technique. This procedure generates conformational changes in the enzyme using substrates (or substrate analogs) of the same, leading it to a more active conformation because the active site has been preformed [74].

Benzoic acid, ibuprofen, and thymol were used as bioimprinting agents. The biocatalysts obtained by immobilization of CALB on Immobead 150 and by immobilization combined with bioimprinting were named as follows:

- CALB-Immo: lipase B from *Candida antarctica* immobilized on Immobead 150,
- CALB-Benz-Immo: lipase B from *Candida antarctica* immobilized on Immobead 150 + bioimprinting with benzoic acid,
- CALB-Ibu-Immo: lipase B from *Candida antarctica* immobilized on Immobead 150 + bioimprinting with ibuprofen,

- CALB-Thy-Immo: lipase B from *Candida antarctica* immobilized on Immobead 150 + bioimprinting with thymol.

Table 2 presents the protein immobilization efficiency for each of the synthesized biocatalysts.

Table 2. Efficiency of protein immobilization in the synthesis of biocatalysts.

Biocatalyst	Immobilization Efficiency (%)
CALB-Immo	33.4 ± 4.7
CALB-Benz-Immo	74.1 ± 4.1
CALB-Ibu-Immo	89.7 ± 6.4
CALB-Thy-Immo	90.0 ± 4.5

The immobilization efficiency was increased when a bioimprinting agent was added. This improvement in immobilization efficiency is directly related to the better contact between the support and the enzymatic solution due to the surfactant effect promoted by the bioimprinting molecules. Since the support is nonpolar in nature, and the immobilization medium is polar, the addition of compounds that behave as surfactants improve the contact between the support and the immobilization medium, producing an increase in the percentage of immobilized protein on Immobead [74,75].

Table 3 shows the conversion of octanoic acid after 24 h of reaction at 50 °C catalyzed by immobilized lipase B from *Candida antarctica*.

Table 3. Esterification of thymol and octanoic acid using immobilized lipase. Reaction conditions: 2 mmol of octanoic acid, 2 mmol of thymol, 15% biocatalyst (with respect to the mass of acid), 50 °C, and 24 h of reaction.

Biocatalyst	Octanoic Acid Conversion (%)
Novozym 435	2.8 ± 0.1
CALB-Immo	4.3 ± 0.3
CALB-Benz-Immo	4.6 ± 0.8
CALB-Ibu-Immo	4.7 ± 0.7
CALB-Thy-Immo	5.3 ± 0.3

The activity of immobilized lipase biocatalysts in the esterification of thymol and octanoic acid was considerably lower than that shown by soluble lipase. The biocatalysts obtained by immobilizing CALB on Immobead 150 showed greater activity than Novozym 435. The bioprinting process generated an increase in enzyme activity. The studied bioimprinting agents are large molecules that are enzyme substrates and could generate important conformational changes before immobilization. The immobilization process fixes that more active conformation and then the bioprinting agent is removed to obtain a more active biocatalyst. Despite the increase in activity due to bioimprinting, the soluble lipase activity was not recovered in any of the cases. The low activity of the immobilized lipase could be associated with two phenomena: on the one hand, the absence of the necessary water for the enzyme to have the conformational changes so that the substrate(s) reach the active site, and, on the other hand, the bonds between the enzyme and the support can seriously limit the conformational changes necessary for the lipase to be active, particularly against such bulky substrates as thymol. With this idea in mind, the esterification reaction was carried out using Novozym 435 under the same conditions as above but using different concentrations of water in the reaction system (10–40% by weight with respect to the mass of octanoic acid). However, no increase in acid conversion was obtained for any of the concentrations tested. It is probable that the cause of the low activity of the immobilized lipase, in comparison with the soluble lipase, is given by the conformational changes that the lipase cannot have due to being fixed to a support.

At this point it is important to take into account that for all the tests carried out with the immobilized lipase it was necessary to establish a protocol to minimize errors due to the adsorption of the acid on the biocatalyst support. Table 4 shows the percentage of acid adsorbed on Novozym 435 after each *n*-heptane wash when a non-reactive adsorption study was performed.

Table 4. Octanoic acid adsorption test results on Novozym 435.

Biocatalyst	Adsorption after the First Wash (%)	Adsorption after the Second Wash (%)
Novozym 435	21.8 ± 3.6	4.4 ± 1.7

Overestimations of the acid conversion of more than 20% can occur if an adequate washing of the biocatalyst is not performed. At least three washes were necessary to achieve the complete quantification of the unreacted acid in the system under study.

2.3. Synthesis of Thymol Octanoate Using CALB

Based on the previous results, lipase B from *Candida antarctica* in its soluble form was selected to optimize the conditions for the synthesis of thymol octanoate.

To assess the potential inhibitory effect on CALB generated by thymol, the thymol/octanoic acid molar ratio was varied between 3:1 and 1:4 (mol/mol). Figure 3 shows the conversion of octanoic acid for thymol/acid molar ratios 1:1 to 3:1 (mol/mol), in which acid is the limiting reagent.

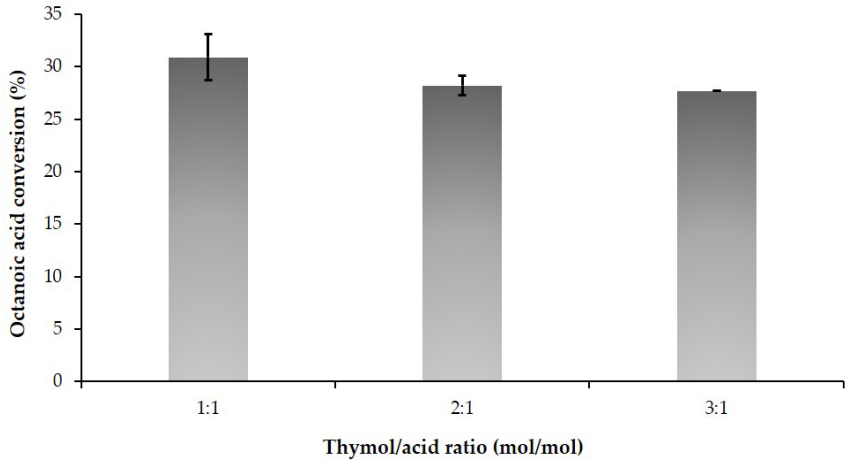

Figure 3. Change of the conversion of octanoic acid as a function of the molar ratio of substrates. Reaction conditions: 2 mmol of octanoic acid, 200 μL of CALB solution (50.5 mg mL^{-1} of protein), 50 °C, 24 h, and magnetic stirring at 200 rpm.

The observed changes in acid conversion (Figure 3) as a function of the variation in thymol mass are not statistically significant. These results would indicate that there is no inhibitory effect of thymol on CALB activity. However, the progress of the reaction could be limited due to steric hindrances that hinder the movement of thymol through a window created by the CALB conformation to the substrate-binding pocket of the lipase and/or the efflux of the ester from the active site to the reaction medium. The esterification of thymol and octanoic acid was performed by feeding both thymol and lipase in stages to avoid any undetected inhibitory effect and to maximize thymol octanoate production the reaction time was extended to 48 h. All of the acid (2 mmol) was fed at the initial time and the following thymol and lipase feeding options were evaluated:

(a) An amount of 2 mmol of thymol and 200 μL of CALB solution at the start of the reaction,

(b) An amount of 2 mmol of thymol at the beginning and CALB solution in two steps (100 μL at the beginning and 100 μL at 24 h)
(c) A volume of 200 μL of CALB solution at the beginning and thymol in two steps (1 mmol at the beginning and 1 mmol at 24 h)
(d) thymol and lipase in two stages (50% at the beginning and 50% at 24 h).

Figure 4 shows the octanoic acid conversion for each of the tests carried out after 48 h of reaction.

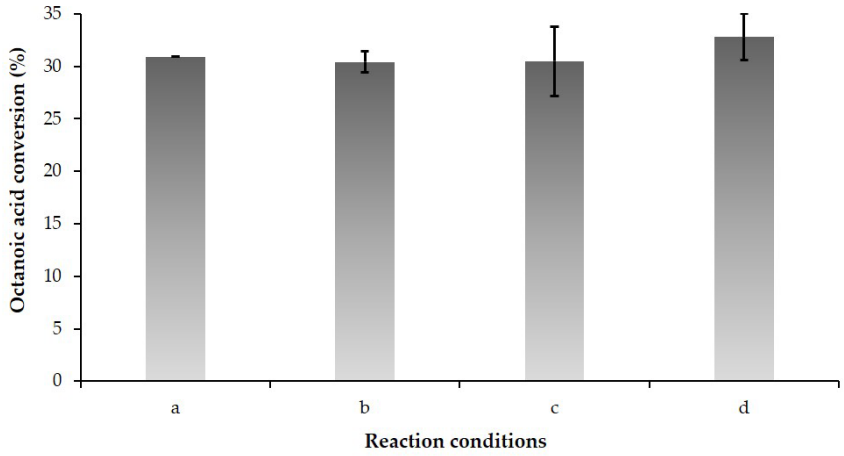

Figure 4. Octanoic acid conversion after 48 h of reaction for different feed conditions of thymol and CALB; (**a**) thymol and CALB solution in one stage, (**b**) thymol in one stage and CALB solution in two stages, (**c**) CALB solution in one stage and thymol in two stages, (**d**) thymol and CALB solution in two stages. Reaction conditions: 2 mmol octanoic acid, 50 °C, magnetic stirring at 200 rpm.

There are no statistically significant changes in acid conversion for any of the conditions studied. Even more, after 48 h of reaction it was not possible to exceed the conversion reached after 24 h of reaction. Probably, in addition to the steric hindrances that limit the arrival of thymol to the active site of lipase, there is inhibition of the enzyme by the product of the reaction.

Figure 5 shows the conversion of thymol in the esterification reaction using thymol/octanoic acid molar ratios ranging from 1:4 to 1:1 (mol/mol). The thymol conversion reached values of the order of 94% when the reaction was carried out with a thymol/acid molar ratio of 1:4 mol/mol. On the other hand, by reducing the concentration of thymol in the reaction system, the conversion of octanoic acid was between 20 and 24%, without major changes compared to systems with higher concentrations of this substrate.

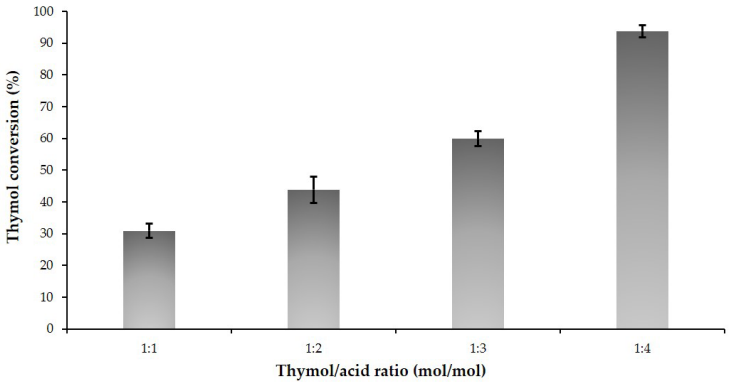

Figure 5. Change of the conversion of thymol as a function of the molar ratio of substrates. Reaction conditions: 2 mmol of octanoic acid, 200 μL of CALB solution (50.5 mg mL^{-1} of protein), 50 °C, 24 h, and magnetic stirring at 200 rpm.

Figure 6 shows the overlay of chromatograms corresponding to the esterification of thymol and octanoic acid. The black line shows the chromatogram corresponding to a sample at the beginning of the reaction, and the red line corresponds to the chromatogram of a sample after 24 h of reaction. In the figure, the peaks corresponding to the substrates and thymol octanoate can be clearly identified.

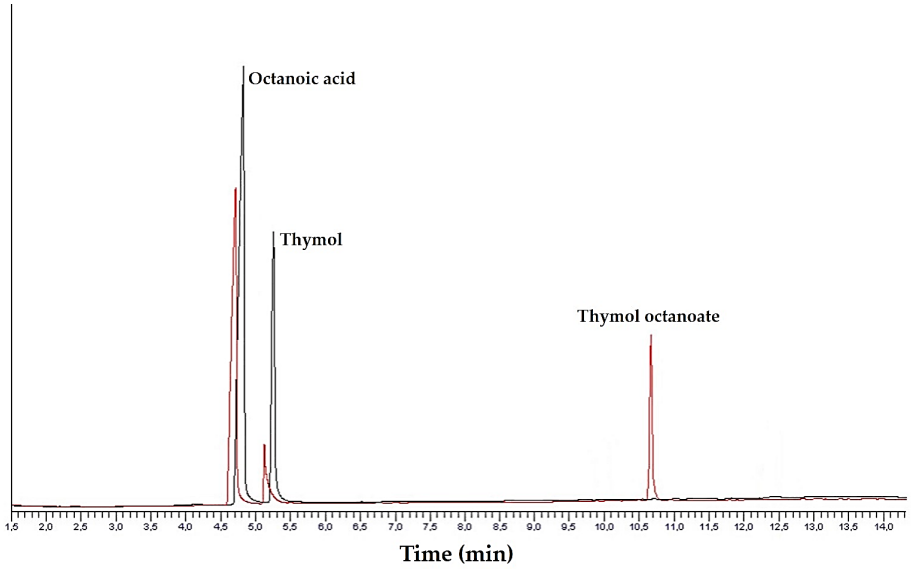

Figure 6. Overlapping of chromatograms corresponding to samples from the beginning and end of the esterification reaction of thymol and octanoic acid. Reaction conditions: 2 mmol of octanoic acid, 0.5 mmol of thymol, 200 μL of CALB solution, 50 °C, and 24 h of reaction. The black line corresponds to the sample at the beginning of the reaction, the red line corresponds to the sample at the end of the reaction.

Figure 7 shows the change in thymol conversion as a function of reaction time when it was carried out using 2 mmol of octanoic acid, a thymol/acid molar ratio 1:4 mol/mol, 200 μL of the CALB solution, 50 °C, and magnetic stirring at 200 rpm.

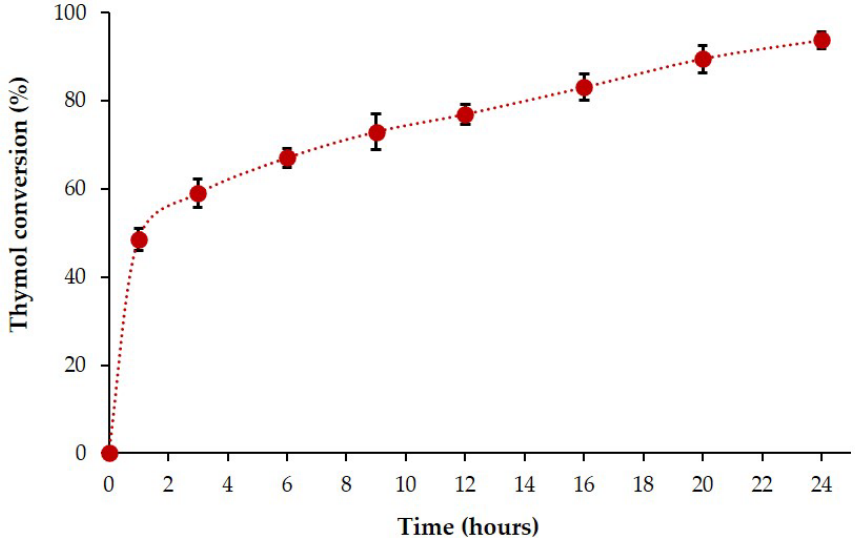

Figure 7. Variation of thymol conversion as a function of reaction time. Reaction conditions: 2 mmol of octanoic acid, 0.5 mmol of thymol, 200 μL of CALB solution, 50 °C, and magnetic stirring at 200 rpm.

The reaction proceeds rapidly in the first hour, reaching approximately 50% conversion of the thymol present in the reaction system. Then there is a significant change in the reaction rate and it continues slowly until reaching a conversion close to 94% after 24 h of reaction. This significant reduction in the reaction rate after the first hour of initiation could be associated with the inhibition of lipase by the product formed and/or the difficulty for the product to exit from the vicinity of the active site into the medium of reaction.

The esterification reaction using low concentrations of thymol was carried out in stages to increase the production of thymol octanoate and to use unreacted acid. For this, the esterification was carried out with a thymol/acid molar ratio of 1:4 mol/mol using 100 μL of the CALB solution. After 24 h of reaction, another 0.5 mmol of thymol was added along with the remaining 100 μL of CALB. The reaction continued for another 24 h. Under these conditions the conversion of thymol was 59.5 ± 4.8%, and 0.62 ± 0.032 mmol of thymol octanoate were generated. This value is practically the same as that obtained by carrying out the reaction with thymol/acid molar ratios of 1:1, 2:1, and 3:1 mol/mol. Table 5 shows the moles of thymol octanoate generated for each of the molar ratios tested. Based on the results reported in Table 5, it seems that the generation of thymol octanoate is limited. The maximum production of the ester is around 0.60 mmol and it could not be exceeded by changing the molar ratio of substrates or by feeding thymol and lipase in stages, using 10.1 mg of soluble CALB. This content of thymol octanoate in the reaction medium could lead to inhibition of the enzyme.

Table 5. Thymol octanoate production for different thymol/octanoic acid molar ratios and residual lipase activity after use. Reaction conditions: 2 mmol of octanoic acid, 200 μL of CALB solution, 50 °C, and 24 h of reaction.

Thymol/Acid Molar Ratio	Thymol Octanoate Produced (mmol)	Residual Activity (%)
3:1	0.56 ± 0.021	51.0 ± 2.0
2:1	0.56 ± 0.015	48.3 ± 1.2
1:1	0.60 ± 0.052	52.8 ± 2.6
1:2	0.46 ± 0.054	90.5 ± 3.2
1:3	0.45 ± 0.044	94.9 ± 1.5
1:4	0.45 ± 0.016	95.9 ± 2.9

Since the compounds present in the reaction system are solubilized and extracted with *n*-heptane and the lipase is in aqueous solution, it is possible to recover it and reuse it in the reaction. Table 5 presents the percentage of enzyme activity retained after the first use. This percentage of residual activity was calculated using Equation (1).

$$Residual_{Activity} = \frac{X_{LR}^{2} \cdot 100}{X_{LR}^{1}} \quad (1)$$

where X_{LR}^{1} is the limiting reagent conversion at the first use of CALB and X_{LR}^{2} is the limiting reagent conversion achieved when the second use of CALB was made.

The lipase retained more than 90% of its initial activity in the reaction systems where thymol/acid molar ratios of 1:4 to 1:2 mol/mol were used. For those reactions where higher thymol concentrations were explored, and where the ester yield was higher, only about 50% of the initial activity was recovered. These results suggest that the synthesis of thymol caprylate could be carried out using low concentrations of thymol (1:4 thymol/octanoic acid molar ratio, for example), recovering and reusing the lipase to reduce production costs. Further studies are necessary to evaluate the role of the enzyme concentration, the number of reuses in which CALB maintains its activity, and the feasibility of separating and purifying the product of interest.

3. Materials and Methods

3.1. Materials

Thymol, lipase B from *Candida antarctica*, lipase from *Rhizomucor miehei*, lipase from *Thermomyces lanuginosus*, lipase from porcine pancreas, silylation reagents, benzoic acid, ibuprofen, and Immobead 150 (an acrylic resin functionalized with epoxy groups, with an average particle diameter of 0.15 mm) were purchased from Sigma-Aldrich (St. Louis, MO, USA). *Candida rugosa* lipase and *Burkholderia cepacia* lipase were supplied by Amano Enzyme Inc. (Nagoya, Japan). Novozym 435, which is a commercial form of *Candida antarctica* lipase B (CALB) immobilized on acrylic resin, was kindly provided by Novo Nordisk A/S (São Paulo, Brazil). Caprylic acid was provided by Fluka (Neu-Ulm, Germany). Absolute ethanol and *n*-heptane were obtained from Dorwil (Buenos Aires, Argentina). Phenolphthalein, potassium hydroxide, and pyridine were purchased from Anedra (Buenos Aires, Argentina). The protein determination kits called Proteínas Totales AA and Proti U/LCR were purchased from Wiener Laboratorios S.A.I.C. (Rosario, Argentina). All products were analytical grade.

3.2. Lipase Immobilization

Immobilization of *Candida antarctica* lipase B was performed following the previously reported methodology using a nominal protein loading of 200 mg g^{-1} of support [74]. For this, a volume of CALB solution containing 10 mg of protein and 2 mL of distilled water pH 6.5 were placed in a 10 mL vial. Then, 50 mg of Immobead was added to the immobilization medium. The immobilization was carried out at 25 °C for 6 h with magnetic stirring at 200 rpm. Finally, the solid was recovered by filtration, washed twice with distilled water, and dried at 30 °C until constant weight.

Immobilization combined with bioimprinting was performed according to the methodology reported by Sanchez et al. [74]. In this case, a volume of CALB solution containing 10 mg of protein, 2 mL of distilled water pH 6.5, and 30 μmol of the bioimprinting agent (benzoic acid, ibuprofen, and thymol) were placed in a 10 mL vial. The system was stirred at 200 rpm and 25 °C for 30 min, then 50 mg of Immobead was added. Immobilization was performed for 6 h. The solid was recovered by filtration, washed twice with distilled water, and dried at 30 °C until reaching a constant weight. Finally, the dried solid was washed with *n*-heptane to remove the bioprinting agent and dried again.

All immobilization procedures were performed in duplicate.

3.3. Protein Determination

The protein content was determined using the Proti U/LCR kit (Wiener Laboratorios S.A.I.C., Rosario, Argentina) for concentrations up to 1.5 mg mL^{-1} and the Proteínas Totales AA kit (Wiener Laboratorios S.A.I.C., Rosario, Argentina) for concentrations from 1.5 mg mL^{-1} up to 170 mg mL^{-1}.

3.4. Immobilization Efficiency

CALB immobilization efficiency was determined by comparing the protein content before immobilization and the protein present in the immobilization supernatant and wash waters. For this, the following equation was used:

$$Im_{eff}(\%) = \frac{Prot_I - Prot_F}{Prot_I} \cdot 100 \quad (2)$$

where $Prot_I$ is the protein content, in mg, at the start of immobilization and $Prot_F$ is the protein content, in mg, in the immobilization supernatant and wash waters.

3.5. Esterification Reaction with Soluble Lipase

The esterification of thymol and octanoic acid was carried out in 10 mL vials in solvent-free systems. In all cases, 2 mmol of acid and 200 μL of enzymatic solution were used.

The concentration of all enzyme solutions was adjusted to 50.5 mg of protein per mL of solution. The thymol content was varied from 0.5 mmol to 6 mmol. Thymol and enzyme feeding was performed at different times in order to minimize lipase inhibition and maximize ester production. The reactions were carried out in thermostatic baths at 50 °C, with magnetic stirring at 200 rpm. The reaction time was varied between 1 and 48 h.

At the end of the reaction, 2 mL of *n*-heptane were added to the vial; it was then shaken and its contents were collected to determine the concentration of free fatty acid using an ethanolic solution of KOH 0.2 mol L^{-1} and phenolphthalein as indicator. Substrate conversion was determined using Equation (3).

$$X_{sust} = \frac{Sust_I - Sust_F}{Sust_I} \cdot 100 \quad (3)$$

where $Sust_I$ is the limiting reagent content at the start of the reaction (mmol) and $Sust_F$ is the limiting reagent content at the end of the reaction.

All reactions were carried out at least in duplicate.

3.6. Esterification Reaction with Immobilized Lipase

The esterification of thymol and octanoic acid was carried out in 10 mL vials in solvent-free systems. In all cases, 2 mmol of acid, 2 mmol of thymol, and 15% biocatalyst with respect to the mass of acid were used. The reactions were carried out at 50 °C, for 24 h and with magnetic stirring at 200 rpm. At the end of the reaction, 2 mL of *n*-heptane were added to the vial, which was shaken and centrifuged at 8000 rpm for 5 min to separate the solid. The supernatant was collected for the determination of the free fatty acid content. The *n*-heptane washing procedure was performed three times to remove the acid adsorbed on the support.

Additionally, the incorporation of water into the reaction system was studied when Novozym 435 was used as the catalyst. For this, the reaction was carried out under the aforementioned conditions with the addition of between 10 and 40% of distilled water (with respect to the mass of acid) at the beginning of the reaction. Octanoic acid conversion was determined using Equation (3).

All reactions were carried out in duplicate.

3.7. Evaluation of Acid Adsorption on Novozym 435

The octanoic acid adsorption assay on Novozym 435 was performed under the same reaction conditions but without the addition of thymol. The test was carried out at 50 °C, with magnetic stirring at 200 rpm, for 24 h. Once this time was reached, 2 mL of *n*-heptane were added to the vial, which was shaken and centrifuged at 8000 rpm for 5 min. The supernatant was collected and the free octanoic acid content was determined by titration. The *n*-heptane wash was performed 3 times and the supernatant was titrated separately in each wash. The percentage of acid adsorption on the biocatalyst was determined as follows:

$$\text{Ad (\%)} = \frac{OA_I - OA_F}{OA_I} \cdot 100 \quad (4)$$

where OA_I and OA_F are the mmoles of octanoic acid at the beginning and at the end of the test, respectively.

3.8. Gas Chromatography Analysis

Samples were diluted with pyridine and silylated with *N*-methyl-*N*-(trimethylsilyl) trifluoroacetamide (MSTFA). The analysis of the samples was performed in a PerkinElmer AutoSystem XL gas chromatograph equipped with on-column injection, a flame ionization detector (FID), and a high-temperature capillary column ZB-5HT Inferno (15 m × 0.32 mm, with a film thickness of 0.10 μm), using H_2 as carrier gas. The temperature detector was maintained at 250 °C. The initial column temperature was maintained at 40 °C for 1

min, increased to 50 °C at a rate of 5 °C min^{-1}, then increased to 100 °C at 15 °C min^{-1}, further increased to 200 °C at 20 °C, and finally maintained there for 2 min.

4. Conclusions

The enzymatic esterification of thymol and octanoic acid to obtain thymol octanoate, a molecule with great application potential due to its biological activity, was studied for the first time. Of the six lipases in their soluble form studied, lipase B from *Candida antarctica* showed the highest activity in the reaction. The activity of the biocatalysts obtained by immobilization of this lipase was reduced, and the bonds between the enzyme and the support could limit the conformational changes necessary for a bulky substrate such as thymol to reach the active site of the lipase.

Different feeding conditions for thymol and lipase were tested to minimize inhibitory effects on enzyme activity and maximize thymol octanoate production. The results obtained suggest that there is inhibition of lipase by the reaction product. Thus, carrying out the reaction with low concentrations of thymol, a thymol conversion of 93.8% was achieved. After its use in the reaction, the lipase maintained more than 90% of its initial activity. This result indicates that the enzyme could be used in successive reaction cycles. Under these conditions, the concentration of thymol octanoate would be below those concentrations that generate important inhibitory effects.

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