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β-Sitosterol Oleate Synthesis by *Candida rugosa* Lipase in a Solvent-Free Mini Reactor System: Free and Immobilized on Chitosan-Alginate Beads

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Abstract: *Candida rugosa* lipase (CRL) was immobilized by the ionic gelling technique using alginate and chitosan as encapsulating agents. An immobilization yield of 99% and an immobilization efficiency of 51% were obtained. Maximum hydrolytic activity for free and immobilized CRL was detected at 40 °C and for synthesis activity at 35 °C. The optimum pH for immobilized and free CRL hydrolysis activity was 8.0. The V_{max} obtained for the hydrolysis reaction was higher for free CRL (4121.4 µmol/min/g) compared to immobilized CRL (2359.13 µmol/min/g). A Vmax of 2.24 µmol/min/g was detected for the synthetic activity of free CRL. The K_m obtained for the hydrolysis reaction was higher (660.02 µmol/L) for immobilized CRL than for free CRL (403.06 µmol/L). For the synthetic activity, a K_m of 234.44 µmol/L was calculated. The conversion of β-sitosterol oleate ranged from 80.85 to 96.84% for free CRL, higher than the maximum found for immobilized CRL (32%). The scale-up (scale factor: 50) with the free CRL was successfully performed, achieving a high conversion value (92%) in a 500 mL bioreactor. This conversion value was within the range predicted by the mathematical model obtained using mini reactors. These mini reactors are good models to test several conditions of enzyme reactions that are intended for large scales.

Keywords: enzymatic esterification; immobilized lipase; mini reactors; green synthesis; phytosterol esters

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1. Introduction

Phytosterols have been used to lower total cholesterol levels. These compounds are triterpenes with a chemical structure similar to cholesterol, including β-sitosterol, campesterol, and stigmasterol [1]. They are usually found in plants rich in lipids, and its consumption has also been related to the prevention of cancer, and with anti-inflammatory and antimicrobial activity [2]. Its insolubility in water and low solubility in oils makes it difficult to include these compounds in food products [3]. The esterification of phytosterols is a good strategy to solubilize them in oily and fatty food systems [4–6]. The synthesis of phytosterol esters can be performed by esterification with fatty acids by chemical or enzymatic methods [7,8]. Lipase-catalyzed phytosterol esters production offers several advantages in relation to chemical synthesis, including mild reaction conditions and catalyst specificity, reducing byproducts formation [9].

Lipases (Triacylglycerol lipase E.C. 3.1.1.3) are able to catalyze ester-bond hydrolysis in triacylglycerols with the release of fatty acids, mono and diglycerides, and glycerol, as well as the synthesis of a variety of esters [10]. The systematic search for articles and patents performed by Pereira et al. [11] with documents from the last 20 years showed

that *Candida rugosa* lipases (CRL) are the most relevant biocatalysts to produce phytosterol esters, being used in more than 50% of the studies. CRL is commercialized in mixtures of isoenzymes (mainly Lip1, Lip2, and Lip3) stabilized with lactose. This disaccharide plays an important role as a water reservoir [12] and has been associated with the high conversion values of the esterification reactions, since it can protect the enzyme in low-water content environments [13]. The active site in CRL is covered by a lid composed of amino acids with amphiphilic properties in a β-helix structure, which is essential for interfacial activation, and consequently for the catalytical enzymatic activity [12], another advantage for esterification reactions.

Immobilized enzymes have been preferentially used, as they usually improve the performance of these biomolecules and reduce process costs by the possibility to reuse the biocatalyst [14]. Different protocols have been tested to immobilize CRL, such as physical adsorption on hydrophobic and hydrophilic supports [15,16] and covalent binding on previously activated supports [17,18]. The synthesis of hexyl butyrate was evaluated in a Box-Behnken design with CRL immobilized by physical adsorption on Diaion HP 20. Higher conversion (95%) was obtained after 8 h at 47 °C, a 1:2 molar ratio and a 17% biocatalyst. CRL-Diaion HP 20 retained 60% of its initial activity after ten cycles [15]. Macroporous resins were used for CRL adsorption during the enzyme catalyzed reaction of phytostanols esterification with the addition of trehalose to protect the enzyme [17]. Zheng et al. [19] immobilized CRL on mixed-mode silica particles via hydrophobic and strong cation-exchange interaction, which remarkably increased its stability at high temperature in comparison to free enzymes.

Microencapsulation using natural polymers—such as gelatin, chitosan, alginate, and agarose—has been tested as an immobilization technique for several enzymes with the advantage of using nontoxic, biocompatible, and biodegradable materials [20–22]. The entrapment of the enzyme in a polymer network promotes an easy way to separate it from the reaction medium and allows substrates and products to pass through [23]. For industrial use, the challenge is to prevent enzyme leakage, since the catalyst does not usually interact with the polymer [22]. For the immobilization of CRL, agarose beads exhibited undesirable swelling, and alginate beads substantially leached [20]. The higher hydrolytic lipase activity in chitosan beads was considered by Betigeri and Neau [20], positive evidence that this polymer is worthy of pursuit to immobilize lipase. Chitosan, acetic acid, and tripolyphosphate were used to create hydrogel beads with CRL entrapped on them. High entrapment efficiency, a high retention of hydrolytic activity, and a minimal release of the entrapped enzyme were achieved by statistical optimization [21]. Our research group optimized lipase entrapment using alginate and chitosan to form the beads [24]. These systems—lipases microencapsulated by natural polymers—are usually tested in hydrolytic reactions and, as far as we know, have not been tested for phytosterol ester synthesis.

The use of miniaturized biological systems that can be easily reproduced in larger scales is important if the process is intended for industrial purposes [25]. That is the case for enzymatic reactions studies, which require several parallel reactors to select optimal reaction conditions and demands a low reagent amount because of the high cost of the biocatalyst. These systems range from small volume flasks and agitated microplates to stirred tank reactor miniatures, with several models available today [26]. However, there are still many enzymatic processes that are investigated in non-scalable systems.

Traditionally, enzymatic synthesis reactions are carried out using an organic solvent, with the advantages of increasing substrate solubility and changing the reaction's thermodynamic equilibrium to favor esterification over hydrolysis [27,28]. However, organic solvents may have some disadvantages, such as the pressurization of vessels, partial denaturation of the enzyme, and dilution of the reaction medium, impacting the efficiency of the process. In addition, they can leave residual substances—that may be toxic to human health—in the final product [11].

In the present study, we used scalable mini reactor systems to investigate the production of β-sitosterol oleate by *C. rugosa* lipase, comparing the free and immobilized catalyst,

without the use of any solvent. The immobilization in chitosan-alginate beads was tested here for the first time for phytosterol ester synthesis.

2. Results and Discussion

Candida rugosa lipase (CRL) was immobilized in alginate and chitosan microcapsules by ionotropic gelation, following the protocol optimized by Pereira et al. [24], with 3.1% (*w/v*) of sodium alginate, 0.2% (*w/v*) of chitosan, 0.14 M of $CaCl_2$, and 1 min of complexation time. For every 10 mL biopolymer solution containing 140 mg of CRL powder (14 mg/mL) and 3.1% alginate, 462 mg of freeze-dried microcapsules were obtained. Therefore, we obtained a microcapsule with around 30% of CRL powder. CRL hydrolysis activity measured before the immobilization process was 11,104.17 ± 1243.73 Units per gram of enzyme protein (U/g). After microencapsulation, an immobilization yield (percentage of enzyme trapped in microcapsules) of 99% was achieved and the immobilized enzyme presented an activity of 1714.53 ± 328.77 U/g. Considering that the enzyme amount (powder) trapped in the microcapsules corresponds to approximately 30% of their total dry mass, we can infer that the maximum expected lipase activity would be, on average, 3321.35 U/g (30% of 11,104.17 U/g). Therefore, we can estimate an immobilization efficiency (percentage of enzymatic activity detected in microcapsules after immobilization in relation to the expected activity value) of 51%. Although the immobilization efficiency was not high, the immobilized lipase activity was still quite expressive, if we compare to other immobilized CRL (41.3 U/g, CRL immobilized in niobium oxide [16]; 33.3 U/g, CRL immobilized by cross-linking with glutaraldehyde followed by entrapment in alginate beads [29], 130 U/g, CRL encapsulated with calix [4]arene-adorned sporopollenin [30], 9500 U/g CRL immobilized in chitosan-coated alginate beads [31]).

2.1. Biochemical Characterization

CRL immobilized in alginate-chitosan microcapsules was evaluated by *p*-nitrophenyl laurate (*p*-NPL) hydrolysis and synthesis reactions in comparison to its free form to characterize its best catalytic activity. The characterizations for both reactions were performed since we intended to develop a universal biocatalyst, one that could be used in both synthesis and hydrolysis processes.

2.1.1. Effect of Temperature and pH on *C. rugosa* Lipase Activity

Free and immobilized CRL activities were tested in different temperatures and pH (Figure 1). The effect of temperature on the hydrolysis activity of free and immobilized CRL was evaluated in the range of 20 to 60 °C (Figure 1a). Maximum hydrolytic activity for both CRL forms was detected at 40 °C, with significant reduction for lower or higher temperatures. This reduction was less intensive for the immobilized lipase, showing that immobilization may have protected the enzyme. Pereira et al. [32] detected an increase in optimal temperature for the immobilized lipase (40 to 50 °C) in comparison to the free lipase (35 °C) for *Y. lipolytica* lipase immobilized in chitosan-alginate beads. The authors also observed that temperatures higher than optimal temperatures led to a drastic reduction in hydrolytic activity. For the synthesis of *p*-NPL from *p*-nitrophenol and lauric acid in hexane, the optimum temperature was detected at 35 °C for the free CRL (Figure 1b), but without significant differences in relation to 30 and 40 °C. Similar optimal temperatures for synthesis reactions can be found in the literature. Dursun et al. [33] covalently immobilized *C. rugosa* lipase and observed optimal temperatures for synthetic activity at 37 °C for the free enzyme and 45 °C for the immobilized enzyme. The optimal temperature for *p*-nitrophenyl acetate synthesis with free *C. rugosa* lipase was 30 °C [34]. No significant synthesis activity was detected for the immobilized CRL.

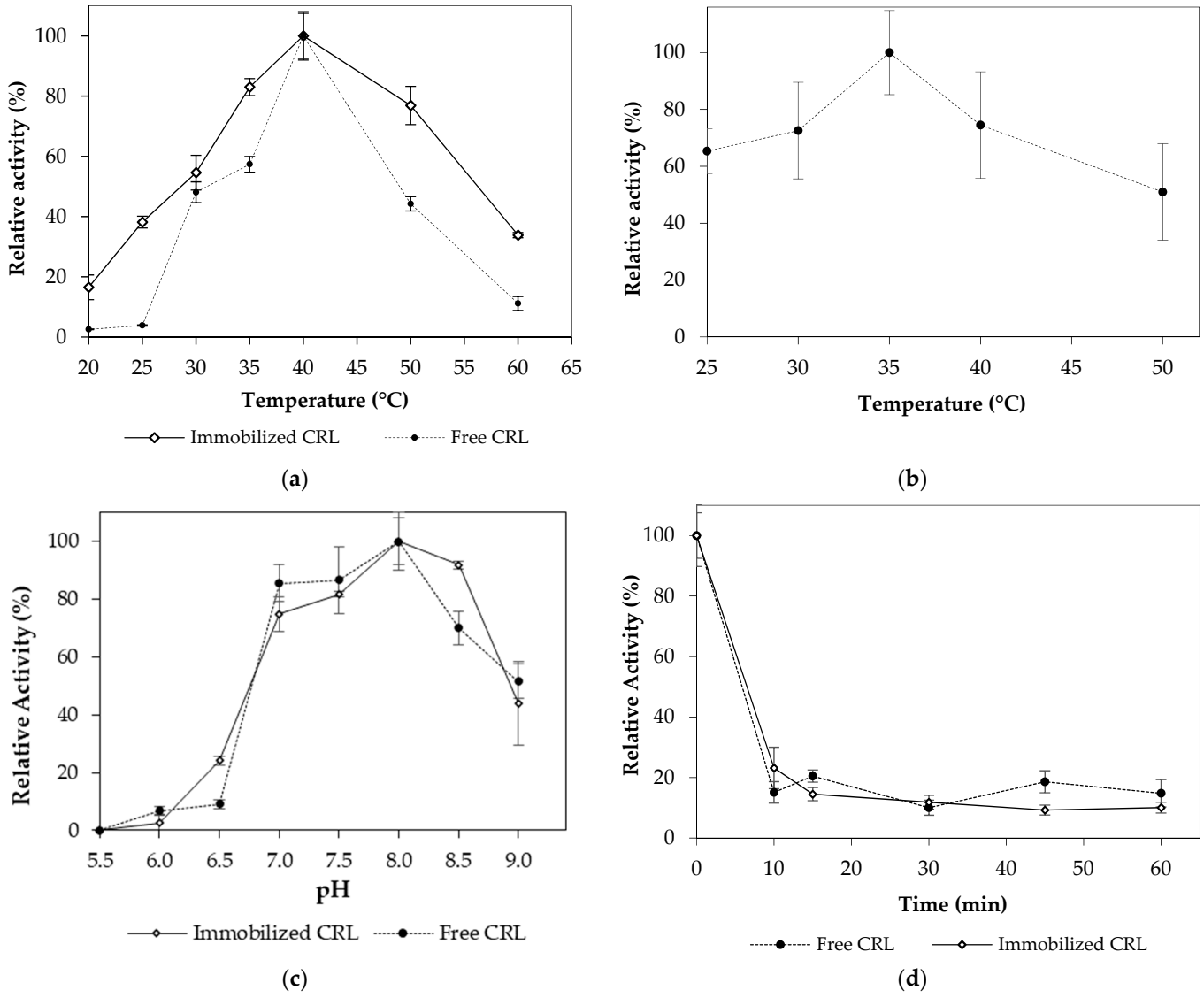

Figure 1. Effect of temperature on the hydrolytic activity of free CRL and immobilized CRL (**a**); effect of temperature on *p*-NPL synthesis activity of free CRL (**b**); effect of pH on the hydrolytic activity of free CRL and immobilized CRL (**c**); and hexane exposure time effect on free and immobilized CRL hydrolytic activity (**d**). The maximum activity obtained was defined as 100%. Note: pH 5.5–6.0—Acetate buffer; pH 6.5–7.5—Phosphate buffer and pH 8.0–9.0—Tris-HCl buffer.

The effect of pH on *p*-NPL hydrolysis free and immobilized CRL is shown in Figure 1c. The highest hydrolytic activity was detected at pH 8.0 for both enzymatic fractions, showing that immobilization did not modify the optimal pH of CRL, as also observed for temperature. However, one could say that CRL immobilized in chitosan-alginate microcapsules could also be used in pH 8.5 without significant difference from pH 8.0, which cannot be said for the free lipase. Therefore, the immobilization in chitosan-alginate beads increased the pH range for hydrolysis reaction performed by CRL. Paula et al. [35] identified a change in the optimum pH of *C. rugosa* lipase from 7.5 to 8.0 after immobilization by covalent bonding. Similar results were also found by Chiou and Wu [36], who observed a change in the optimum pH of lipase from *C. rugosa* from 8.0 to 9.0 after immobilization on chitosan.

Due to the low activity observed for the synthesis of *p*-NPL (free lipase: 2.43 ± 0.36 U/g —100%; immobilized lipase: no activity), free and immobilized CRL were put in contact with hexane, the solvent used for the synthesis reaction to dilute the substrate, and after a certain time, *p*-NPL hydrolysis activity was measured. As seen in Figure 1d, more than

80% of the enzymatic activity was lost after exposure for 10 min in hexane under reaction conditions (40 °C and 200 rpm). This may have happened because some lipases are unstable when placed in contact with organic solvents such as hexane. Some of the reasons include: denaturation, reduced solubility, changing pH, and removing water from the hydration layer of the enzyme.

The linearized Arrhenius equation was used to determine the apparent activation energies (E_a) for the free and immobilized CRLs with the hydrolytic and synthetic activity values obtained for each temperature. Activation energy is inversely related to the reaction rate (activity); that is, the higher the activation energy, the slower the reaction. In this work, E_a values of 66.69 KJ/mol and 29.15 KJ/mol for the hydrolysis activity performed by the free and immobilized CRL, respectively, were calculated. This showed that there was a reduction in the energy required for the formation of the enzyme-substrate complex with the immobilization process. E_a values for *Y. lipolytica* lipase immobilized in alginate-chitosan beads were also inferior (14.28 KJ/mol) to that of the free lipase (19.31 kJ/mol) (Pereira et al. [32]). For the synthesis activity, an E_a of 14.07 KJ/mol was calculated for free CRL.

2.1.2. Kinetic Parameters

The influence of substrate concentration on free and immobilized CRL activity was evaluated. Non-linear adjustments were performed using the Michaelis-Menten kinetic model, resulting in the apparent kinetic parameters: maximum reaction rate (V_{max}) and Michaelis-Menten constant (K_m) (Table 1).

Table 1. Kinetic parameters for free *C. rugosa* lipase (CRL) and immobilized CRL (dried chitosan-alginate microcapsules containing CRL).

Parameters	Immobilized CRL		Free CRL	
	Hydrolysis	**Synthesis**	**Hydrolysis**	**Synthesis**
V_{max} (μmol/min/g) [a]	2359.13	-	4121.84	2.24
K_m (μmol/L) [b]	660.02	-	403.06	234.44

[a] V_{max}: maximum reaction rate; [b] K_m: Michaelis-Menten constant.

The maximum reaction rate (V_{max}) obtained for the hydrolysis reaction with the free CRL was 1.75 times higher than with the immobilized CRL, which shows that CRL in its free form is more active. The entrapment of the enzyme in a polymeric matrix leads to diffusion restrictions of the substrate, or to the reduction of the enzymatic flexibility necessary for the formation of the enzyme-substrate complex. Similar results were observed by Pereira et al. [32] and by Monajati et al. [37] by immobilizing lipase and L-asparaginase, respectively. For the synthesis activity of free CRL, a very low V_{max} was obtained (2.24 μmol/min/g); no results for the immobilized CRL were obtained, due to the low activity values determined. Ozyilmaz [34] reported a V_{max} of 2750 U/g for the synthetic activity of CRL in hexane with *p*-nitrophenol and palmitic acid, but the hydrolysis activity of this CRL was much higher (1170 U/mg) than the one used in the present work (>2 U/mg). The author obtained higher V_{max} for CRL covalently immobilized onto silica gel via glutaraldehyde (2970 U/g) and via hydrophobic spacer arm (3550 U/g). The K_m values obtained for the hydrolysis reaction indicated that the entrapment of the enzyme reduced its affinity for the substrate because of the higher value calculated for the immobilized CRL in relation to the free CRL. Brígida et al. [38] also found an increase in K_m when immobilizing *C. antartica* lipase in coconut fiber. For synthesis activity, a low K_m was detected. For the synthesis of butyl butyrate using immobilized *C. rugosa* lipase activated by chitosan reinforced nanocellulose derived from raw oil palm leaves, Elias et al. [39] found a lower affinity for butanol (K_m = 137 mM) and butyric acid (K_m = 142.7 mM). Ozyilmaz [34] obtained a K_m of 1300 μM for the free CRL in synthesis reaction.

2.2. β-Sitosterol Oleate Production Using Free and Immobilized *C. rugosa* Lipase (CRL)

Parameters such as temperature, substrate molar ratio, enzyme loading, reaction time, and agitation are known to exert great influence on biocatalysis [11]. In this context, all these variables were considered for β-sitosterol oleate synthesis by free and immobilized CRL using experimental designs. Reactions were carried out in 10 mL mini reactors in a solvent-free reaction medium, since it was identified that the use of hexane was harmful for CRL. Preliminary tests were performed to screen β-sitosterol to oleic acid molar ratios (from 1:2 to 1:12) to evaluate its solubilization. No phytosterol solubilization was observed for 1:2, 1:3, 1:4, and 1:6 molar ratios (data not shown), which eliminated these proportions from the study. β-sitosterol remained soluble at the lowest reaction temperature (40 °C) for the other molar ratios (1:8; 1:10, and 1:12), which were chosen for the experimental design (Table 2).

Table 2. Fractional Factorial Design (FFD) to increase the esterification of β-sitosterol oleate catalyzed by free and immobilized *C. rugosa* lipase (CRL).

Run	Real Values (Corresponding Coded Levels)						Conv.$_{free}$ (%) [7]	Conv.$_{imb}$ (%) [8]
	Molar Ratio (mol/mol) [1]	[E_{imb}] (%) [2]	[E_{free}] (%) [3,*]	T (°C) [4]	RT (min) [5]	S (rpm) [6]		
1	$8_{(-1)}$	$1_{(-1)}$	$0.3_{(-1)}$	$40_{(-1)}$	$30_{(-1)}$	$800_{(+1)}$	25.14 ± 4.73	21.16 ± 5.60
2	$12_{(+1)}$	$1_{(-1)}$	$0.3_{(-1)}$	$40_{(-1)}$	$30_{(-1)}$	$200_{(-1)}$	29.75 ± 5.23	11.64 ± 4.40
3	$8_{(-1)}$	$5_{(+1)}$	$1.5_{(+1)}$	$40_{(-1)}$	$30_{(-1)}$	$200_{(-1)}$	24.40 ± 2.57	21.97 ± 6.99
4	$12_{(+1)}$	$5_{(+1)}$	$1.5_{(+1)}$	$40_{(-1)}$	$30_{(-1)}$	$800_{(+1)}$	33.06 ± 6.77	23.85 ± 6.93
5	$8_{(-1)}$	$1_{(-1)}$	$0.3_{(-1)}$	$50_{(+1)}$	$30_{(-1)}$	$200_{(-1)}$	17.35 ± 6.61	19.29 ± 5.75
6	$12_{(+1)}$	$1_{(-1)}$	$0.3_{(-1)}$	$50_{(+1)}$	$30_{(-1)}$	$800_{(+1)}$	21.17 ± 6.18	17.54 ± 6.30
7	$8_{(-1)}$	$5_{(+1)}$	$1.5_{(+1)}$	$50_{(+1)}$	$30_{(-1)}$	$800_{(+1)}$	21.38 ± 3.09	24.70 ± 0.91
8	$12_{(+1)}$	$5_{(+1)}$	$1.5_{(+1)}$	$50_{(+1)}$	$30_{(-1)}$	$200_{(-1)}$	24.87 ± 4.33	18.89 ± 1.24
9	$8_{(-1)}$	$1_{(-1)}$	$0.3_{(-1)}$	$40_{(-1)}$	$360_{(+1)}$	$200_{(-1)}$	30.91 ± 4.72	23.84 ± 6.70
10	$12_{(+1)}$	$1_{(-1)}$	$0.3_{(-1)}$	$40_{(-1)}$	$360_{(+1)}$	$800_{(+1)}$	23.01 ± 3.64	18.85 ± 5.20
11	$8_{(-1)}$	$5_{(+1)}$	$1.5_{(+1)}$	$40_{(-1)}$	$360_{(+1)}$	$800_{(+1)}$	82.13 ± 3.14	22.27 ± 5.70
12	$12_{(+1)}$	$5_{(+1)}$	$1.5_{(+1)}$	$40_{(-1)}$	$360_{(+1)}$	$200_{(-1)}$	84.01 ± 0.32	25.41 ± 5.51
13	$8_{(-1)}$	$1_{(-1)}$	$0.3_{(-1)}$	$50_{(+1)}$	$360_{(+1)}$	$800_{(+1)}$	40.54 ± 4.62	22.37 ± 2.60
14	$12_{(+1)}$	$1_{(-1)}$	$0.3_{(-1)}$	$50_{(+1)}$	$360_{(+1)}$	$200_{(-1)}$	36.97 ± 0.90	21.80 ± 7.70
15	$8_{(-1)}$	$5_{(+1)}$	$1.5_{(+1)}$	$50_{(+1)}$	$360_{(+1)}$	$200_{(-1)}$	83.42 ± 4.68	24.24 ± 2.29
16	$12_{(+1)}$	$5_{(+1)}$	$1.5_{(+1)}$	$50_{(+1)}$	$360_{(+1)}$	$800_{(+1)}$	74.25 ± 0.06	20.13 ± 3.80
17 (C [9])	$10_{(0)}$	$3_{(0)}$	$0.9_{(0)}$	$45_{(0)}$	$195_{(0)}$	$500_{(0)}$	69.90 ± 1.41	26.35 ± 7.52
18 (C [9])	$10_{(0)}$	$3_{(0)}$	$0.9_{(0)}$	$45_{(0)}$	$195_{(0)}$	$500_{(0)}$	59.49 ± 6.63	24.93 ± 6.64
19 (C [9])	$10_{(0)}$	$3_{(0)}$	$0.9_{(0)}$	$45_{(0)}$	$195_{(0)}$	$500_{(0)}$	54.01 ± 5.34	24.91 ± 6.01

[1] Molar Ratio: mol of oleic acid per mol of β-sitosterol; [2] Immobilized Enzyme load ([E_{imb}] (% dry mass)); [3] Free Enzyme load ([E_{free}] (% dry mass)); [4] Temperature (T, °C); [5] Reaction Time (RT, min); [6] Stirring (S, rpm); [7] β-sitosterol oleate conversion (Conv.$_{free}$, %); [8] β-sitosterol oleate conversion (Conv.$_{Imb}$, %); [9] Centre Point (C).
* 1 g of microcapsules contains approximately 0.3 g of *C. rugosa* lipase.

β-sitosterol to β-sitosterol oleate conversion varied from 17 to 85% when free CRL was used, demonstrating that the experimental conditions tested strongly influenced conversion. For CRL immobilized in microcapsules, no large variations were detected for β-sitosterol oleate conversion (from 11 to 27%). Furthermore, it was noted that the results were much lower than those obtained for the free CRL. To investigate the significance of the results, an analysis of variance (ANOVA) was performed, and the significance of the variables was verified using Fisher's statistical test (F test), considering a significance level of 10%. The Pareto diagrams depict the most significant variables and those not statistically significant in the range of the study (Figure 2).

Enzyme loading and reaction time significantly influenced β-sitosterol oleate conversion for both enzymes, showing a positive effect for the free (Figure 2a) and the immobilized (Figure 2b) CRL, indicating that higher enzyme loading and higher reaction times can improve conversion. However, the amount of enzyme used for a certain reaction strongly affects the technical-economic viability of biocatalytic processes, since these biocatalysts still have a high market value. Similarly, the reaction time greatly influences the viability of the process, due to the high costs for its maintenance [11]. Therefore, these variables must be optimized.

Temperature and stirring were not significant to β-sitosterol oleate conversion in the range studied at a significance level of 10% for both CRL forms (Figure 2a,b). Temperature

control contributes to the solubilization of reagents, to the increased molecular collision between enzyme and substrate, and even reduces mass transfer limitations [40]. Another important point is that enzymes have optimal temperatures, and temperatures that are very different from these can reduce their activity or even stop it. Despite that, temperature was not an important variable, probably because both CRLs have a wide optimal temperature range and the reagent solubilization was achieved at the lower temperature. Stirring is also of great importance in biocatalytic processes, contributing to the homogenization of the reaction system and also to increasing the collision between enzyme and substrate. However, for the present system, it seems that 500 rpm was already enough to cause this benefit.

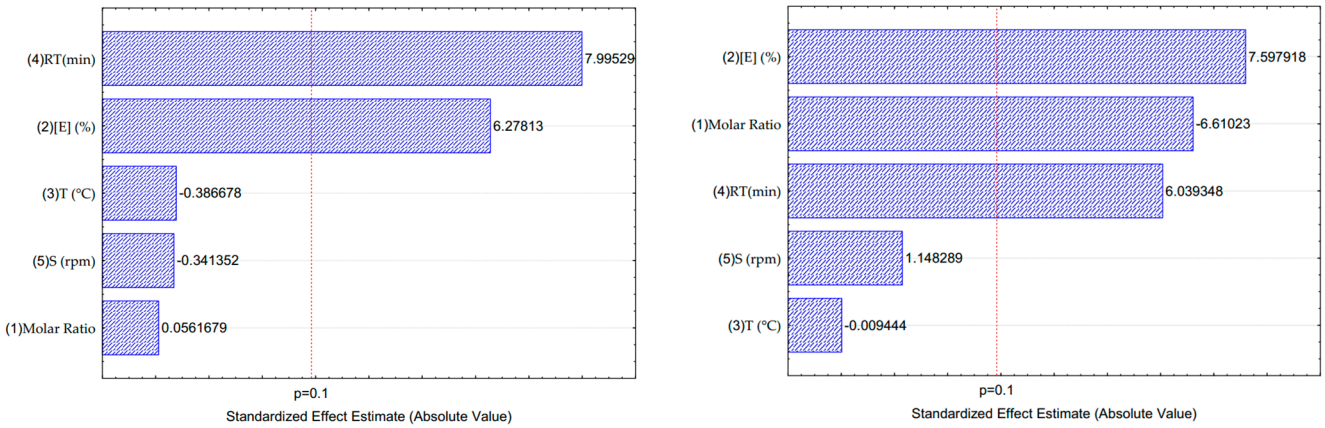

Figure 2. Pareto diagram for the estimated effect of each variable of the fractional factorial design: (**a**) β-sitosterol oleate conversion with free CRL (Conv.$_{free}$, %); (**b**) β-sitosterol oleate conversion with immobilized CRL (Conv.$_{Imb}$, %). CRL: *C. rugosa* lipase; [(1)] Molar Ratio: mol of oleic acid per mol of β-sitosterol; [(2)] Enzyme load ([E] (% dry mass)); [(3)] Temperature (T, °C); [(4)] Reaction Time (RT, min); [(5)] Stirring (S, rpm).

The oleic acid/ β-sitosterol molar ratio was only significant for β-sitosterol oleate conversion with the immobilized form of CRL, with a negative effect (Figure 2b). As the immobilized biocatalyst can be more subject to mass transfer problems [14], increasing the amount of oleic acid, which is a viscous liquid, might hinder the catalytic effect of CRL, especially because it is imprisoned in a microcapsule and the increase in viscosity may make it difficult for the reagent to enter the capsule. Pereira et al. [32] characterized alginate/chitosan microcapsules with *Yarrowia lipolytica* lipase entrapped, and found a drop in the V_{max} value of this biocatalyst; they associated this effect with a diffusional substrate constraint through the support. This might also be the reason for a lower conversion with CRL immobilized in microcapsules (<27%) in comparison to the free enzyme (84%).

Since it was not possible to reduce the oleic acid amount because a lower oleic acid/β-sitosterol molar ratio (<6:1) impaired the phytosterol solubilization, we decided to verify whether the conversion could be improved if the substrates and the enzyme were put in contact for a prolonged period (96 h). However, a conversion of no more than 32% was achieved after 24 h (Figure 3), indicating that the system was already at its best. Increasing the enzyme load was also not possible, due to the low density of the microcapsules, which, in concentrations greater than 6%, would impair the reaction medium homogenization (data not shown). Thus, to increase the reaction conversion, it may be necessary to study the immobilization parameters for *C. rugosa* lipase. Some of the possible parameters to investigate include alginate, chitosan, and calcium chloride concentrations, complexation time, and other encapsulating polymers.

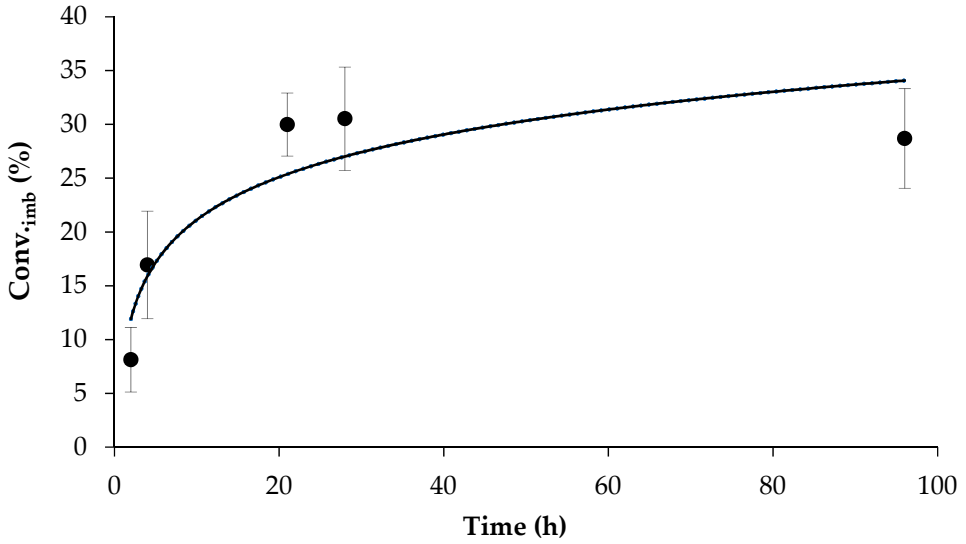

Figure 3. β-sitosterol oleate conversion by immobilized CRL. Reaction conditions: oleic acid to β-sitosterol Molar Ratio (8:1); Temperature (40 °C); Immobilized Enzyme load (5% dry mass); and Stirring of 500 rpm.

Therefore, as β-sitosterol oleate conversion by immobilized CRL was low, an experimental optimization was proposed for the free lipase. As the only variables that significantly influenced β-sitosterol to β-sitosterol oleate conversion were Enzyme load (E, %) and Reaction Time (RT, min), they were selected for the optimization strategy using Central Compound Rotational Design (CCRD). For these experiments, higher enzyme loads and longer reaction times were used, as shown in Table 3. Other variables, such as the oleic acid to β-sitosterol molar ratio, Temperature, and Stirring, were fixed at 8:1, 40 °C, and 500 rpm, respectively.

Table 3. Central Composite Rotational Design (CCRD) to increase β-sitosterol oleate esterification catalyzed by free *C. rugosa* lipase (CRL).

Run	Real Values (Corresponding Coded Levels)		$Conv._{free}$ (%) [3]
	$[E_{free}]$ (%) [1]	RT (min) [2]	
1	$0.9_{(-1)}$	$240.0_{(-1)}$	80.99 ± 3.27
2	$0.9_{(-1)}$	$720.0_{(+1)}$	90.24 ± 6.53
3	$2.7_{(+1)}$	$240.0_{(-1)}$	86.87 ± 5.85
4	$2.7_{(+1)}$	$720.0_{(+1)}$	96.26 ± 0.40
5	$0.54_{(-1.41)}$	$480.0_{(0)}$	80.85 ± 2.42
6	$3.06_{(+1.41)}$	$480.0_{(0)}$	85.07 ± 6.96
7	$1.8_{(0)}$	$140.6_{(-1.41)}$	91.50 ± 2.09
8	$1.8_{(0)}$	$819.4_{(+1.41)}$	92.15 ± 4.90
9 (C [4])	$1.8_{(0)}$	$480.0_{(0)}$	96.84 ± 0.14
10 (C [4])	$1.8_{(0)}$	$480.0_{(0)}$	95.76 ± 0.33
11 (C [4])	$1.8_{(0)}$	$480.0_{(0)}$	94.28 ± 1.22

[1] Free Enzyme load ($[E_{free}]$ (% dry mass)); [2] Reaction Time (RT, min); [3] β-sitosterol oleate conversion with free CRL ($Conv._{free}$, %) [4] Centre Point (C).

β-sitosterol oleate conversion ranged from 80.85 to 96.84% (Table 2), much higher than the $Conv._{free}$ obtained with the fractional factorial design (Table 1), evidencing the evolution toward better reaction conditions with the statistical designs. Analysis of variance was performed considering a significance level of 5%. The quadratic effect of RT, as well as the interaction between the enzyme load and the reaction time, were not significant and, therefore, were eliminated from the model. The ANOVA for the reduced model is presented in Table 4.

Table 4. Analysis of variance (ANOVA) for the Central Composite Rotational Design (CCRD) to increase the esterification of β-sitosterol oleate catalyzed by the free *C. rugosa* lipase (CRL).

Factor	DF [1]	Sum of Square	Mean Square	*F*-Value [2]	*p*-Value	R^2
						0.8129
(1) [E] (%) (L)	1	39.9298	39.9298	24.1984	0.038928	
[E] (%) (Q)	1	191.3228	191.3228	115.9461	0.008515	
(2) RT (min) (L)	1	47.8341	47.8341	28.9886	0.032808	
Lack of Fit	5	61.0145	12.2029	7.3952	0.123389	
Pure Error	2	3.3002	1.6501			
Total SS	10	343.4015				

[1] DF: degree of freedom; [2] F-value: Test for model variance and residual (error) variance comparison. (L) Linear term; (Q) Quadratic term; R^2: coefficient of determination.

Note that there was no significant lack of fit ($p > 0.05$) and a high R^2 (0.8129) was obtained for the model, which proves that the model can be used to predict the conversion rate of β-sitosterol to β- sitosterol oleate by free *C. rugosa* lipase. The model obtained using the real variables is shown in Equation (1).

$$\text{Conv.}_{\text{free}}\ (\%) = 62.14 + 27.63[\text{E}_{\text{free}}] - 6.98[\text{E}_{\text{free}}]^2 + 0.010\text{RT} \quad (1)$$

where $\text{Conv.}_{\text{free}}$ (%) is the β- sitosterol oleate conversion with the free *C. rugosa* lipase, $[\text{E}_{\text{free}}]$ is the enzyme load, and RT is the reaction time.

The model was used to generate the response surface plot (Figure 4). Due to the non-significance of the quadratic term of the reaction time variable, the critical values for β-sitosterol into β-sitosterol oleate conversion by free lipase from *C. rugosa* were not determined. However, it shows that increasing the reaction time beyond 500 min does not promote a relevant increase in the conversion. It is also noted that the use of an adequate enzyme load is crucial for obtaining high conversion rates without wasting the biocatalyst. This can be observed in the present study, in which enzyme loads above 2% do not improve process efficiency.

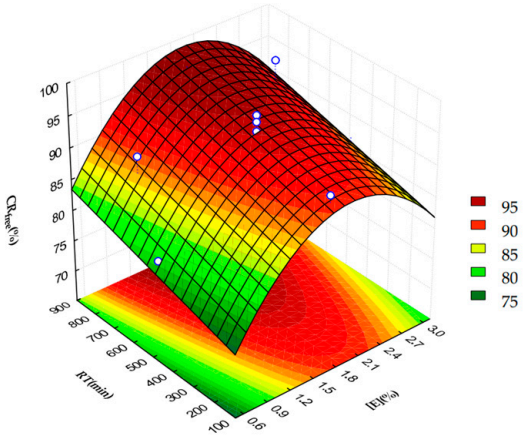

Figure 4. Response surface plots for β-sitosterol oleate conversion rate catalyzed by free CRL ($\text{Conv.}_{\text{free}}$, %), as a function of Enzyme load ([E], %) and Reaction Time (RT, min). White circles correspond to the experimental data.

To validate the obtained model, some reaction conditions were chosen, and the conversion was compared to its predicted values (Table 5).

Table 5. Predicted and observed β-sitosterol to β-sitosterol oleate conversion in the model validation process.

Run	[E] (%) [1]	RT (min) [2]	Predicted Values	Observed Values Real
1	0.60	200	78.20	69.13 ± 11.26
2	0.90	300	84.35	81.24 ± 4.34
3	1.20	400	89.24	92.56 ± 0.42
4	1.50	500	92.88	93.79 ± 1.23

[1] Enzyme load ([E], %); [2] Reaction Time (RT, min).

The observed values for the conversion of β-sitosterol oleate followed the same tendency as the predicted values, with similar values, which demonstrate that the model was validated.

2.3. Scale-Up of β-Sitosterol Oleate Production

To verify if the process of β-sitosterol oleate synthesis by the free CRL was scalable, the same reaction performed in 10 mL-glass conical flasks with 5 mL of reaction volume was tested in a 500 mL bioreactor (Multifors, INFORS, Basel, Switzerland) with 250 mL of reaction volume, a 50-times volume increase. The results obtained at an oleic acid to β-sitosterol molar ratio of 8:1, a temperature of 40 °C, a free enzyme load of 1.8% dry mass, and stirring at 500 rpm (best reaction conditions for the 10 mL flasks) are shown in Figure 5.

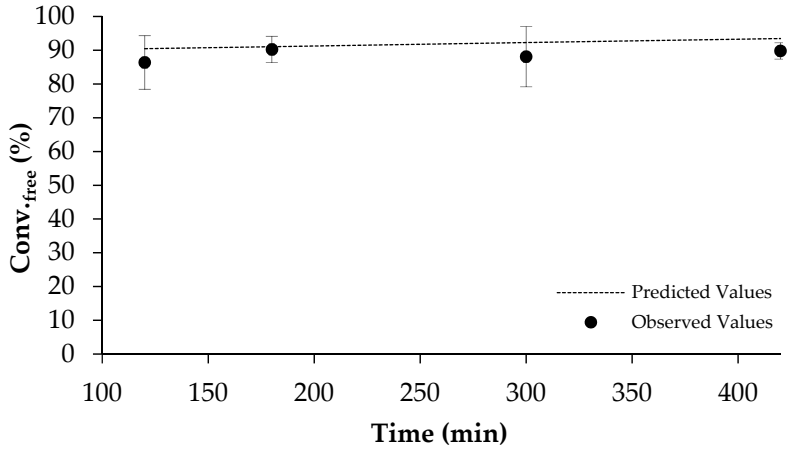

Figure 5. β-sitosterol oleate conversion catalyzed by free CRL ($Conv._{free}$ (%)) carried out in a 500 mL bioreactor. Reaction conditions: molar ratio (8:1); temperature (40 °C); free enzyme load (1.8% dry mass); and stirring at 500 rpm.

The increase in the enzymatic reaction scale was successful, as shown by the results of Figure 5, which depict high conversion values (above 80%) that were very close to those predicted by the mathematical model obtained in experimental design using mini reactors. These results are very important, as they confirm the possibility of developing this process at a higher production scale. Cui et al. [41] studied the production of phytosterol ester in a bioreactor (5 L) with water activity control using *Yarrowia lipolytica* lipase immobilized on inorganic support as a catalyst and a solvent-free system. The best result observed by the authors was a conversion of 91.1% in 78 h of reaction. Xu et al. [42] studied the phytosterol esters synthesis in a continuous flow bioreactor (1 mL/min) using immobilized lipase from *C. rugosa* and observed a high conversion (93.6%). Furthermore, the authors reported that the bioreactors were able to produce 1564 g of phytosterol esters/g of catalyst in a continuous processing period of 30 days. The results of the present study are close to the literature examples, demonstrating the possibility of using CRL for β-sitosterol oleate

synthesis on a large scale, but the immobilization procedure must be better investigated to enable its reuse.

3. Materials and Methods

3.1. Materials

Lipase from *Candida rugosa* (Manufacturer code: 62316; powder, yellow-brown, ≥2 U/mg), sodium alginate, chitosan, p-nitrophenyl laurate, p-nitrophenol, dimethylsulfoxide, acetic acid, Tris-HCl, hexane, chloroform, methanol, β-sitosterol, and oleic acid was acquired from Sigma Aldrich, Barueri, Brazil. Other materials: calcium chloride, sodium acetate, potassium phosphate, ethanol, acetone, and sodium hydroxide, were obtained from Kasvi (Paraná, Brazil).

3.2. Immobilization of C. rugosa Lipase (CRL) in Chitosan-Alginate Beads

C. rugosa lipase (CRL) was immobilized in chitosan-alginate beads, according to the optimal conditions described by Pereira et al. [24]. The biopolymer solution was prepared by solubilizing 3.1% (*w/v*) of sodium alginate in 10 mL of a free lipase solution (14 mg/mL). Then, the biopolymer solution was dripped into 100 mL of a solution of calcium chloride (0.14 M) and chitosan (0.2% (*w/v*)) using a 20 mL syringe and the system was kept under magnetic stirring (500 rpm) for 1 min. Microcapsules were collected using a sieve, frozen, and then freeze dried (LC 1500, Terroni, São Carlos, Brazil). Immobilization yield was determined by analyzing the difference in *Candida rugosa* lipase activity (U/mg of protein) detected in the calcium chloride + chitosan solution (gelling solution) before and after the microencapsulation process. The activity remaining in this solution is considered not encapsulated. Principally, in the immobilization, the enzyme is mixed with sodium alginate and dripped into a gelling solution (calcium chloride + chitosan). When the capsules are formed, the enzyme is mostly retained inside them, and the activity in the solution (*CRLresidual*) is determined. Furthermore, a blank sample consisting of the addition of the same amount of enzyme (used in the immobilization) directly to the calcium chloride + chitosan solution (without alginate) is prepared, and its activity is determined. This is considered 100% of activity that could be immobilized (*CRLinitial*). The immobilization yield consists of the difference between these activities (Equation (2)).

$$IY(\%) = \frac{CRLinitial - CRLresidual}{CRLinitial} 100 \quad (2)$$

Immobilization efficiency was determined by quantifying the encapsulated *CRL* activity after it was freeze dried (Equation (3)).

$$IE(\%) = \frac{CRLimb}{CRLinitial * f} 100 \quad (3)$$

where CRLimb is the enzymatic activity measured with the microcapsules after encapsulation and drying, CRLinitial is the enzymatic activity measured before the encapsulation process, and f is the matching factor (0.3). An amount of 1 g of microcapsules contains approximately 0.3 g of C. rugosa lipase.

3.3. Biochemical Characterization

3.3.1. Effect of Temperature and pH on *C. rugosa* Lipase (CRL) Activity

The optimal temperature of free and immobilized *C. rugosa* lipase was obtained from *p*-nitrophenyl laurate (*p*-NPL) hydrolysis and *p*-NPL synthesis, using a temperature range of 30 to 60 °C. Potassium phosphate buffer (50 mM, pH 7.0) was used to determine the optimal temperature. For optimal pH, different buffers were used: pH 4.0 and 5.0 sodium acetate buffer (50 mM); pH 6.0 and 7.0, potassium phosphate buffer (50 mM); pH 8.0 and 10.0 Tris-HCl buffer (50 mM). For these experiments, the temperature was maintained at

40°C. The activity values were used to calculate the apparent activation energy (E_a) using the linearized Arrhenius equation (Equation (2)) [43].

$$\ln K = \ln A - \frac{E_a}{R} \times \frac{1}{T} \quad (4)$$

where K is the catalyst reactivity constant, A is the frequency of the Arrhenius collision factor, E_a is the activation energy (KJ/mol), R is the universal gas constant (8.314×10^{-3} KJ/mol.K), and T is the absolute temperature (K).

3.3.2. Kinetic Parameters

The Michaelis-Menten constant (K_m) and the maximum reaction rate (V_{max}) were obtained from the *p*-nitrophenyl laurate (*p*-NPL) hydrolysis and synthesis by free and immobilized *C. rugosa* lipase (CRL). Substrate concentrations ranging from 20 to 1000 µM were used for the hydrolysis, and 125 to 750 µM were used for the synthesis. Reactions were performed as described in Sections 3.6.1 and 3.6.2. K_m and V_{max} were calculated from a non-linear regression of the reaction rate versus substrate concentration data using the Michaelis-Menten kinetics model.

3.4. β-Sitosterol Oleate Production Using Free and Immobilized C. rugosa Lipase (CRL)

Esterification reactions were carried out in 10 mL mini enzymatic bioreactors (Figure 6), with 5–8 mL of reaction medium. Mini bioreactors were developed in a cylindrical jacketed glass tank with a capacity of 10 mL of reaction medium. Stirring was performed using a magnetic bar and a magnetic stirrer with multiple positions (Model SP-10009/S—SPLabor, Sao Paulo, Brazil). The temperature was controlled by external heating using a thermostatic bath with external water circulation (Model: NT248—Novatecnica, Piracicaba, Brazil). The reactions were performed in batch mode.

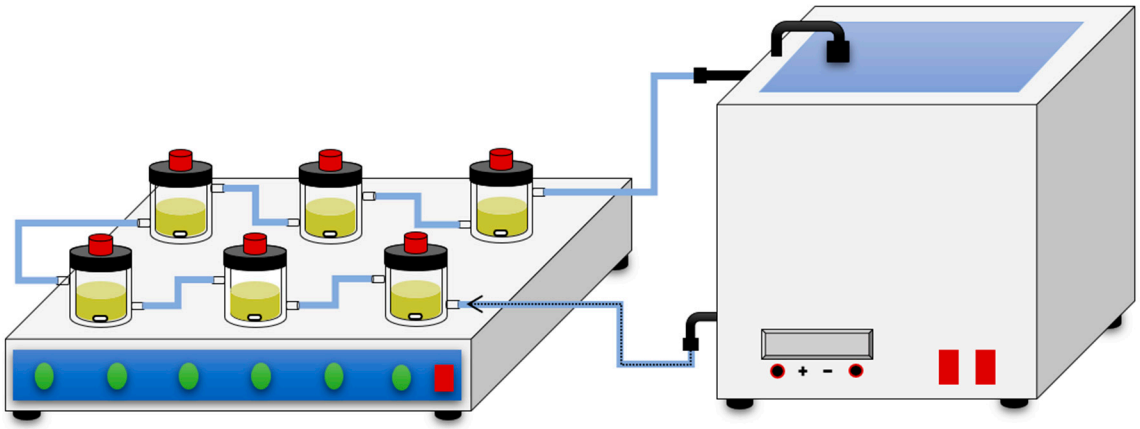

Figure 6. Mini reactors used for β-sitosterol oleate production by free and immobilized *C. rugosa* lipase CRL.

β-sitosterol and oleic acid were added to the bioreactors, followed by the addition of different concentrations (% dry mass) of free or immobilized *C. rugosa* lipase (CRL). The reactions were carried out with controlled magnetic stirring and temperature, and pre-defined reaction times. After the pre-defined reaction times, 0.1 mL of the sample was collected and added to 0.9 mL of chloroform. The resulting mixture was filtered through a 0.22 µm filter and stored for further analysis of β-sitosterol by Gas Chromatography. The conversion (Conv.) was calculated according to Equation (5).

$$\text{Conv. } (\%) = \left(\frac{C_1 - C_2}{C_1} \right) \times 100 \quad (5)$$

where C_1 and C_2 are the β-sitosterol concentrations (mM) before and after the reaction, respectively.

3.4.1. Fractional Factorial Design (FFD)

A 2^{5-1} FFD was used to evaluate the reaction conditions for β-sitosterol oleate conversion (Conv., %) with free and immobilized CRL. The effects of five independent variables, oleic acid to β-sitosterol Molar ratio (Molar ratio, mol of oleic acid/mol β-sitosterol); Enzyme load (E, %); Temperature (T, °C); Reaction Time (RT, min); and Stirring (S, rpm) on the Conv. were evaluated. Table 1 shows the level values for each parameter studied. Effects were considered significant when the p-value was less than 0.10.

3.4.2. Central Composite Rotational Design (CCRD)

A Central Composite Rotational Design (CCRD) was performed to increase β-sitosterol oleate conversion by free CRL. The independent variables were Enzyme load (E, %) and Reaction Time (RT, min). The new limits defined for each parameter are shown in Table 2. The results were analyzed using Analysis of Variance (ANOVA), considering a significance level of 5%. A second-order polynomial model with all linear, quadratic, and interaction coefficients was used to estimate β-sitosterol oleate conversion (Equation (6)).

$$\text{Conv.} = \beta_0 + \sum \beta_i X_i + \sum \beta_{ii} X_i^2 + \sum \beta_{ij} X_i X_j + \sum \beta_{ijk} X_i X_j X_k \quad (6)$$

where β_0 is the general process constant; β_i, β_{ii} are the linear and quadratic effects of X_i; and β_{ij} and β_{ijk} are the effect of the interaction between X_i and X_j and X_i, X_j and X_k, respectively.

3.5. Scale-Up of β-Sitosterol Oleate Production

The scale-up of β-sitosterol oleate production was performed in a 500 mL cylindrical glass stirred tank bioreactor (Multifors, INFORS, Bottmingen, Switzerland) with 250 mL of reaction medium, increasing volume by 50 times. Stirring in this bioreactor is performed by a six-bladed Rushton impeller with magnetic drive (radial flow impeller 4.0 cm above the vessel bottom) and temperature controlled by external heating. The reaction conditions were defined in the optimization process with the mini reactors, which were: molar ratio (8:1); temperature (40 °C); free enzyme load (1.8% dry mass); and stirring at 500 rpm. After the reaction, samples were collected and diluted 10 times in chloroform for GC analysis.

3.6. Analytical Procedures

3.6.1. Hydrolytic Activity

The hydrolytic activity of the free *C. rugosa* lipase was determined based on the hydrolysis reaction of p-nitrophenyl laurate (*p*-NPL) at 40 °C. The product formed during the reaction, *p*-nitrophenol (*p*NP), was monitored at 410 nm for 100 s in a spectrophotometer (Shimadzu (Kyoto, Japan) model UV-180). For *C. rugosa* immobilized lipase in microcapsules (containing the free enzyme), the hydrolytic activity was determined using mini reactors containing 25 mL of *p*-NPL solution and 5 mg of microcapsules, the system being kept under magnetic stirring for 5 min at 40 °C. The reaction was monitored by measuring the absorbance (410 nm) at 30 s intervals. One unit of hydrolysis activity was defined as the amount of *C. rugosa* lipase required to release 1 µmol of *p*-nitrophenol per minute under the assay conditions.

3.6.2. Synthetic Activity

Synthetic activity was determined as described by Ozyilmaz [34]. The reaction system was formed by an organic medium with 2 mL of hexane containing 1 mM of *p*-nitrophenol (*p*NP) and 50 mM of lauric acid. The reaction was started by adding 10 mg of free *C. rugosa* lipase or 30 mg of immobilized *C. rugosa* lipase to the reaction system, which was kept under orbital agitation at 200 rpm, 40 °C (Ecotron, INFORS HT, Switzerland) for 15 min. After the reaction was finished, 1 mL of solution was collected from the mixture and

added to 3.5 mL of 25 mM NaOH solution to recover unreacted *p*NP. Quantification was performed by measuring the absorbance at 410 nm in a spectrophotometer, and the *p*NP concentration was estimated by a standard curve of *p*NP at different concentrations, prepared in hexane and extracted with 3.5 mL of 25 mM NaOH solution. The difference in *p*NP concentration between the non-catalyzed and catalyzed medium was used to calculate the amount of *p*NP that participated in the esterification reaction. Activity was expressed as μmol *p*NP min^{-1} mg $catalyst^{-1}$.

3.6.3. β-Sitosterol Concentration by Gas Chromatography (GC)

The β-sitosterol concentration consumed during synthesis reactions was determined by GC in a GC-2010 gas chromatograph (Shimadzu, Japan) equipped with a split/splitless inlet, in an DB-5HT capillary column (15 m × 0.32 mm ID and 0.10 μm film thickness) and a flame ionization detector (FID). The temperature of the sample injector and the flame ionization detector was 320 °C and 350 °C, respectively. The temperature of the chromatographic column started at 210 °C for 2 min and was heated up to 320 °C at a speed of 10 °C /min and maintained for 15 min. Subsequently, the temperature was heated up to 350 °C at a speed of 10 °C/min.

3.7. Statistical Analysis

Experimental data were statistically analyzed with STATISTICA 7.1 software (StatSoft, Inc., Tulsa, OK, USA). The obtained models were statistically verified by means of analysis of variance (ANOVA) and the significance was determined by Fisher's statistical test (F test). The best experimental conditions for the conversion rate were determined using the surface response methodology. The variance explained by the model was observed by the determination coefficient (R^2).

4. Conclusions

C. rugosa lipase was microencapsulated by ionotropic gelation, characterized, and used in the β-sitosterol oleate synthesis. A high immobilization yield (99%) was obtained after microencapsulation, and a good immobilization efficiency (51%) was estimated. Immobilization of the enzyme did not change its optimal temperature and pH; however, a reduction in the maximum reaction rate was observed. A high β-sitosterol oleate conversion was obtained (>95%) for the free CRL when mini reactors were used. The scale-up with the free CRL using the conditions defined using the mini reactors led to a high conversion value (>92%). Therefore, this study demonstrates that *C. rugosa* lipase is an excellent biocatalyst for the β-sitosterol oleate synthesis. In addition, the use of mini reactors in synthesis reactions proved to be very efficient for the definition of important variables in the esterification process, enabling an enzymatic process scale-up.

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