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# Novozym® 435 and Lipozyme® RM IM as Biocatalysts for Benzyl Benzoate Synthesis

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**Abstract:** With the ever-increasing demand for clean technology in the industrial sector, natural methods, such as enzyme-catalyzed, represent a sustainable alternative to industrial chemical processes. In this context, the synthesis of benzyl benzoate ester using commercial immobilized lipases was evaluated. For this, a kinetic study was carried out to determine the reaction time (24 h) and enzyme concentration (10 wt%). Then, a $2^2$ full factorial design was proposed to evaluate the effect of molar ratio (benzyl alcohol to benzoic anhydride) and temperature on conversion of benzyl benzoate in the presence of tert-butanol as solvent. For the Novozym® 435, maximum conversion (32%) was achieved at 60 ºC, using a molar ratio of 1:5 (alcohol to anhydride). A maximum conversion of 51% was obtained for Lipozyme® RM IM at 40 ºC and the molar ratio of 1:5. The benzyl benzoate showed moderate antimicrobial action against *S. aureus* (MIC = 0.05 mg $\mu L^{-1}$). With the results, the conclusion was that the methodology of design of experiments was adequate for the proposed system and allowed the optimization of the production of benzyl benzoate.

**Keywords:** enzymatic synthesis; Novozym® 435; Lipozyme® RM IM; benzyl benzoate; lipase



## 1. Introduction

In recent years, there has been a tendency for consumers to prefer foods, ingredients, and additives labeled as natural as an important attribute when choosing the product, leading them to spend billions of dollars on them [1-4]. According to United States law, compounds such as esters obtained by enzymatic catalysis are labeled as natural, thus satisfying this trend by consuming natural products [5-7].

The biotechnological processes have clear environmental advantages compared to inorganic acids generally used as catalysts in chemical synthesis. In this way, the use of enzymes, which can be reused in biotechnological processes, reduces waste production [8,9]. In addition, these processes have proven to be a competitive alternative to chemical methods due to their high catalytic efficiency, moderate operating conditions, and the selectivity of natural catalysts [10,11], which guarantee higher yields close to room temperature. These characteristics result in quality products, energy savings, and the absence or reduction in the incidence of by-products.

The industrial use of lipases allows the development of technological processes, as well as energy savings and the minimization of thermal degradation, which are probably the major advantages of replacing conventional chemical technologies with biological processes. The high specificity of the lipases, the use of mild temperatures, and solvent-free reaction conditions make these processes commercially favorable and can replace the chemical catalysis of aromatic esters [12-15].

The benzyl benzoate is an ester that has very diversified and long-lasting applications [16-20], being very efficient as acaricidal [21-23] and considered vital in the treatment of scabies [24-26]. Recently, a study suggested that BRD-2 (Host Bromodomain protein) inhibitors could potentially block where the COVID-19 envelope protein E binds and is a possible drug target against COVID-19 [27]. According to the results obtained by Aydin *et al*. [27] using the AutoDock, benzoate esters, including benzyl benzoate, showed good binding affinities for BRD-2.

In this way, more investigation can be done aiming new applications for this ester as an antifungal and antimicrobial agent, since some studies have been shown that the emulsification of this compound reduces the irritation of the skin and eyes [24], and the encapsulation may guarantee a safe application for cosmetics and foods. The synthesis of benzyl benzoate by enzymatic route is still a poorly explored field, and although some studies have shown that there is a possible route by transesterification and acylation reactions [28-30], currently, there is no optimization of the process variables. Therefore, the objective of this work was to optimize the synthesis of benzyl benzoate using two commercial immobilized enzymes of microbial origin (Novozym® 435 and Lipozyme® RM IM) and the evaluation of its antimicrobial activity.

## 2. Materials and Methods

### *2.1. Substrates.*

The reagents used for the reactions were benzoic anhydride (98%, Acros - Brazil) and benzyl alcohol (Neon - Brazil) as substrates and tert-butanol (99%, Vetec - Brazil) employed as solvent. All the reagents used were of analytical grade, with no additional treatment.

### *2.2. Biocatalysts.*

Two commercial lipases of microbial origin were used: Novozym® 435, produced from *Candida antarctica* fraction B, immobilized in macroporous acrylic resin; and Lipozyme® RM IM, obtained from the *Rhizomucor miehei* fungus, immobilized in phenolic resin. Both enzymes were kindly provided by Novozymes S.A. (Araucária - Brazil).

### *2.3. Determination of reaction time.*

To define the reaction time using the commercial immobilized enzymes cited in item 2.2, a previous kinetic study was carried out with a constant agitation rate of 150 rpm, the temperature of 60 ºC, the molar ratio between the substrates of 1:1 (alcohol to anhydride), and 10 wt% (total substrates mass) of the enzyme. All reactions were performed in a 2 mL closed reactor in the presence of tert-butanol (1 mL). Aliquots were taken periodically at intervals of 6, 12, 18, 24, 36, and 48 hours. Benzyl benzoate conversion was determined using gas chromatography (GC) analysis. All assays were performed in triplicate.

*2.4. Effect of biocatalyst concentration on reaction kinetics.*

To define the enzyme concentration used to evaluate the effect of temperature and alcohol to anhydride molar ratio on benzyl benzoate production (item 2.5), a kinetic study was performed using Novozym® 435 and Lipozyme® RM IM, with reaction samples taken at intervals of 1, 2, 3, 6, 12, 18, 24, 36 and 48 hours. Concentrations for each enzyme were 2.5, 5, and 10 wt% (total substrates mass), at a fixed temperature of 60 ºC, agitation rate of 150 rpm, and benzyl alcohol to benzoic anhydride molar ratio of 1:2.5. Benzyl benzoate conversion was determined by each sample's gas chromatography (GC) analysis. All assays were performed in triplicate.

*2.5. Optimization of benzyl benzoate production.*

To evaluate the effect of temperature (40 to 60 °C) and substrates molar ratio (1:1 to 1:5) in the production of benzyl benzoate, a $2^2$ full factorial design was carried out with triplicate of the central point, totaling 11 experiments for each enzyme studied [31]. Reactions were conducted in a shaker incubator under constant agitation (150 rpm) and enzyme concentration (10 wt% - total substrates mass) over a period of 24 hours with tert-butanol as an organic solvent. All results were performed using online software Protimiza Experimental Design (https://experimental-design.protimiza.com.br/), considering a significance level of 95% ($p<0.05$).

*2.6. Evaluation of biocatalyst stability.*

At the end of each reaction, the enzyme was recovered from the reaction medium by filtration. After washing with n-hexane, solvent excess was removed, and the biocatalyst was left in the oven for about 1 h at 40 °C [32] and then used in the next reaction. To analyze the stability of the enzymes used, enzymatic syntheses were performed at a temperature of 40 °C, 150 rpm of agitation, 10 wt% (total substrates mass) of the enzyme, and alcohol to anhydride molar ratio of 1:5 in the presence of tert-butanol. Reaction time for the two enzymes was set at 24 hours. At the end of each batch, conversion into benzyl benzoate was determined by gas chromatography (GC). All assays were performed in triplicate.

*2.7. Benzyl benzoate purification.*

Benzyl benzoate purification was performed by means of adsorption column chromatography (20 x 1.5 cm) containing Silica Gel (Sigma-Aldrich) as the stationary phase. The mobile phase, in turn, consisted of a mixture of ethyl acetate and cyclohexene in the ratio of 1:14 (v/v) acidified with acetic acid (2%, v/v). The procedure involved the application of a 1 mL crude sample to the upper portion of column with constant elution of the mobile phase to separate the components of the sample. Samples were collected in 2 mL flasks at pre-set times, and the fractions of benzyl benzoate were analyzed using thin-layer chromatography (TLC - Sigma-Aldrich - Brazil). Benzyl benzoate was determined by the value of the ratio when compared to a standard of the substance. After the aliquots with greater benzyl benzoate conversion were separated, the solvent was evaporated under reduced pressure, giving a purity of 98%.

*2.8. Determination of benzyl benzoate conversion.*

The quantification of the ester produced was carried out based on the work of Chiaradia *et al*. [33], in which Shimadzu model GC-17A used the gas phase chromatography technique with FID detector. Analysis was performed using an INOWAX fused silica capillary column (30 m x 0.25 mm x 0.25 μm), with the following temperature programming: 3 °C/min (100-135 °C), 135-250 °C (10 °C/min), 250 °C (15 min), injector temperature of 250 °C and detector temperature of 275 °C. The injection mode selected was the split ratio of 1:100, using $N_2$ as the carrier gas. The sample volume was 40 μL of the reactional medium diluted ethyl acetate (1:10).

The conversion of benzyl benzoate was performed by monitoring the reduction in the signal area of the limiting agent (benzyl alcohol), as well as the benzyl benzoate peak.

*2.9. Evaluation of antimicrobial activity.*

The antibacterial activity of the purified benzyl benzoate was assessed against the Gram-positive *Staphylococcus aureus* (ATCC 25923) and the Gram-negative *Escherichia coli* (ATCC 25922) bacteria. The broth micro-dilution technique was used to determine the minimum inhibitory concentration (MIC), with 96-well microplates recommended by the Clinical and Laboratory Standards Institute – CLSI [34]. Samples of the purified benzyl benzoate, diluted at 200 mg $mL^{-1}$ in 10% dimethylsulfoxide (DMSO), were placed in the first microwells before being transferred to the adjacent microwells, containing Müller-Hinton broth, with the aim of obtaining two-fold serial dilution. The bacterial inoculum (0.1 mL) used had a concentration of $5x10^8$ UFC $mL^{-1}$ (colony forming units per mL) of the microorganism to be tested. Some wells from each microplate were reserved for executing negative controls containing DMSO solution. The bacteria under study were incubated with the commercial antibiotic amoxicillin (1 mg $mL^{-1}$ in DMSO solution) for the positive control. The microplates were aerobically incubated at 35 °C ± 1 °C, for 24 hours. After incubation, bacterial growth was verified by adding 10 μL to each microwell of a resazurin (7-hydroxy-3H-phenoxazin-3-one-10-oxide, Sigma-Aldrich - Brazil) solution of 6.75 mg $mL^{-1}$, noting the presence of the reddish bacterial bud at the bottom of each microwell. The MIC was defined as the last ester concentration capable of inhibiting bacterial growth.

## 3. Results and Discussion

*3.1. Determining reaction time for benzyl benzoate production.*

The kinetics of enzymatic catalysis change depending on the reagents, the biocatalyst, and quantity. Consequently, it becomes one of the most important process variables during the synthesis processes. Thus, knowing the reaction kinetics is fundamental for optimization studies, always aiming at the highest product yield with the least amount of biocatalyst and less reactional time. To determine the reaction time for benzyl benzoate synthesis, a previous kinetic test was performed using Novozym® 435 and Lipozyme® RM IM enzymes. In this step, 1 mL of tert-butanol was added in the following conditions: 1:1 of benzyl alcohol and benzoic anhydride and 10% by enzyme weight, 150 rpm of agitation, and temperature of 60 °C. At predetermined times, reaction samples were collected for up to 48 hours. At each interval, the conversion of the product was measured. The results are shown in Figure 1.

According to the results of enzymatic kinetics with Novozym® 435 and Lipozyme® RM IM (Figure 1), the highest conversion for Lipozyme® RM IM of 30.32% was observed in 12 hours. For Novozym® 435, conversion of 17.15% was observed in 48 hours, but the time used for the experimental design was 24 hours because it presented a 16.61% conversion. The results observed with both lipases are superior to those observed by Gryglewicz *et al*. [28] for the synthesis of benzyl benzoate using Novozym® 435 when comparing the short reaction times of the present study. Meneses *et al*. [30], in their preliminary tests for benzyl benzoate synthesis using an excess of benzoic anhydride (9 times) and tert-butanol as a solvent, observed a 30% conversion.

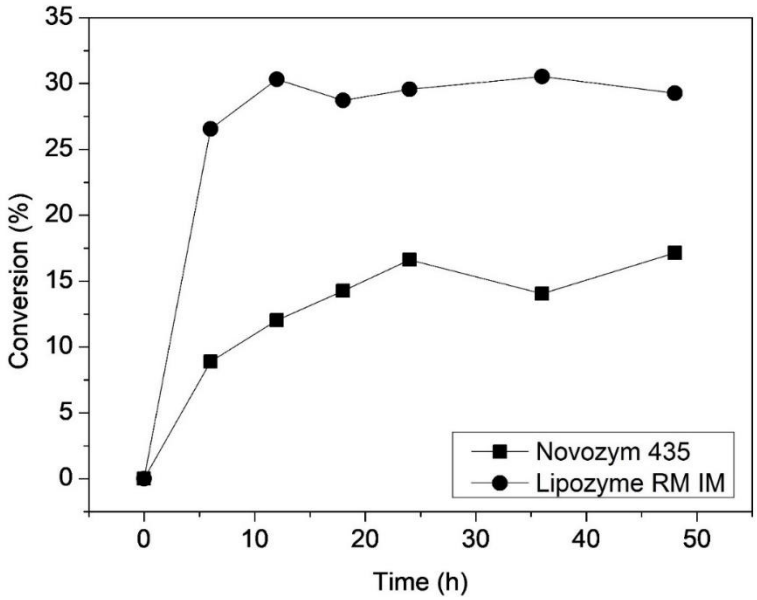


**Figure 1.** Kinetics for enzymatic production of benzoate production. Reaction conditions: temperature of 60 ºC, benzyl alcohol to benzoic anhydride molar ratio of 1:1, agitation of 150 rpm, 10% enzyme (based on the mass of substrates), and 1 mL tert-butanol. For all the assays, the standard deviation was ≤ 1.5%.

*3.2. Effect of enzyme concentration on benzyl benzoate synthesis*

A kinetic study was also carried out to evaluate the effect of enzyme concentration under the following conditions: temperature of 60 ºC, agitation rate of 150 rpm, and alcohol to anhydride molar ratio of 1:2.5, with enzyme concentration varying between 2, 5, and 10% by weight. This evaluation was performed for the enzymes Novozym® 435 and Lipozyme® RM IM.

In Figure 2, the condition which led to the best performance in the production of benzyl benzoate was that one that used a higher concentration of Novozym® 435 (10% by weight). This figure shows that the increase in catalyst content can positively affect the synthesis of benzyl benzoate. Similar behavior was observed for lipase Lipozyme® RM IM (Figure 3), and the higher conversion of the ester was obtained with the highest enzyme concentration (10% by weight). As it is known, the enzyme kinetics is directly related to the number of active sites available, thus using a greater mass of biocatalyst resulted in high product conversions.

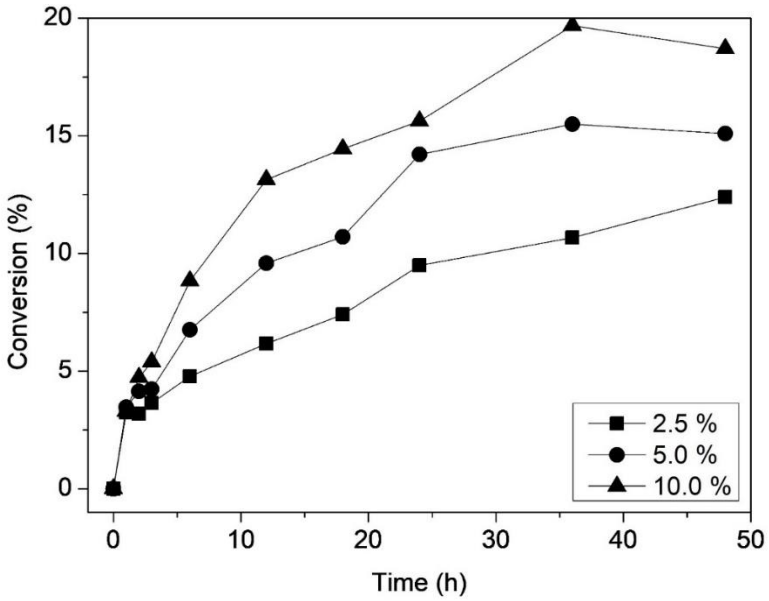


**Figure 2.** Evaluation kinetics of effect of Novozym® 435 concentration on benzyl benzoate conversion. The reactions were performed at 60 °C, 150 rpm of agitation using benzyl alcohol to benzoic anhydride molar ratio of 1:2.5. For all the assays, the standard deviation was ≤ 1.5%.

Comparing the reaction times of 36 and 48 hours, at 10 wt% concentration of Novozym® 435, there was a slight drop in the conversion of benzyl benzoate (Figure 2). When using Lipozyme® RM IM at a concentration of 10%, the conversion to benzyl benzoate at 36 and 48 hours remained constant (Figure 3). This reduction in product conversion can occur when an excessive amount of enzyme on the reaction is used, which can favor the formation of the particles agglomerates of the immobilized enzymes, preventing substrate access to the active site, thus contributing to the reduction in product conversion [35,36]. In addition, on the outer surface of the particles, enzymes are exposed to high concentrations of reactants, but mass transfer within the enzyme support can limit the entry of reactants reducing product synthesis.

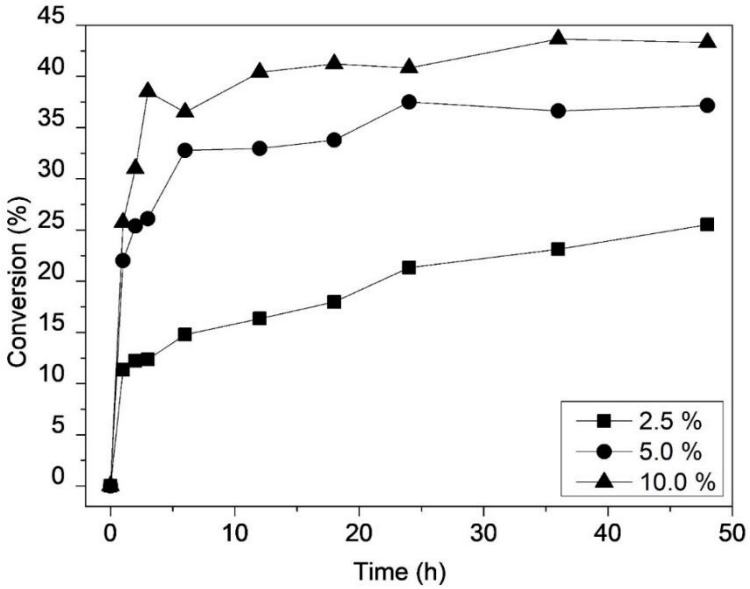


**Figure 3.** Evaluation kinetics of effect of Lipozyme® RM IM concentration on the synthesis of benzyl benzoate. The reactions were performed at 60 °C, 150 rpm of agitation using benzyl alcohol to benzoic anhydride molar ratio of 1:2.5. For all the assays, the standard deviation was ≤ 1.5%.

In these assays, possible substrate inhibition may have occurred due to excess benzyl anhydride that has been used. Benzoic anhydride can act as a lipase inhibitor, just as lipase was inhibited by acetic anhydride in synthesizing isoamyl acetate [8].

*3.3. Optimization of benzyl benzoate production.*

After determining reaction time and enzyme concentration for benzyl benzoate synthesis, the influence of temperature and molar ratio of the substrate in the process was performed. Therefore, a $2^2$ full factorial design was performed, keeping the enzyme concentration, agitation, and reaction time constant. All assays were performed in the presence of tert-butanol. Table 1 shows the matrix of $2^2$ full factorial designs with the values of each assay for the conversion of benzyl benzoate. The highest production (32%) of benzyl benzoate using Novozym® 435 was achieved in the condition with a molar ratio of 1:5 and temperature of 60 °C (assay 4, Table 1), whereas for Lipozyme® RM IM the highest production (50%) occurred in the assay 3 (Table 1), corresponding to the conditions of 40 °C and the molar ratio of 1:5.

**Table 1.** Matrix of the $2^2$ full factorial design (coded and real values) performed for optimization of benzyl benzoate production using the Novozym® 435 and Lipozyme® RM IM lipases. Reaction conditions: agitation of 150 rpm, 10% enzyme (total substrates mass), 1 mL tert-butanol, and 24 hours of reaction time.

| Assay | Temperature (ºC) | Molar Ratio[a] | Conversion (%) | |
|---|---|---|---|---|
| | | | Novozym® 435 | Lipozyme® RM IM |
| 1 | -1 (40) | -1 (1:1) | 10 | 21 |
| 1* | -1 (40) | -1 (1:1) | 9 | 21 |
| 2 | 1 (60) | -1 (1:1) | 9 | 21 |
| 2* | 1 (60) | -1 (1:1) | 10 | 20 |
| 3 | -1 (40) | 1 (1:5) | 22 | 50 |
| 3* | -1 (40) | 1 (1:5) | 22 | 51 |
| 4 | 1 (60) | 1 (1:5) | 32 | 49 |
| 4* | 1 (60) | 1 (1:5) | 32 | 49 |
| 5 | 0 (50) | 0 (1:3) | 19 | 43 |
| 6 | 0 (50) | 0 (1:3) | 19 | 44 |
| 7 | 0 (50) | 0 (1:3) | 19 | 44 |

[a] Benzyl alcohol to benzoic anhydride.
* Genuine replicates.

When an overall evaluation of the assays is carried out under the conditions studied, better performance of Lipozyme® RM IM was observed. This lipase showed a 50% higher conversion when compared to tests carried out under the same conditions using Novozym® 435. Only in the trial with the highest conversion for Novozym 435 (assay 4, Table 1) did this difference show a drop of approximately 35%. Higher conversions using Lipozyme® RM IM may be associated with several factors, such as the enzyme-producing microorganism, the type of immobilization and support, adjustment of the substrate to the active site, and the diffusion of the substrates, especially benzoic anhydride, through the pores of the support [30].

The experimental design results were analyzed statistically using the analysis of variance (ANOVA) to evaluate the effect of variables on the esterification reaction (Table 2). Therefore, to assess whether there are differences between the means, the *F test* was performed. When Novozym® 435 and Lipozyme® RM IM were used, a *calculated F* of 172 and 19 times greater than the *tabulated F*, respectively, was observed, which means there are statistical differences. The percentage of the total variance model was also possible to verify, evaluated by $R^2$, which presented the value of 99% for the reactions with Novozym® 435 and 92% for Lipozyme® RM IM.

Based on ANOVA, it can be concluded that the model generated by the results of Table 1 is predictive [31]. With these results, it was possible to generate the models demonstrated by Equations 1 and 2. Therefore, the coded models expressed by Equations 1 and 2 were used to generate the response surfaces for synthesis using Novozym® 435 (Figure 4) and the effect of molar ratio for Lipozyme® RM IM (Figure 5).

**Table 2.** Analysis of variance (ANOVA) for the benzyl benzoate production using the Novozym® 435 and Lipozyme® RM IM lipases.

| Variation source | Sum of Squares | Degrees of Freedom | Mean Square | $F_{calc}$ |
|---|---|---|---|---|
| **Novozym® 435** | | | | |
| Regression | 712.50 | 3 | 237.50 | 746.42 |
| Residuals | 2.22 | 7 | 0.31 | |
| Total | 714.70 | 10 | | |
| **Lipozyme® RM IM** | | | | |
| Regression | 1682.00 | 1 | 1682.00 | 95.37 |
| Residuals | 158.72 | 9 | 17.63 | |
| Total | 1840.72 | 10 | | |

Novozym® 435 *tabulated* $F_{0.95;3;7}$= 4.34.

Lipozyme® RM IM *tabulated* $F_{0.95;1;9}$= 5.11.

The results of the experimental design (Table 1) suggest that it is convenient to use the highest molar ratio of benzyl alcohol to benzoic anhydride (1:5) to obtain the highest conversions (Table 1, assays 3 and 4). This variable had a significant positive effect for both lipases used (Equation 1 and 2). Meneses *et al*. [30] concluded that an excess of alcohol is essential for the solubilization of the benzoic anhydride, thus facilitating its diffusion through the pores of the enzyme support. On the other hand, the use of excess methyl benzoate in relation to alcohol in alcoholysis reactions, such as transesterification, resulted in greater conversion of benzoate esters [28,29].

There was a significant positive effect (Equation 1) on the temperature effect when Novozym® 435 was used; that is, higher temperatures (60 °C) led to greater conversion into the product. However, for Lipozyme® RM IM the temperature did not show a significant effect, where both the assays performed at 40 °C and 60 °C showed very close conversion into the product (50 and 49%, respectively). Similar to the results obtained in this work, Meneses *et al*. [30] also observed that temperature (40 to 60 °C) did not significantly affect the production of benzyl benzoate within 24 hours of reaction. However, they observed a higher initial reaction speed with increasing temperature.

$$Y_1 = 18.45 + 2.50\,T + 8.75\,RM + 2.50\,T\,RM \quad (1)$$

$$Y_2 = 37.55 + 14.50\,RM \quad (2)$$

where: $Y_1$ is the conversion of benzyl benzoate to Novozym® 435 (%); $Y_2$ is the conversion of benzyl benzoate to Lipozyme® RM IM (%); T is the temperature (°C), and RM is the molar ratio (alcohol to anhydride).

As can be seen in Figure 4, the increase in temperature and molar ratio increases the conversion of benzyl alcohol in benzyl benzoate using Novozym® 435. Figure 5 shows the effect of increasing the molar ratio on the production of benzyl benzoate for Lipozyme® RM IM. To analyze the effects of temperature, enzyme concentration, substrate molar ratio, and solvent volume, Horchani *et al*. [37] used immobilized S*taphylococcus aureus* lipase to synthesize eugenol benzoate. According to the statistical results analyzed by the response surface, a great increase in the conversion of the benzyl ester in high levels of enzyme and molar ratio of the reaction substrates was observed. However, there was no significant increase

when a lipase amount above 295 IU was used, which could be related to the agglomeration of particles of the immobilized enzymes [36].

As the temperature did not significantly affect the conditions tested for Lipozyme® RM IM, any temperature within the range studied could be used. Then, the enzymatic synthesis can be conducted at mild temperatures, resulting in savings in the esterification process.

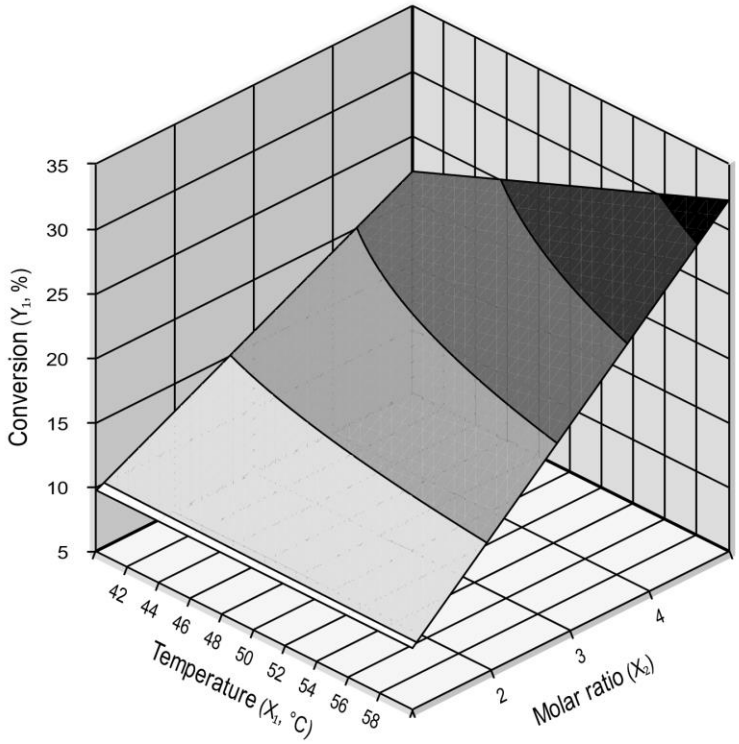


**Figure 4.** Response surface of benzyl benzoate production as a function of temperature and substrates molar ratio using Novozym® 435. Experimental data and conditions are shown in Table 1.

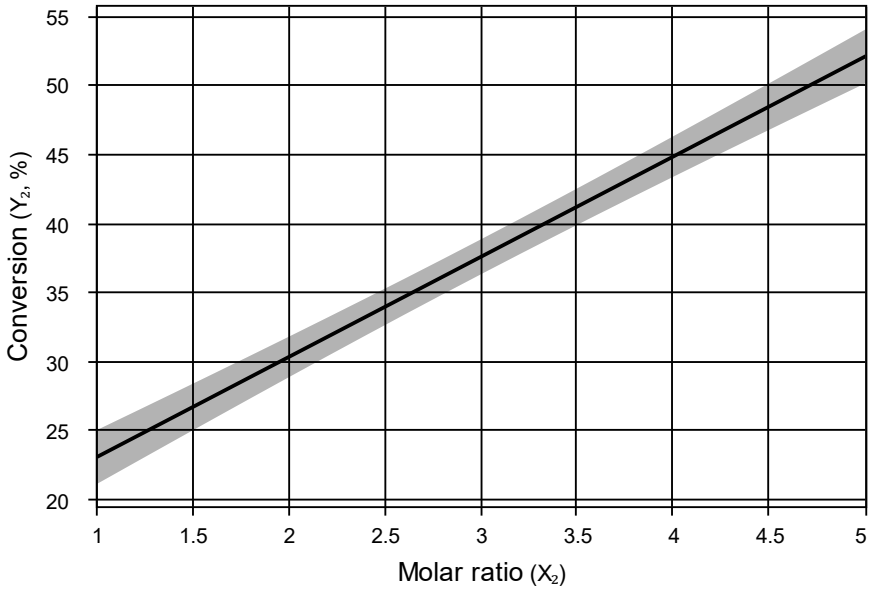


**Figure 5.** Benzyl benzoate production as a function of substrates molar ratio using Lipozyme® RM IM. Experimental data and conditions are shown in Table 1.

*3.4. Enzyme reuse cycles.*

According to Dhake *et al*. [38], enzymatic reuse is one of the main components to be able to apply the enzymatic synthesis on a large scale. Despite this advantage, the use of

immobilized enzymes as biocatalysts in the reaction is still an expensive process due to the high cost of the enzyme. Therefore, the application of enzymatic reuse in the industrial sector can become an important factor in the project's economic viability [39,40].

As such, the reuse of lipases was studied to synthesize benzyl benzoate under optimized reaction conditions in the experimental design. For these experiments, the condition that produced the best benzyl benzoate conversion using Novozym® 435 was that one from the assay 4 (Table 1), while for Lipozyme® RM IM, it was the assay 3 (Table 1). At the end of each cycle, the enzymes were recovered by filtration, washed with n-hexane, and filtered under a vacuum. They were then kept in a laboratory oven at 40 °C for one hour and placed in a desiccator for 24 hours [32]. This procedure was repeated for both enzymes. All assays were performed in triplicate. At the end of each cycle, conversion to benzyl benzoate was measured. Five cycles of use for each enzyme were evaluated, and the results are shown in Figure 6.

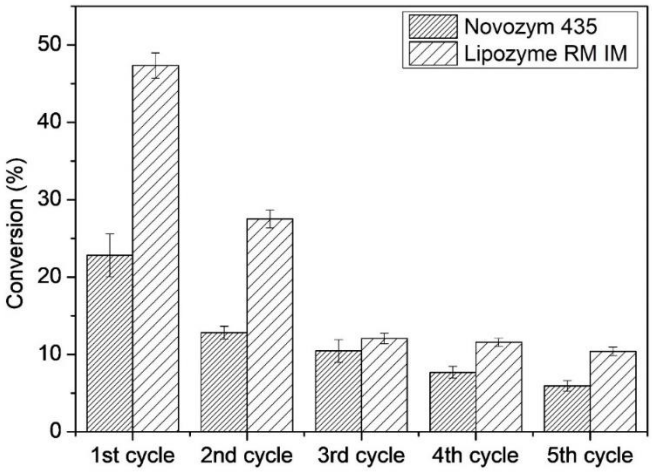


**Figure 6.** Cycles of reuse of Novozym® 435 and Lipozyme® RM IM lipases for benzyl benzoate production. Experimental conditions for Novozym® 435: temperature of 60 ºC, benzyl alcohol to benzoic anhydride molar ratio of 1:5, agitation of 150 rpm, 10% enzyme (total substrates mass), 1 mL of tert-butanol and 24 hours of reaction time. Experimental conditions for Lipozyme® RM IM: temperature of 40 ºC, benzyl alcohol to benzoic anhydride molar ratio of 1:5, agitation of 150 rpm, 10% enzyme (total substrates mass), 1 mL of tert-butanol and 24 hours of reaction time.

During the cycles, particularly from the first to the second, it was noted that there was a decline in the percentage of ester produced for both enzymes. This decrease in the benzyl benzoate conversion rate was approximately 40% when comparing the two enzymes' first cycles. However, from the third cycle on, it was noted that desired product conversion became constant (variation of approx. 2%).

According to Lerin *et al*. [32] and de Sousa *et al*. [41], the reasons for the reduction of catalytic activity during the cycles may be caused by the deactivation of the enzyme due to thermal effects, presence of substrates, long time reaction, product conversion and the decrease of the integrity of the support, which can affect the protein binding with the support. In addition, according to Gomes *et al*. [42], the increase in temperature leads to an increase in the speed of the reaction per enzyme unit. However, the use of the enzyme at an elevated temperature for a prolonged time may result in enzyme deactivation, reducing product conversion.

*3.5. Evaluation of antimicrobial activity.*

After purification, the benzyl benzoate was evaluated in relation to its antibacterial activity against Gram-positive *S. aureus* and Gram-negative *E. coli* bacteria through the

Minimum Inhibitory Concentration (MIC), using the broth micro-dilution technique. Comparing the antimicrobial activity evaluation techniques of disc diffusion with broth micro-dilution, the latter has the advantage of gains in space-saving, the quantity of reagent used, and the efficiency of results due to the number of plates prepared from a series of antimicrobial dilutions, besides the quantification of the generated result. Identification of bacteria inhibition takes place by observing the change in color: pink indicates the growth of the bacteria, while the closer to blue the color, the greater the inhibition.

The MIC is the lowest concentration of benzyl benzoate capable of inhibiting the growth of the *S. aureus* and *E. coli* bacteria as identified by the wells of a bluish color. In this study, MIC against *S. aureus* was 0.05 mg $\mu L^{-1}$. As per the criteria set forth by Machado et al. [43], benzyl benzoate's moderate antibacterial activity against *S. aureus*. However, there was no antibacterial activity detected against *E. coli*. According to Morris *et al*. [44], of the 521 fragrance materials analyzed, 44% were identified as having significant antimicrobial activity against at least one of the test organisms (*S. aureus, E. coli*, and *Candida albicans*). However, benzyl benzoate showed weak activity for the tested microorganisms.

## 4. Conclusions

The optimization of the production of benzyl benzoate through the methodology of design of experiments (DOE) was successfully used in this work, showing itself to be an excellent methodology for the optimization of bioprocesses that seek to reduce environmental impacts in the production of biocompounds. Based on the results obtained, optimum production (51%) was achieved using Lipozyme® RM IM and, for Novozym® 435, a conversion of 32% in 24 hours of reaction time. The benzyl benzoate showed moderate antimicrobial activity against the Gram-positive bacteria *S. aureus*.

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## Conflicts of Interest

The authors declare no conflict of interest.

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