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Exploring Whole Cells of *Rhizopus oryzae* for Efficient Synthesis of Emollient Esters: Bioesters for Cosmetics

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Production of esters, organic compounds with emollient, emulsifying, and aromatic properties is growing due to high demand and their applications in the cosmetics industry. The synthesis of these compounds involves the reaction between a carboxylic acid and an alcohol, which can be catalyzed by lipases. In this context, the objective of this study was to produce mycelium-bound lipases of the fungus *Rhizopus oryzae* through submerged cultivation, with the aim of applying them in the synthesis of ethylene glycol oleate, ethylene glycol stearate, and isoamyl palmitate esters, which are relevant to the cosmetic industry. The influence of solvent usage (heptane), biocatalyst mass, and acid/alcohol molar ratio was evaluated in the esterification reactions. In the synthesis of the three tested esters, and in the presence of heptane solvent, an equimolar ratio (1:1) of each acid and alcohol with a concentration of 0.01 mol L^{-1} was utilized. The reactions were performed in agitated Erlenmeyer flasks at 40 °C with an agitation speed of 200 rpm. The lipolytic biomass of *Rhizopus oryzae* showed better performance in the synthesis of isoamyl palmitate, achieving a maximum conversion of 83% in 24 h of reaction using 0.30 g of lipolytic biomass and the solvent heptane. In addition, it was observed that higher concentrations of catalyst biomass (0.45 g) and lower molar ratios (1:1.5) of acid to alcohol resulted in a higher conversion of isoamyl palmitate ester, reaching conversions of 48.59 and 83.11%, respectively.

Keywords: esterification, mycelium-bound lipases, *Rhizopus oryzae*, emollient esters

Introduction

The industrial application of esters has become extremely important for current researchers, as these compounds are widely required, particularly in the cosmetic, food, and pharmaceutical industries, due to their aromatic, emulsifying, and emollient properties. Although these compounds can be extracted from plant and animal species, obtaining them on a large scale requires a significant amount of raw material and involves high costs, making the synthesis of these compounds a more economically viable alternative.[1]

Esters can be produced on a laboratory and industrial scale through esterification reactions, which occur between a carboxylic acid and an alcohol, in the presence or absence of catalysts. To obtain a product of the highest possible quality and reduce costs, the use of chemical or biological catalysts is interesting to optimize the process by increasing the reaction rate and consequently reducing the time involved.[2-5]

One of the industries most interested in the development of new technologies for ester synthesis is the personal care, perfumery, and cosmetics sector, which achieved a turnover of $1.52 billion in 2022, showing a growth of 9.4% compared to the turnover recorded in 2021 ($1.39 billion) according to data collected by the Brazilian Association of the Personal Hygiene, Perfumery, and Cosmetics Industry.[6] Furthermore, this survey also reveals that in this year, the export rate ($776.5 million) exceeded the import rate ($741 million) of these products, indicating Brazil's significant participation in the global market.

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This growth is attributed, among other factors, to the fact that these industries are embracing the development of new processing technologies, primarily focusing on sustainability.[7]

Lipase-catalyzed esterification has gained prominence due to the increased use of organic esters in biotechnology and the chemical industry. Several studies[3,5] have been conducted using microbial lipases, different alcohols, and reaction systems. Factors such as enzyme and substrate concentrations, molar ratio, pH, temperature, mixing rates, and water content play an important role in esterification yield.[8,9] In this scenario, the use of enzymes as catalysts for the reaction is interesting, as they are not only bio-sustainable but also have other advantages over chemical catalysts, such as high specificity, operation under milder conditions, and no generation of unwanted by-products.[1,9]

Despite all the advantages, the use of enzymes entails a high cost related to the production, purification, and even enzymatic immobilization process. However, this obstacle can be overcome by using biomass from fungal species that produce mycelium-bound lipase, such as *Mucor circinelloides*,[10] *Penicillium citrinum*,[11] and especially *Rhizopus oryzae*,[12] the focus of this work. These lipases can catalyze hydrolysis, esterification, and transesterification reactions without the need for immobilization because the produced lipase remains active even after obtaining and death of the fungal biomass, allowing the use of intact cells of these fungi as a biocatalyst for various industrial applications, thus reducing the high cost involved in enzymatic catalysis.[13]

Considering the limited number of studies involving the application of intact lipolytic cells in esterification reactions, particularly for the production of emollient esters of interest to the cosmetics industry, this study aimed to evaluate the synthesis of different emollient esters (ethylene glycol oleate, ethylene glycol stearate, and isoamyl palmitate) in a solvent-free system, catalyzed by lipases attached to the mycelium in submerged cultivation of the fungus *Rhizopus oryzae*. The study also aimed to investigate the influence of enzyme concentration and alcohol/acid ratio on the process to achieve a better esterification conversion.

Experimental

Materials

Sabouraud agar (HiMedia, Kennett, USA), soy peptone (HiMedia, Kennett, USA), magnesium sulfate heptahydrate (Vetec, Duque de Caxias, Brazil), olive oil (Carbonell, Córdoba, Spain), gum arabic powder (Dinâmica, Indaiatuba, Brazil), disodium phosphate dibasic (Dinâmica, Indaiatuba, Brazil), sodium hydroxide (Vetec, Duque de Caxias, Brazil), ethyl alcohol (70% v/v) (Vetec, Duque de Caxias, Brazil), acetone (Synth, Diadema, Brazil), phenolphthalein (Synth, Diadema, Brazil), isoamyl alcohol (Dinâmica, Indaiatuba, Brazil), palmitic acid (HiMedia, Kennett, USA), ethylene glycol (Dinâmica, Indaiatuba, Brazil), oleic acid (Dinâmica, Indaiatuba, Brazil), heptane (Synth, Diadema, Brazil) were used as received.

Cultivation of *Rhizopus oryzae* on solid medium

The strain used was *Rhizopus oryzae* CCT3759 (*R. oryzae*) obtained from the André Tosello Foundation for Research and Tropical Technology (Campinas-SP, Brazil). To obtain and maintain active spores of the culture, fungal cells were previously inoculated on Sabouraud agar medium under aseptic conditions. The spore culture was incubated at 30 °C for 72 h or until reaching the highest sporulation status. The cells were washed with 10 mL of sterile distilled water to obtain a spore suspension under aseptic conditions for subsequent inoculation.

Submerged cultivation of *Rhizopus oryzae*

The culture medium and inoculation method used were established by Reis *et al.*[12] The culture medium consisted of 30 g L^{-1} of vegetable oil, 70 g L^{-1} of soy peptone, 1 g L^{-1} of $NaNO_3$, 1 g L^{-1} of KH_2PO_4, and 0.5 g L^{-1} of $MgSO_4.7H_2O$, all previously autoclaved (121 °C for 15 min). After autoclaving, the Erlenmeyer flasks were cooled, and olive oil (3% m/v) was added in a sterile manner. The cultures were conducted in 250 mL Erlenmeyer flasks containing 100 mL of autoclaved medium and inoculated with a suspension of 1×10^6 spores at 30 °C with orbital agitation at 180 rpm. The spore concentration was determined by counting the cells in a Neubauer chamber using an Olympus® binocular microscope (Hicksville, USA). At the end of the cultivation, the produced biomass was separated from the medium by vacuum filtration, washed with water and acetone, and quantified for hydrolytic activity and moisture content by drying the wet biomass in a microwave oven (180 W for 5 min) according to the methodology of Pereira and Kilikian.[14] Subsequently, the fungal biomass was stored at 4 °C before use. Moisture content was calculated using equation 1, where DW represents the dry mass (final), WM represents the wet mass (initial), and MC represents the moisture content.

$$DW = (1 - MC) \times WM \quad (1)$$

Biochemical properties of lipase bound to the mycelium of the fungus *R. oryzae*

The biochemical properties of the mycelium-bound lipase from the fungus *R. oryzae* include its temperature and pH optima. Studies conducted by Reis *et al.*[12] provided insights into these properties, revealing that the lipase exhibits an optimal temperature of 40 °C and an optimal pH of 6.0.

Determination of lipolytic activity

Lipolytic activity was determined using the olive oil emulsion hydrolysis method proposed by Marotti *et al.*[11] The fatty acids formed were titrated with 20 mmol L^{-1} NaOH solution, using phenolphthalein as an indicator. One unit of activity was defined as the amount of enzyme that releases 1 µmol of fatty acid *per* min of reaction, under assay conditions (0.30 g of biomass or 1 mL of culture broth at 37 ºC reaction temperature, 100 mmol L^{-1} sodium phosphate buffer and pH of 7.0 in 5 min of reaction).

Effect of solvent on esterification reactions

Reactive media were prepared for the synthesis of three different esters: ethylene glycol oleate (EGO) (oleic acid/ethylene glycol), ethylene glycol stearate (EGS) (stearic acid/ethylene glycol), and isoamyl palmitate (IPA) (palmitic acid/isoamyl alcohol). The esterification reactions of the biomass were conducted in sealed Duran flasks containing an equimolar ratio (1:1) of each acid and alcohol, and a concentration of 10 mmol L^{-1} for both reagents. After adding the reagents, the flask volume was completed with heptane, which acted as the organic solvent, to reach a total volume of 20 mL of reaction medium. The reactions were performed in duplicates, with 0.30 g of dried biomass, and a control without the presence of a catalyst to evaluate the spontaneous formation of esters. All flasks were incubated in a shaker at 40 °C with orbital agitation at 200 rpm. After 24 h of reaction, aliquots were taken from each assay and quenched by adding 10 mL of a 1:1 ethanol/acetone solution. The same protocol was adopted for the esterification reactions in the absence of solvent. Heptane was not added to the reaction medium, keeping it at a concentration of 10 mmol L^{-1} for each reagent. The amount of produced esters was quantified by titration with 20 mmol L^{-1} NaOH using phenolphthalein as an indicator.

One unit of esterification activity was defined as the amount of enzyme required to form one micromole of ester *per* min under the assay conditions.[15] The percentage of esterification was determined using equation 2, where FFA_0 is the initial free fatty-acids (FFA) concentration (mol L^{-1}), and FFA_t is the residual FFA concentration after a certain time t (mol L^{-1}).[16]

$$\text{Esterification}(\%) = \frac{FFA_0 - FFA_i}{FFA_0} \times 100 \quad (2)$$

Effect of concentrations of mycelium-bound lipases on the synthesis of isoamyl palmitate

An esterification reaction was performed in duplicate using a mixture of palmitic acid and isoamyl alcohol (1:1) in the presence of heptane. Three different concentrations of mycelium-bound lipases were used, corresponding to 0.15, 0.30, and 0.45 g of dry biomass. Aliquots were taken periodically to measure the esterification percentage during the 24 h reaction.

Effect of the molar ratio of acid to alcohol on the production of isoamyl palmitate

After selecting the emollient ester with the highest esterification percentage and optimal lipase concentration, a new study was conducted to investigate the influence of increasing the alcohol concentration relative to the acid on the reaction rate and conversion. Therefore, a new batch of esterification reactions was performed in duplicate using solutions containing palmitic acid and isoamyl alcohol in the presence of heptane as a solvent. The molar ratios tested were 1:1, 1:1.25, 1:1.5, 1:2, 1:6, and 1:12, while maintaining a fixed dry biomass of 0.30 g. Aliquots were taken at 1, 2, 4, 6, 8, 10, and 24 h to monitor the reaction behavior in ester synthesis.

Results and Discussion

Submerged cultivation of *Rhizopus oryzae*

First, the cultivation of the fungus *R. oryzae* was performed to produce the lipolytic biomass to be used as a biocatalyst in the esterification reactions. Figure 1a shows the submerged cultivation after 72 h, and Figure 1b depicts the biomass obtained after vacuum filtration. The average fungal dry mass obtained in each cultivation was 1.6 to 2 g.

Effect of solvent use on the synthesis of ethylene glycol oleate, ethylene glycol stearate and isoamyl palmitate

Initially, in order to prove the adsorption capacity of the reagents by the fungal biomass, a test with a biomass inactivated by an alcohol/acetone solution (1:1) was added

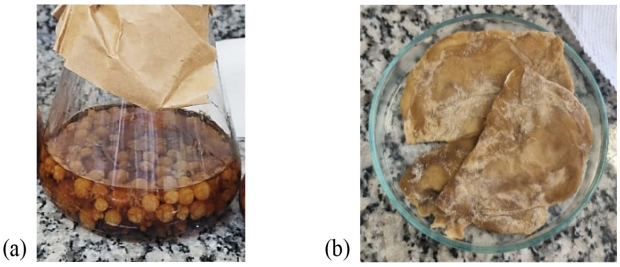

Figure 1. (a) *R. oryzae* after 72 h of cultivation and (b) the filtered biomass.

to the reaction medium containing an equimolar ratio (1:1) of each acid and alcohol, and a concentration of 10 mmol L^{-1} for both reagents for the synthesis of EGO, EGS, and IPA. The results indicated that there was no conversion to any of the esters of interest in this study, so the other reactions could proceed to evaluate the biomass containing active lipases in the esterification reactions. In order to determine the activity provided for the esterification reaction, the activities of the biomass that were applied as a biocatalyst were evaluated. Table 1 shows the values referring to moisture and enzymatic activity present in the biomass used in the execution of this experiment.

The results from Table 1 show that both biomass samples used in the esterification reactions presented high moisture content, with values of 40.57 and 60% moisture for the solvent and solvent-free systems, respectively. The hydrolytic activities obtained for the systems with and without solvent were 122.03 and 100.80 U g^{-1}, and since 0.30 g of biomass was used as a biocatalyst in each reaction, an activity of 36.60 and 30.25 U was provided for the respective reaction systems.

After determining the activity of the biomass containing the lipase, esterification reactions were performed to form the following esters: EGO, EGS, and IPA. The reactions were performed in agitated flasks at 40 °C with a shaking speed of 200 rpm, using 0.30 g of dry biomass as the catalyst for both the solvent and solvent-free systems. The results obtained regarding the influence of the solvent heptane on the reaction of each ester are described in Figure 2.

Considering the results presented in Figure 2, a distinct behavior is observed among the evaluated esters in this study, which is due to the molecular structure of the acids and alcohols used, as well as the biochemical properties of the lipase when in contact with them and the reaction conditions, such as the application of an organic solvent.

According to Figure 2a, the results of the esterification reactions with solvent show that the assay without biocatalyst exhibited higher conversion values for the synthesis of EGO. After 6 h of reaction, the conversion reached 27.11%, while the system containing the biocatalyst achieved a maximum conversion of only 22.04%. Therefore, the lipolytic cells of *R. oryzae* were not efficient in converting EGO under the proposed conditions in this study. The same behavior was observed in the synthesis of EGS, where, despite a higher maximum conversion with the use of the catalytic biomass (6.14%

Table 1. Moisture, hydrolytic activity and activity offered for the synthesis of esters (EGO, EGS and IPA) in the presence of heptane

System	Moisture / %	Activity / (U g^{-1})	Activity for esterification / U
Solvent	40.57	122.03 ± 15.23	36.60
No solvent	60.0	100.80 ± 12.50	30.25

EGO: ethylene glycol oleate; EGS: ethylene glycol stearate; IPA: isoamyl palmitate.

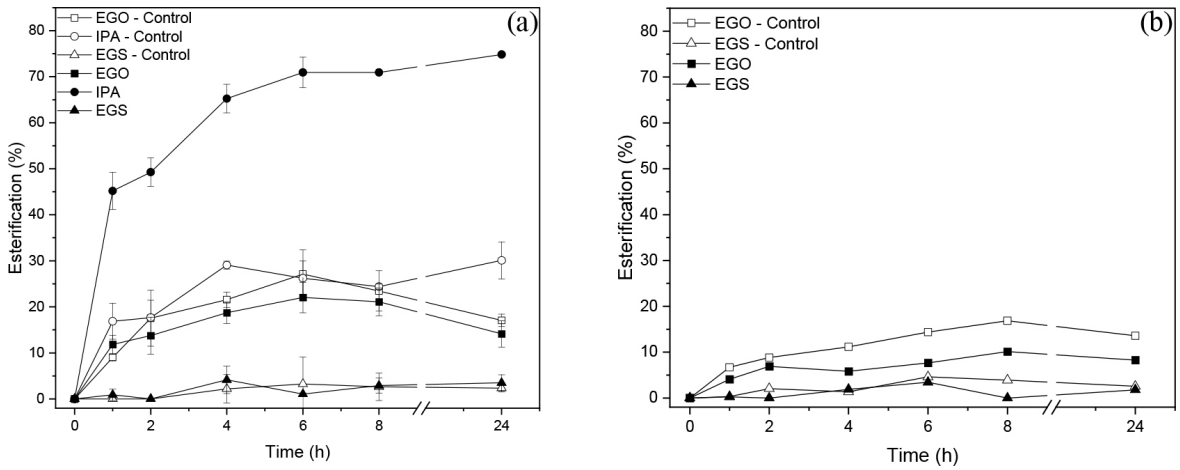

Figure 2. Production of emollient esters (ethylene glycol oleate-EGO, ethylene glycol stearate-EGS, and isoamyl palmitate-IPA) with solvent (heptane) (a) and without solvent (b).

compared to 3.24% for the control), the conversion profile was not consistent throughout the reaction time. The conversions obtained for EGO and EGS are likely to have occurred through a spontaneous reaction between alcohol and acid, without the action of the enzymes involved in the process.

For the ester IPA, the lipolytic biomass showed better performance in converting this ester under the adopted conditions with the use of heptane as a solvent in the reaction. After 24 h of reaction, the control samples reached a conversion of 30.08%, while the reaction containing the biocatalyst achieved a maximum conversion of 74.84%. Furthermore, it is noteworthy that after 1 h of reaction, the esterification conversion had already reached 45.20%, indicating high efficiency of the lipases attached to the mycelium of *R. oryzae* in the early h of the reaction.

Regarding esterification in the absence of a solvent, difficulties were encountered in the synthesis of IPA, as the palmitic acid (solid) did not dissolve in isoamyl alcohol, making it impossible to perform the reaction experiment. To overcome this obstacle, one alternative would be to increase the alcohol concentration and raise the temperature of the reaction medium close to the melting temperature of the palmitic acid (62.9 °C). However, the solubility of palmitic acid did not significantly improve to achieve complete dissolution. Another alternative would be to further increase the alcohol concentration relative to the acid, but this would require a high consumption of reagents. Therefore, the attempt to produce IPA in the absence of a solvent was considered unfeasible due to the adopted proportions of acid and alcohol. Thus, only EGO and EGS were synthesized without the use of a solvent since these reaction components could adequately solubilize, allowing the occurrence of the reaction.

The analysis of the results (Figure 2b) shows that, under the adopted experimental conditions, the lipolytic biomass of *R. oryzae* did not demonstrate satisfactory performance in converting EGO and EGS, as the non-enzymatically catalyzed control assays presented higher conversion values. The maximum esterification percentages were achieved with 16.52% for the synthesis of EGO, after 8 h of non-biocatalyzed reaction with *R. oryzae* biomass, and 4.59% for the synthesis of EGS, after 6 h of non-lipolytic *Rhizopus oryzae*-catalyzed reaction. After 24 h of reaction, the control samples without biomass as biocatalysts showed a conversion 1.5 times higher than those containing the biomass. In the synthesis of EGO, the final esterification percentage was 8.26% in the reactions with the biomass, while for the synthesis of EGS, a lower percentage of approximately 2% was obtained for the enzymatic catalysis reaction.

The low conversion of EGO and EGS catalyzed by the produced lipase may be related to factors such as the low affinity of the enzyme produced for the acid and alcohol used in the reaction, the activity provided for the reaction, and even the moisture present in the biomass. For esterification reactions, it is desirable for the biomass to have the least possible amount of residual moisture, with values below 5-10%, as excess moisture tends to negatively affect the conversion of esterification reactions, inducing hydrolysis reactions.[17] However, to achieve these moisture levels, more sophisticated equipment such as a lyophilizer would be required.

Effect of mass variation on lipolytic biomass synthesis of isoamyl palmitate

Since the lipase attached to the mycelium of the fungus *R. oryzae* showed better performance in the synthesis of IPA using heptane as an organic solvent, subsequent experiments were conducted under these conditions to better evaluate the performance of the lipolytic biomass in catalyzing esterification reactions. First, the influence of biomass mass was evaluated. For this experiment, a new biomass was produced through submerged fermentation, and the values of moisture and activity are detailed in Table 2.

Analyzing Table 2, it was determined that the moisture content in the biomass used in this experiment was 17.94%. Although this percentage indicates a relatively high amount of water in the biomass, it is lower than what was observed in the previous experiments. Therefore, it is expected that esterification reactions will be favored over hydrolysis reactions.[17]

The average activity obtained by the fungal biomass was 103.53 ± 21.99 U g^{-1}, which is similar to the activity obtained in the previous experiments. The reactions were performed in duplicate, with 0.15, 0.30, and 0.45 g of dry biomass, providing hydrolytic activities of approximately 10.35, 31.06, and 46.60 U g^{-1}, respectively. A control

Table 2. Percentage of moisture and activity (U) related to the biomass of mycelium-bound lipases used in the synthesis of isoamyl palmitate

Biomass / g	Moisture / %	Activity / (U g^{-1})	Activity for esterification / U
0.10			10.35
0.30	17.94	103.53 ± 21.99	31.06
0.45			46.60

without the presence of lipase enzyme was also evaluated. The results obtained for the percentage of esterification at each time interval are shown in Figure 3.

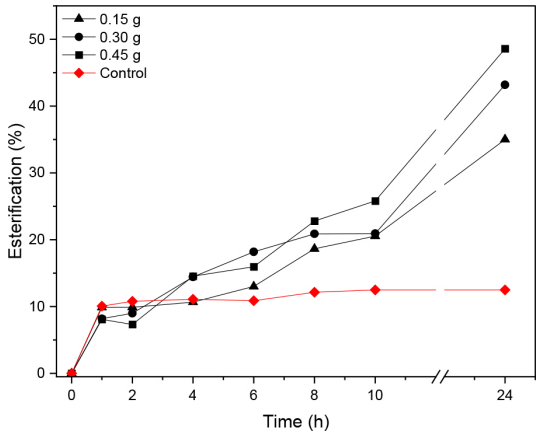

Figure 3. Influence of biocatalyst mass on the percentage of isoamyl palmitate ester conversion.

In Figure 3, it can be observed that in the first 2 h of reaction, the use of mycelium-bound lipases did not achieve higher conversions compared to the control assay, reaching esterification percentages lower than the 10% presented by the control. However, after 4 h of reaction, the conversions obtained for each evaluated biomass mass were higher than the esterification conversions of the control at all evaluated reaction times.

The control experiment achieved a maximum ester conversion of 12.49%, and after 24 h of reaction, it was possible to observe that in the experiments containing 0.45 g of fungal biomass, a higher ester conversion was achieved, reaching 48.59%. However, the experiments with 0.15 and 0.30 g reached esterifications of 35 and 43.19% in 24 h of reaction, respectively. These results suggest that, within the adopted experimental parameters, the increase in fungal biomass, that is, the increase in activity provided for the esterification reaction, favors the conversion of IPA.

Similar results were obtained by Lee *et al.*[18] and Shin *et al.*,[19] who verified the influence of the amount of biocatalyst biomass employed in the synthesis of esters. They also observed the same behavior of increased ester conversion with the increase in biocatalyst concentration, which is directly related to the increase in esterification reaction rates.

Effect of acid:alcohol molar ratio on the production of isoamyl palmitate ester

In the study of the effect of the acid:alcohol molar ratio, a biomass with an average moisture content of 33.20% was used. The biomass presented considerably lower moisture than the ones used in previous experiments, making it more suitable for esterification reactions. Regarding lipolytic activity, the average found in the mycelium-bound lipases was 105.50 U g^{-1}, indicating a value very close to those found in the other experiments, which ranged from 100.80 to 122.03 U g^{-1}. The activity provided for each assay was 31.65 U. Figure 4 presents the results obtained in the production of IPA by varying the acid:alcohol ratios of 1:1, 1:1.25, 1:1.5, 1:2, 1:6, and 1:12.

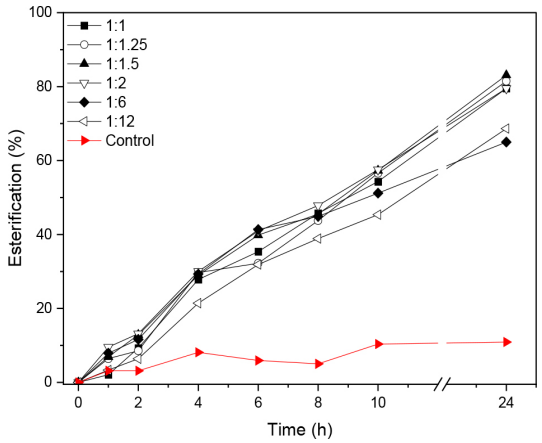

Figure 4. Influence of acid/alcohol concentration on the percentage of isoamyl palmitate ester conversion.

Analyzing the results obtained in the synthesis of isoamyl IPA, it is possible to observe that the performance of the mycelium-bound lipases from *R. oryzae* as catalysts had a positive impact. In the control samples, which did not contain biomass, the maximum esterification percentage after 24 h was only 10.91%. In contrast, the samples containing the biomass showed percentages ranging from 65 to 83.11%, representing a conversion up to eight times higher compared to the control.

In experiments with lower proportions of isoamyl alcohol (1:1, 1:1.25, 1:1.5, and 1:2), higher conversion of the ester were observed. After 10 h of reaction, only one experiment (1:12) had not reached 50% conversion. After 24 h of reaction, the maximum conversion values ranged from 79.50% to approximately 83%, with the 1:1.5 ratio showing the best performance of the biocatalyst.

With the increase in the proportion of isoamyl alcohol to 1:6 and 1:12, the maximum conversion obtained were 65 and 68.64%, respectively, representing an average decrease of approximately 15% compared to the assays with lower concentrations of isoamyl alcohol. Studies reported by Stergiou *et al.*,[8] propose that an increase in the alcohol concentration relative to the acid can positively influence the rate of product formation, as an increase in the quantity of one of the reactants tends to shift the reaction equilibrium towards product formation.

Conclusions

The results obtained in this study can be considered promising and have contributed to the investigation of mycelium-bound lipases applied in biocatalysis. Based on the reported data, it can be concluded that IPA showed the best results, reaching 83.13% esterification after 24 h of reaction, with an acid:alcohol molar ratio of 1:1.5 and using 0.30 g of dry biomass as the catalyst. Furthermore, it was observed that appropriate proportions of isoamyl alcohol were crucial to achieve high conversion of the ester, while an excess of alcohol had an inhibitory effect on the reaction. Further studies are required to optimize the reaction conditions and enhance the efficiency of *R. oryzae* lipolytic biomass in ester synthesis. These results can contribute to the optimization of IPA synthesis process, leading to a more efficient production of this compound catalyzed by intact cells.

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Author Contributions

Arthur O. Preto was responsible for data curation, formal analysis, investigation, resources, visualization and writing original draft; Willian S. M. Reis for data curation, investigation, visualization, writing (original draft, review and editing); Ana K. F. de Carvalho for data curation, formal analysis, investigation, visualization, writing (original draft, review and editing); Ernandes B. Pereira for conceptualization conceptualization, data curation, formal analysis funding acquisition, investigation, project administration, resources, visualization, writing (original draft, review and editing).

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