Synthesis of cetyl ricinoleate catalyzed by immobilized Lipozyme® CalB lipase in a solvent-free system.

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Abstract

A green process has been performed for the synthesis of the emollient ester cetyl ricinoleate with a new immobilized derivative of *Candida antarctica* lipase B, which has been prepared by physical adsorption of Lipozyme® CalB L on a macroporous anionic exchange resin (Lewatit® MonoPlus MP 64). An immobilized CalB lipase with protein content over 30 mg/g has been obtained and it has been successfully used as biocatalyst to produce cetyl ricinoleate from esterification of ricinoleic acid with cetyl alcohol. Influence of amount of biocatalyst and temperature was studied in the open-air reactor, and optimal values could be fixed in 2 mg and 70ºC respectively. Biocatalyst storage stability study was developed in this reactor and it was showed the high storage stability of the immobilized derivative, because it keeps 100% of its enzymatic activity after eight months. Studies of recovery and reuse of the immobilized derivative were performed in the vacuum reactor, and it was proved the posibility of using the same biocatalyst in three consecutive reaction cycles without apparent loss of activity. Finally, the characterization of the cetyl ricinoleate obtained in the vacuum reactor

demonstrated that the product obtained after only 3 or 4 hours of reaction meets manufacturers' specifications.

Key-words: *Candida antarctica* lipase B, enzymatic synthesis, solvent-free, cetyl ricinoleate, cosmetics.

1. Introduction

Natural products are on the rise, and biotechnology has already arrived in the field of cosmetic ingredients [1], like biocatalytic produced emollient esters, which meet the consumers demand for non-synthetic high quality products and conform to the legislation related to cosmetic formulations. Emollient esters are used as oil phase in a wide range of cosmetic emulsions because they significantly help to protect, moisturize and smooth the skin [2]. This family of compounds is produced through the esterification of a fatty acid with a long-chained alcohol [3].

The conventional industrial process requires temperatures over 160°C and non-selective contaminating catalysts [4-6], which lead to raw products that need to undergo a host of reprocessing steps because they do not have enough quality in terms of purity, colour and smell.

The enzymatic synthesis runs at mild temperatures and supplies ultra-pure, odor and colorless products, decreasing side reactions and contaminating wastes [7]. The most widely used enzyme is *Candida antarctica* lipase B (CalB) which has the capability to become adsorbed on hydrophobic interfaces and to act on insoluble substrates. When it is used in its immobilized form, enables the recovery and the reuse of the biocatalyst, in order to make the process economically profitable [8, 9]. In addition, in most of cases, the immobilization of lipases enhances enzyme activity and can modify the substrate selectivity and enantioselectivity, reduce the effects of inhibitors and extend the utility of enzyme reactions to a range of non physiological environments, such as extreme temperatures, non-aqueous solvents, ionic fluids and supercritical CO_2 [10, 11].

In the bibliography, it has been widely described the use of Novozym® 435 in esterification reactions and, in some of these works [12], it has been confirmed the need of centrifugation during the recovery of the enzyme in order to efficiently separate the lipase from the reaction medium. This separation step could be avoided by using an immobilized derivative with a higher particle size.

This work focuses on a new immobilized derivative of *Candida antarctica* lipase (Lipozyme® CalB L) obtained by physical adsorption on a macroporous anionic exchange resin, Lewatit® MonoPlus MP 64. The synthesis of an emollient ester has been studied using this biocatalyst in a solvent-free system, which enables to obtain a product free of toxic solvents [8]. Finally, the activity of this immobilized lipase is tested against the well-known commercially available form of immobilized CalB, Novozym® 435.

The emollient chosen for this research is cetyl ricinoleate (CR), ester of ricinoleic acid with cetyl alcohol. CR is a non-comedogenic product widely used in cosmetic industry. It is usually found in the formulation of hand and face creams, body lotions, solar products, decorative cosmetics and lipsticks [13]. The scheme of the esterification reaction is showed in Figure 1.

2. Materials and methods

2.1. Chemicals

Lipozyme® CalB L (liquid formulation of *Candida antarctica* lipase B, 12.5 mg Lowry protein/mL) and Novozym® 435 (immobilized *Candida antarctica* lipase B), were kindly provided by Novo Nordisk AS (Copenhagen, Denmark). Immobilization support was Lewatit® MonoPlus MP 64 (an anionic exchange resin from Fluka). Ricinoleic acid (~80%) and cetyl alcohol were from Aldrich and Fluka, respectively.

2.2. Immobilization of CalB L

First, 5 g of support (Lewatit® MonoPlus MP 64) were mixed with a 50 mL of a 20 mg/mL ricinoleic acid suspension and stirred at 120 rpm in an orbital shaker 24 hours, in order to increase the hydrophobicity of the support. After that, the activated support was transferred to a jacketed column reactor (2.5 i.d. and 30 cm length), which is equipped with a sintered glass plate placed 5 cm from the bottom. The support was washed twice with 25 mL of deionized water. Then, 25 mL of enzymatic solution (50%

v/v), prepared with Lipozyme® CalB L and phosphate buffer 0.1 M, pH 7, were added and circulated for 48 h at 4ºC. The supernatant solution was recovered and the immobilized derivative was rinsed with 2 x 25 mL of phosphate buffer 0.1 M, pH 7, in order to remove loosely bound enzyme. Finally, it was washed twice with 12.5 mL of acetone and air dried, decreasing its weight to 3.6 g after that. The immobilized derivative was stored at 4ºC until use.

The amount of immobilized protein was determined from the difference between the protein available in the enzymatic solution and that present in the supernatant and buffer washings. The protein analysis was made by Lowry's method [14].

2.3. Open-air reactor experiments

The enzymatic reaction was carried out in an open-air jacketed glass reactor (250 mL, total volume) equipped with an overhead stirrer provided with a two-bladed propeller (axial flow). First, 11.04 g of ricinoleic acid and 8.96 g of cetyl alcohol (molar ratio 1:1), was added to the reactor. Biocatalyst amounts in a range of 0.2 - 8 g and temperatures between 50ºC and 70ºC were used.

The acid value (AV), which represents the number of milligrams of potassium hydroxide necessary to neutralize free acids contained in 1 g of sample [15], was used as an index of the reaction extension. The results, presented graphically afterwards, are the mean of three measurements and include error bars (± standard deviation).

2.4. Vacuum reactor experiments

For the vacuum reactions, a Parr 5100 series low-pressure reactor was used. The reaction vessel (100 mL, total volume) is glass-jacketed. The experimental conditions for the vacuum series were: 20 g of substrates in equimolecular proportion, 60ºC, 350 rpm, continuous dry N_2 input (25 cm^3/s), 213 hPa and 1 g of biocatalyst.

2.5. Storage and reuse stabilities of the biocatalyst

The storage stability of immobilized Lipozyme® CalB L after 0, 4 and 8 months stored at 4 ºC was tested in the open-air reactor, with 20 g of substrates (molar ratio 1:1), 1 g of biocatalyst, 60ºC and a vertical stirrer at 350 rpm.

The reuse stability of the biocatalyst was studied by running up to three batch reactions with the same immobilized derivative. In this case, the reactions were carried out under vacuum with 20 g of substrates (molar ratio 1:1), 1 g of biocatalyst, 60ºC, 350 rpm and 25 cm^3/s dry N_2 input. After each use, the immobilized derivative was recovered by filtration, rinsed with acetone and air dried.

3. Results and discussion

3.1. Lipase immobilization

The selection of the immobilization support was based on foregoing research with other lipases (*Candida rugosa* [16, 17], *Rhizopus arrhizus* [17, 18], *Rhizopus oryzae* [17, 19] and *Mucor javanicus* [16]). Those studies showed that this support has a high mechanic stability, which enables immobilized-derivative reuse. Besides, its bead size (0.59±0.05 mm), density (1.04 mg/L) and porosity (61-66%) facilitates the separation of the immobilized enzyme from the product and avoids centrifugation.

In this case, the surface of the support should be hydrophobic, to ensure the interfacial activation of the lipase. Therefore, previous treatment of the support with ricinoleic acid was carried out to get a suitable hydrophobicity. When no support activation was performed, the catalytic activity of the immobilized derivative obtained was very low, which was demonstrated by using this immobilized derivative in the esterification reaction. In this case, a decrease of 5 units in acid value for 180 h of reaction was observed, while the ricinoleic acid activated one yielded a decrease of 95 units for the same reaction time.

Furthermore, the removal of the water contained in the immobilized derivative by the acetone washings noticeably improved the reaction rate.

Table 1 shows the results of the immobilization experiments under optimized conditions (average of six independent experiments). According to the recommendation of the European Federation of Biotechnology Section on Applied Biocatalysis (ESAB) [20], these results are average values corresponding to every immobilization, along with their standard deviation.

An immobilized CalB L lipase with protein content higher than 30 mg/g of dry derivative has been obtained. This value is comparable to those described in the bibliography for the immobilization by adsorption of the same enzyme on similar supports, which cover a range between 8.8 mg/g [21] and 290 mg/g [22]. A good immobilized derivative must provide not only high protein content, but also an appropriate activity and stability. Thus, this work proves the suitability of the immobilized lipase for the synthesis of CR.

3.2. Synthesis of CR in an open-air stirred tank reactor

3.2.1. Influence of the amount of biocatalyst

The influence of the amount of enzyme, between 0.2 g (0.99%) and 8 g (28.57%), was first studied in an open-air reactor. Figure 2 shows the variation of the acid value over time. The error bars are not often clearly visible due to the very low value of the standard deviation, but it can be seen that reaction rate increased noticeably using amounts of immobilized lipase ranging from 0.5 to 2 g. A further increase of biocatalyst amount (4 g) did not lead to such a great improvement in the reaction rate, probably due to the fact that the water removal from the reaction medium is the limiting step. This effect was more evident when 8 g of biocatalyst were used. An acid value close to 3 mg KOH/g was always reached after 24 h of reaction.

3.2.2. Influence of the reaction temperature

The temperature dependence of CalB lipase is very much determined by the conditions studied [23]. Therefore, three temperature values were assayed to study its influence on

the biocatalytic synthesis of CR. Results are showed in Figure 3, where a slight increase in reaction rate as the temperature was increased can be observed. After 24 h, the CR obtained at 70ºC had an acid value of 2.71 mg KOH/g. The optimum temperature will be a compromise between operational and economic requirements.

3.2.3. Synthesis of CR in a vacuum reactor

In order to achieve complete consumption of substrates and shift the equilibrium towards product, the water produced in the process must be removed. For that reason a vacuum reactor with a continuous dry N_2 input was used. Figure 4 shows the results of two experiments conducted under the same operational conditions; one was performed in the open-air reactor while the other one in the vacuum reactor. As it can be observed, the variation of acid value over reaction time was very similar in both cases. However, after 24 h, the vacuum-obtained CR reached an acid value of 1.98 mg KOH/g, lower than 3.63 mg KOH/g, final acid value obtained in the open-air reactor. These results proved the important influence of water removal on the reaction equilibrium.

3.2.4. Biocatalysts reuse and storage stabilities

According to ESAB [20] recommendations, the storage and operational stabilities are an important part of the essential information needed to reproduce and understand the results obtained with immobilized enzymes. With the purpose of determining whether Lipozyme® CalB L immobilized on Lewatit® MonoPlus MP 64 lost activity during storage, three CR synthesis reactions were carried out. One of them was performed using a freshly-prepared immobilized derivative, while the other two were carried out using the biocatalyst stored at 4°C for 4 and 8 months, respectively. The results proved that the immobilized derivative studied in this work had high storage stability since it kept 100% of its enzymatic activity after eight months (Figure 5). This result is of great importance when using the immobilized enzyme in large-scale production, since it is not necessary to obtain the immobilized biocatalyst just before its use.

In order to make the enzymatic synthesis economically profitable, it is very important the use of the biocatalyst in a continuous process or, where appropriate, its repeatedly

reuse in batches [20]. Figure 6 displays the results of three consecutive reactions of CR synthesis using the same immobilized derivative. As shown in Figure, the activity of the derivative is maintained for at least three consecutive cycles without apparent loss of activity. The reusability of the immobilized derivative has important consequences for the economy of the process. As the only treatment to be done on the derivative before reuse is to wash with acetone, spending on biocatalyst will be the result of dividing its cost by the number of cycles that can be used.

3.2.5. Comparison of the immobilized Lipozyme® CalB L and Novozym® 435

Novozym® 435 is a commercial preparation of *Candida antarctica* lipase (CalB) immobilized by Novozymes. The immobilized enzyme consisted of bead-shaped particles with a diameter in the range of 0.3–0.9 mm. The enzyme is delivered with a water percentage of 1–2% (w/w) and according to the literature, with an enzyme loading is 20% (w/w) [22]. Since this immobilized derivative is widely used for many applications, it has been used in this work as a reference to check the applicability of the new immobilized enzyme.

Two CR synthesis reactions were performed in the vacuum reactor under the same operating conditions, adding the appropriate amount of each biocatalyst that contains the same immobilized protein (70.5 mg Lowry protein). In this way, we added 2 g of Lipozyme® CalB L (35.2 mg protein/g dry derivative) and 0.35 g of Novozym® 435 (20% w/w [22]). As observed in Figure 7, reactions catalyzed by both immobilized derivatives evolved similarly. The final acid values were very similar (1.21 mg KOH/g for Lipozyme® CalB L and 1.32 mg KOH/g for Novozym® 435).

These results confirm that the biocatalyst described in this work may be a feasible alternative to the well-known Novozym® 435, because it presents similar catalytic properties, with the additional advantage that the recovery of the new immobilized derivative from the product is much faster, due to its mean bead size and its density, which let to avoid the centrifugation. The final choice will be hence conditioned by economic studies, which should be done in the next future.

3.2.6. Characterization of the CR obtained

Although there are not regulations for cosmetic ingredients, most of the manufacturers agree on a series of specifications which make CR adequate for its effective use in cosmetic formulations. Table 2 lists the characteristics of the CR synthesized in this work (vacuum reactor, 60°C, 1 g of immobilized derivative) and the recommended values.

As observed, the enzymatic CR presented similar characteristics to those specified by the manufacturers. These results make the enzymatic product an attractive alternative to the chemically obtained CR because its biotechnological origin allows labeling it as "natural product".

4. Conclusions

A green process has been developed for the biocatalytic synthesis of CR, with a new immobilized derivative of CalB. The obtained biocatalyst has a high protein content, as well as high activity and stability. Its activity is comparable to the widely known Novozym® 435. The biocatalytic synthesized CR presents similar characteristics to the commercially available CR ones.

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Table 1. Coupling parameters for the immobilization of Lipozyme® CalB L by physical adsorption on Lewatit® MonoPlus MP 64.

	Protein concentration (mg prot/mL)	**Protein solution volume (mL)**	**Protein (mg)**
Solution offered	12.50 ± 0.79	25.00 ± 0.00	312.54 ± 19.71
Supernatant	6.58 ± 0.39	23.55 ± 0.58	154.74 ± 7.68
First wash	1.01 ± 0.18	24.62 ± 0.73	24.84 ± 4.38
Second wash	0.28 ± 0.05	22.5 ± 1.21	6.15 ± 1.41
Protein offered (mg)	**Immobilized protein (mg)**	**Immobilized protein (%)**	**Protein loading (mg/g dry derivative)**
312.54 ± 19.71	126.81 ± 17.32	40.46 ± 3.81	35.23 ± 4.90

Table 2. Characteristic parameters of the enzymatic CR and the CR commercialized by two companies.

Parameter	Enzymatic CR	Commercial CR	
		JEEN International Corporation	Ambuja SOLVEX PVT. LTD.
Acid value (mg KOH/g)	1.98	< 3	< 1
Hydroxyl value (mg KOH/g)	96.36	90-105	90-110
Saponification value (mg KOH/g)	111.96	95-110	100-110
Iodine index (g I_2/100 g)	42.13	45-55	40-60
Water content (%)	0.33	< 0.5	< 0.05

Figure

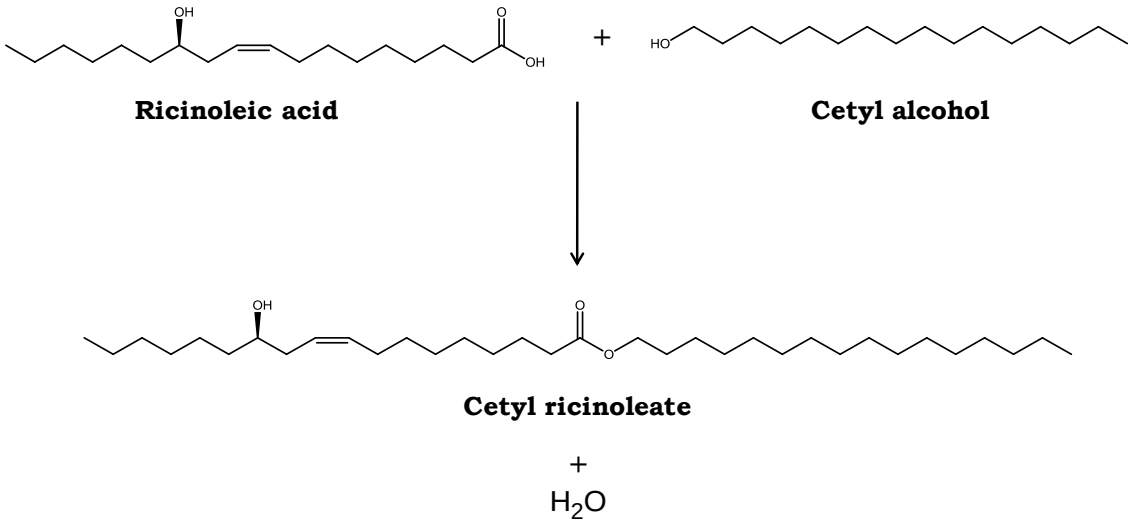

Figure 1. Scheme of esterification reaction to obtain cetyl ricinoleate.

Figure

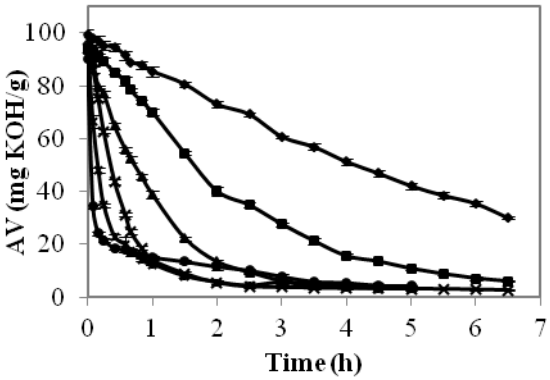

Figure 2. Influence of the biocatalyst amount in CR enzymatic synthesis, 60ºC, 350 rpm. 0.2 g (♦); 0.5 g (■); 1 g (▲); 2 g (×); 4 g (*); 8 g (●).

Figure

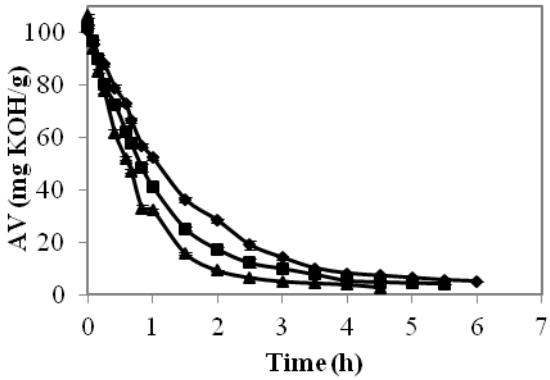

Figure 3. Influence of the reaction temperature in CR enzymatic synthesis,1 g IME, 350 rpm. 50ºC (♦); 60ºC (■); 70ºC (▲).

Figure

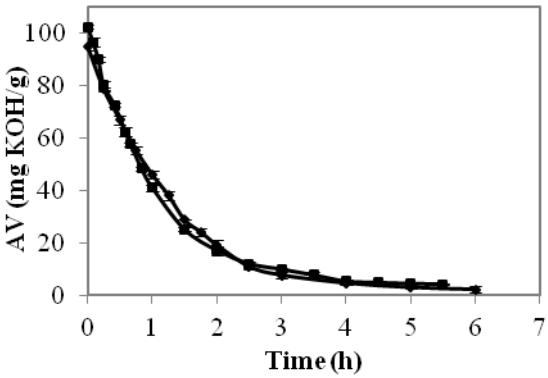

Figure 4. Comparison of the CR enzymatic synthesis conducted in the vacuum reactor and the open-air reactor, 1 g IME, 60ºC, 350 rpm. Vacuum reactor (♦); open-air reactor (■).

Figure

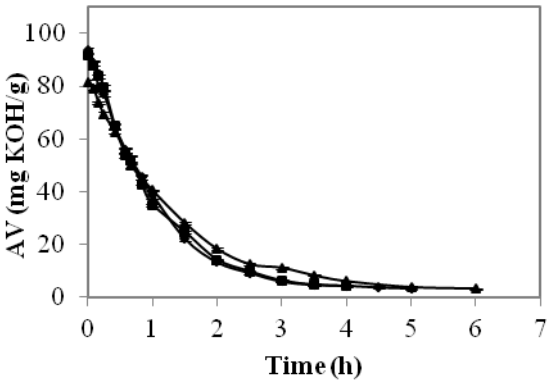

Figure 5. Storage stability study for Lipozyme® CalB L immobilized on Lewatit® MonoPlus MP 64, 1 g IME, 60 ºC, 350 rpm. Freshly-prepared (♦); 4 months-stored (■); 8 months-stored (▲).

Figure

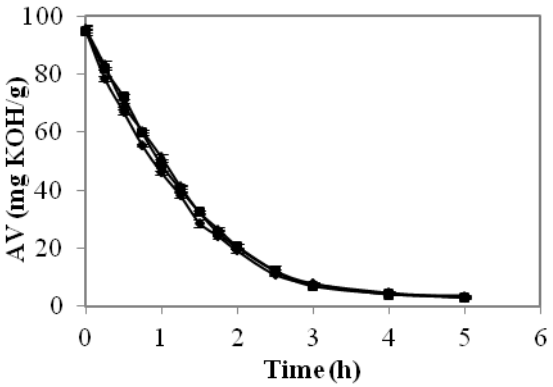

Figure 6. Operational stability study for Lipozyme® CalB L immobilized on Lewatit® MonoPlus MP 64, 1 g IME, 60 ºC, 350 rpm. First use (♦); second use (■); third use (▲).

Figure

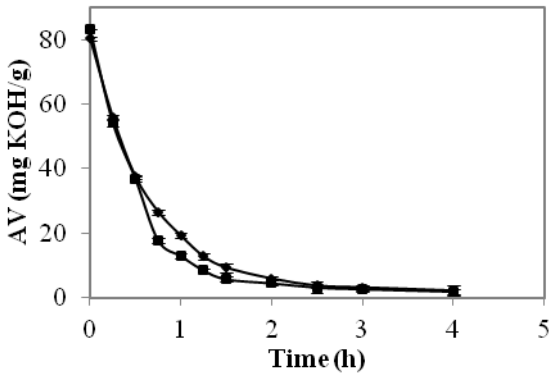

Figure 7. Comparison between immobilized Lipozyme® CalB L and Novozym® 435, 20 g substrates (1:1 molar ratio), 60 ºC, 350 rpm. Novozym® 435 (♦); Lipozyme® CalB L (■).

Highlights

1. An immobilized CalB lipase with protein content over 30 mg/g has been obtained.
2. This immobilized CalB lipase was used as biocatalyst to produce cetyl ricinoleate.
3. The highest reaction rate value was obtained at 70ºC.
4. The vacuum-obtained cetyl ricinoleate reached an acid value of 1.98 mg KOH/g.
5. The derivative can be used three consecutive cycles without loss of activity.

*Graphical Abstract (for review)