STUDIA UBB CHEMIA, LXVIII, 3, 2023 (p. 129-139)
(RECOMMENDED CITATION)
DOI:10.24193/subbchem.2023.3.08

ENTRAPMENT OF LIPASE B FROM CANDIDA ANTARCTICA IN TAILORED SOL-GEL MATRICES FOR THE SYNTHESIS OF BENZYL DECANOATE – IMPORTANCE OF USING AN ADDITIVE

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ABSTRACT. Benzyl decanoate was successfully synthesized by the direct esterification of decanoic acid with benzyl alcohol mediated by lipase B from *Candida antarctica* immobilized by entrapment in a sol-gel matrix in a solvent-free system, using a ternary mixture of silane precursors and glycerol as additive. The most active (LS-6, 92.1% conversion after 90 minutes) and stable biocatalyst (95% of the initial activity after 7 reaction cycles) yielded 2.2 g of benzyl decanoate in a scaled-up process (91% isolation yield).

Keywords: *benzyl decanoate, esterification, solvent free system, lipase, additive, sol-gel, biocatalysis, aroma ester*

INTRODUCTION

Preparation of natural flavors starting from natural substrates through bioprocesses is industrially applied for their marketing as natural products in the European Union and US. Many flavors and fragrances esters can be enzymatically obtained using lipases that catalyze esterification or transesterification reactions. Nowadays, flavors have an important place in the world market for food additives produced *via* chemical synthesis. Aromatic esters are flavor esters obtained *via* direct extraction from plants or fruits,

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however, disadvantages like low product yield or purity make this technique expensive and difficult to apply industrially [1-4]. Enzymatic synthesis is an alternative because of milder reaction conditions and high chemo-, regio- and stereoselectivity [5]. Since different enantiomers or regioisomers could present different sensorial properties, enzymes are good tools, suitable to obtain the desired compound at industrial level as well [6]. Furthermore, in order to improve the control of the reactions and significantly reduce the risk of product contamination with proteins, enzyme immobilization can be employed [7]. There are many known techniques that can improve lipases' activity and stability, including adsorption to solid supports, covalent attachment, or entrapment in polymeric materials. Silica-based carries are used for enzyme immobilization by covalent attachment to solid supports, encapsulation or even by adsorption [7]. Moreover, in sol-gel entrapment of enzymes, some compounds could be used as additives (for example crown ethers, ionic liquids, deep eutectic solvents, glycerol, polyvinyl alcohol, etc.) for maintaining lipase activity, mainly in esterification reactions and/ or for protecting the enzyme structure during the immobilization process from harsh pH conditions and from alcohols resulted during sol-gel matrix formation. The advantages of this immobilization method are larger surface-to-volume *ratio*, high porosity of the obtained carriers, and high mass transfer [6, 8-10].

Lipases belong to the hydrolase family, enzymes responsible for hydrolyzing ester bonds. Because of their versatility, strong regio- and enantioselectivity, high stability in various conditions and extensive specificity for multiple substrates, these biocatalysts are frequently used in biochemical processes [2]. Lipases are appropriate for a wide range of industrial applications, including biodiesel manufacturing, pharmaceutical, food, and cosmetic technologies [10]. One recent study shows the synthesis of thymol octanoate, a promising hybrid molecule with various biological activities, mediated by lipase B from *Candida antarctica* [11].

Lipase B from *Candida antarctica* (CaL-B) is one of the most known versatile lipases. With an isoelectric point of 6.0 and a molecular weight of 33 kDa [10], the enzyme catalyzes esterification reactions through its active site which contains the catalytic triad Ser-His-Asp/Glu [2]. An essential structural feature of CaL-B is the presence of a "lid" composed of two flexible polypeptide helices which allows the enzyme to have an open and closed conformation. The lid plays a crucial role in interfacial activation mechanism and promotes the enzyme susceptibility to esterification reactions while allowing immobilization on hydrophobic supports [10].

Due to their biotechnological applications, lipases require a high degree of recovery and increased stability under the process conditions (pH, temperature, pressure, organic solvent) [6]. In this regard, immobilization techniques like adsorption, covalent attachment, cross-linking, inclusion, sol-

gel encapsulation or affinity-tag binding on supports can be used [1]. All these techniques exceed the limitations of soluble enzymes by potentially increasing catalytic activity and selectivity while lowering the production cost and allowing the automation of the process.

For this study the sol-gel encapsulation method was chosen for CaL-B immobilization, considering that the technique potentially enhances the enzyme's properties such as mechanical resistance, operation and long-term stability, thermostability and tolerance to various solvents [10]. Sol-gel encapsulation involves the use of silica-based carriers to attach enzymes through covalent binding or adsorption and presumes the formation of a silane matrix through a polymerization process that involves acidic or basic hydrolysis of silane compounds such as tetraethoxysilane (TEOS) or tetramethoxysilane (TMOS) [5].

In this work, we report the lipase catalyzed solvent-free preparation of benzyl decanoate mediated by sol-gel entrapped lipase B from *Candida antarctica*. Six biocatalysts were prepared with and without glycerol as additive and tested in the direct esterification of benzyl alcohol with decanoic acid and compared with the commercially available Novozym 435. The obtained biocatalysts were characterized by their synthetic and hydrolytic activities and recyclability in order to study the effect of additives during the encapsulation process and to prove their potential for an industrial scale-up. The optimum biocatalyst was used in a scale-up reaction (starting from 1 g of benzyl alcohol) for obtaining benzyl decanoate in order to further prove the novel biocatalysts' suitability for a potential industrial application.

RESULTS AND DISCUSSION

Lipase immobilization by sol-gel entrapment. Hydrolytic and synthetic activity assays

Six biocatalysts (LS-1 - LS-6) were successfully prepared by entrapping lipase B from *Candida antarctica* (CaL-B) *via* sol-gel method. Tetraethoxysilane and *n*-propyltriethoxysilane were used for all biocatalysts in the same molar *ratio* while three silane precursors with different hydrophobic groups (octyltriethoxysilane, *n*-hexyltriethoxysilane and phenyltriethoxysilane, all were added in a 1.6 molar *ratio*). Three catalysts were prepared without the addition of any additive (LS-1, LS-2 and LS-3), whilst for three biocatalysts glycerol was added as an additive (LS-4, LS-5 and LS-6). For the novel biocatalysts the hydrolytic and synthetic activities were evaluated and presented in **Table 1**.

As indicated in **Table 1**, the amount of biocatalyst obtained is improved when glycerol was added as additive during the immobilization step, as higher quantities were obtained for LS-4, LS-5 and LS-6 compared to their counterparts

obtained without glycerol addition (LS-1, LS-2 and LS-3), whereas the enzyme loading decreases for all biocatalysts when glycerol was added. This was to be expected, since the starting enzyme solution had the same concentration for all biocatalysts and the quantity of biocatalyst is higher for LS-4-6 than LS-1-3, therefore the *ratio* between the starting amount of enzyme and total mass of biocatalyst will be smaller.

The addition of glycerol led to an increased hydrolytic activity of LS-4-6. It can be seen that LS-5, formed from HTEOS, *n*-PTEOS and TEOS as silane precursors and glycerol as additive (4% aqueous solution, 100 µL) showed the highest hydrolytic activity, which makes the biocatalyst the most suitable for application in aqueous reaction systems. On the other hand, the synthetic activity of biocatalysts LS-4 and LS-5 was negatively influenced by the addition of glycerol, since LS-1 and LS-2 presented higher values for the synthetic activity. The only biocatalyst showing an increased synthetic activity when using glycerol was LS-6 (approximately 60% higher than its counterpart LS-3). However, LS-1, formed from OTEOS, *n*-PTEOS and TEOS as silane precursors without glycerol, showed the highest synthetic activity, making it the most suitable to be used in hydrophobic media, which was to be expected due to highly hydrophobic octyl chain on the support's surface.

Table 1. Enzyme loading, hydrolytic and synthetic activity assays for the novel biocatalysts

Sol-gel code	Silane precursors [1.6:0.4:1 molar *ratio*]	Additive	Quantity obtained [mg]	Enzyme loading [μg_{enzyme}/ $mg_{biocatalyst}$]	Hydrolytic activity [U]	Synthetic activity [mmol/min*g_{enzyme}]
LS-1	OTEOS: *n*-PTEOS:TEOS	No	36.3	0.31	12.55	8.14
LS-2	PhTEOS: *n*-PTEOS:TEOS	No	10.2	1.10	15.68	6.35
LS-3	HTEOS: *n*-PTEOS:TEOS	No	59.1	0.19	18.81	2.08
LS-4	OTEOS: *n*-PTEOS:TEOS	Yes	51.2	0.22	21.94	1.64
LS-5	PhTEOS: *n*-PTEOS:TEOS	Yes	13.6	0.82	25.07	4.75
LS-6	HTEOS: *n*-PTEOS:TEOS	Yes	61.9	0.18	28.2	3.32

To conclude, the addition of glycerol during the CaL-B entrapment *via* the sol-gel method enhances the enzymes' activity in aqueous media, whereas in hydrophobic media the enzymes' activity is negatively influenced, probably due to the increased viscosity around the entrapped enzyme.

Biocatalyst screening for the synthesis of benzyl decanoate

Experiments were performed in duplicate with the media results and standard deviations being presented. All six prepared biocatalysts (LS-1 - LS-6) were tested for the synthesis of benzyl decanoate by direct esterification of decanoic acid with benzyl alcohol in a solvent-free system. A commercially available form of CaL-B (Novozym 435) was also used as biocatalyst in order to evaluate the performances of the novel biocatalysts (**Figure 1**).

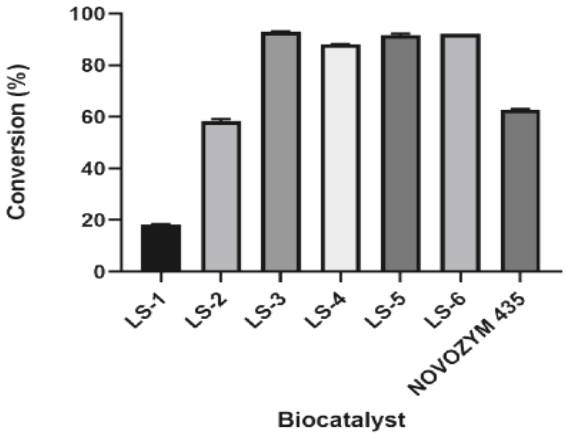

Figure 1. Results obtained for the enzymatic synthesis of benzyl decanoate, mediated by the six novel biocatalysts, in comparison with Novozym 435. Reaction conditions: 20 mg benzyl alcohol, 2 equiv. decanoic acid, 2 mg CaL-B, 30 °C, 600 rpm, 90 minutes.

The results obtained in this part of the study show that LS-1 performed poorly in the biosynthesis of benzyl decanoate with a conversion value of only 18.1% after 90 minutes. LS-2 and Novozym 435 delivered satisfactory results (58.9% and 62.8% conversion values, respectively). LS-4 and LS-5 showed excellent activity since conversion values of 88.2% and 91.1%, respectively, were obtained. The highest conversion values were registered when LS-3 and LS-6 were used as biocatalysts as they yielded the desired aroma ester with 92.9% and 92.1% conversion values, respectively. The beneficial effect of using HTEOS as one of the silane precursors in the silane matrix can be seen, since CaL-B shows great activity towards benzyl decanoate formation (in the presence or absence of glycerol).

Reusability studies

In order for a biocatalyst to be considered for a potential industrial application it should be cheap, stable, easy to recover and reusable. For this, the reusability grade of the newly prepared biocatalysts was evaluated for the production of benzyl decanoate and the obtained results can be seen in **Figure 2**.

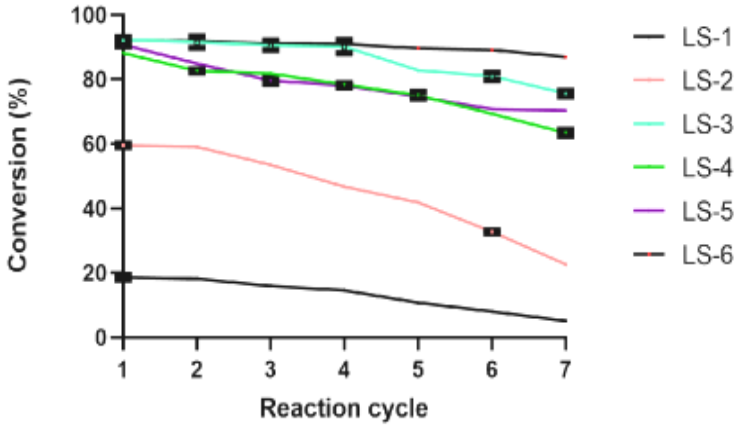

Figure 2. Evaluation of the recyclability of the newly developed biocatalysts. Reaction conditions: 20 mg benzyl alcohol, 2 equiv. decanoic acid, 2 mg CaL-B, 30 °C, 600 rpm, 90 minutes per reaction cycle.

LS-1 showed poor activity towards benzyl decanoate formation and lost almost 50% of its initial activity after the 7th reaction cycle. LS-2 also registered a relatively low conversion value for the synthesis of benzyl decanoate and also lost more than half of its initial activity after the 7th reaction cycle. LS-4 and LS-5 were more active than LS-1 and LS-2 (similar compositions, LS-4 and LS-5 contain also glycerol as additive) and also registered a loss of initial activity, initially a 5% loss of initial activity after the 1st reaction cycle and reaching ~25% loss of initial enzymatic activity after the 7th reaction cycle, however, it wasn't as significant as for the two previously mentioned biocatalysts.

LS-3 maintained all its initial activity even after the 4th reaction cycle, but the conversion value suffered a drop after the 5th reaction cycle and reached a value of 75% after the 7th reaction cycle. On the other hand, LS-6 maintained almost all its initial activity even after the 6th reaction cycle, before reaching a conversion value of 86.9% after the 7th reaction cycle. Even though both biocatalysts, LS-3 and LS-6, delivered excellent results towards

benzyl decanoate formation, the biocatalyst containing glycerol as additive (LS-6) proved to be more stable, as it maintained almost 95% of its initial activity even after the 7th reaction cycle, therefore making it suitable for a potential industrial application.

CONCLUSIONS

Benzyl decanoate was successfully synthesized *via* an enzymatic approach with lipase B from *Candida antarctica* immobilized in a sol-gel matrix starting from benzyl alcohol and decanoic acid. The sol-gel matrix obtained from hexyltriethoxysilane: *n*-propyltriethoxysilane and tetraethoxysilane (in a 1.6:0.4:1 molar *ratio*) gave the most active biocatalyst. The benefits of glycerol as additive in the immobilization step were proven, since by using the LS-6 biocatalyst (containing glycerol as additive) yielded benzyl decanoate with a conversion value of 92.1% and lost approximately only 5% of its initial activity after the 7th reaction cycle. The scale-up of the proposed process yielded 2.2 g of benzyl decanoate (η=91%) in just 90 minutes of reaction time. Benzyl decanoate obtained by this enzymatic method can be considered a natural aroma ester, since the reactants can be found in nature and an enzyme is used as catalyst, as per Regulation No. 1334/2008 of the European Parliament and of the Council and LS-6 can be considered a viable candidate for a potential industrial application.

EXPERIMENTAL SECTION

Materials

CaL-B as lyophilized powder and Novozym 435 were purchased from Novozymes (Denmark). Isopropylic alcohol, *n*-hexyltriethoxysilane (HTEOS), tetraethoxysilane (TEOS), *n*-propyltriethoxysilane (*n*-PTEOS), *n*-octyltriethoxysilane (OTEOS), phenyltriethoxysilane (PhTEOS), glycerol, decanoic acid, 3-methyl-2-benzothiazolinone hydrazine hydrochloride hydrate (MBTH), sodium sulfate, benzyl alcohol and HPLC grade methanol and water were purchased from Merck (Germany). 4-Nitrophenylpalmitate and 4-nitrophenol were products of Fluka (Switzerland). Sodium sulfate, bovine serine albumin (BSA), ammonium iron(III) sulfate, arabic gum and sodium deoxycholate were purchased from Alfa Aesar (USA). Benzyl decanoate was previously synthesized at the Enzymology and Applied Biocatalysis Research Centre. All solvents and reagents were freshly distilled and dried by standard methods before use.

The mixture of benzyl alcohol and benzyl decanoate was analyzed by reverse phase HPLC (RP-HPLC) with an Agilent 1260 Infinity series (USA) equipped with a UV-Vis detector using a Phenomenex Gemini NX-C18 (150 x 4.5 mm; 5 µm) chromatographic column and methanol: water 80:20 (v/v %) at 1 mL/min flow as eluent. For the rigorous evaluation of the conversion values, a relative response factor (RF=0.8549) was determined by analyzing samples of known concentration (for both benzyl alcohol and benzyl decanoate).

For the quantitative spectrophotometric determination of enzyme load through BCA method, using the Pierce® BCA Protein Assay Kit (Thermo Fisher Scientific Inc., USA) and for the evaluation of the novel biocatalysts synthetic and hydrolytic activities an Agilent 8453 UV-Vis spectrophotometer equipped with thermostat was used.

The enzymatic reactions were perfected using IKA C-MAG HS 7 (Germany) magnetic stirrers equipped with temperature controllers. The vacuum needed for the removal of the water formed in the system was ensured by using a Heidolph (Germany) vacuum pump.

Lipase immobilization by sol–gel entrapment

CaL-B was immobilized in sol-gel according to the recipe previously described [10]. Six samples were prepared using lyophilized enzyme powder to prepare 0.5 mL enzymatic solution (22.4 mg enzyme/mL), which was mixed with 0.1 mL *iso*-propanol and 0.05 mL NaF 1M solution. The additive (100 µL glycerol, 4% aqueous solution) was added to three of these samples (LS-4, LS-5 and LS-6) while the other three biocatalysts (LS-1, LS-2 and LS-3) were prepared without any additive. After stirring, silane precursors (OTEOS, PhTEOS, and HTEOS) were added keeping the silane precursors OTEOS, PhTEOS, HTEOS: *n*-propyltriethoxysilane: tetraethoxysilane molar *ratio*s of 1.6:0.4:1, the detailed components for each of the prepared biocatalysts can be found in **Table 1**. Each mixture was added in 4 mL glass vials, stirred and left for 24 hours at room temperature. The obtained gels were washed and vacuum filtered with 7 mL *iso*-propyl alcohol, 5 mL distilled water, 5 mL *iso*-propyl alcohol and 2.5 mL of *n*-hexane. The obtained biocatalysts were dried under advanced vacuum (17 mbar) for 1 hour and left at room temperature for 24 hours. After drying, the resulted products were crushed in a mortar and the biocatalysts were stored at 4 °C in glass vials.

Hydrolytic activity assay of the novel biocatalysts

The hydrolytic activity of the biocatalysts was determined using as model reaction the hydrolysis of 4-nitrophenylpalmitate (0.75 mM in *iso*-propanol). The reactions were perfected in Sorensen buffer which was prepared

by mixing 90 mL phosphate buffer solution (0.2 M, pH 8) with 207 mg sodium deoxycholate and 100 mg arabic gum. The reactions were perfected in 2 mL cuvettes in which 10 mg biocatalyst, 900 µL Sorensen buffer and 100 µL substrate solution were added, incubated at 37 °C, 800 rpm for 10 minutes. After 10 minutes, the samples were centrifuged and were analyzed at 410 nm.

Synthetic activity assay

The synthetic activity of the biocatalyst (expressed as the amount of released acetaldehyde *vs* the reaction time and the amount of enzyme) was evaluated using a fast and sensible colorimetric method [12] based on the *n*-butanol transesterification with vinyl acetate (2 equiv.) in *n*-hexane at 30 °C and 1000 rpm. The released acetaldehyde was quantified after derivatization with MBTH as hydrochloride salt, when the formed aldazine is further converted by oxidative coupling with another MBTH molecule in the presence of ammonium iron(III) sulfate into a blue tetraaza-pentamethylene cyanine with a maximum absorption at 598 nm.

Biocatalyst screening for the synthesis of benzyl decanoate

For the lipase mediated synthesis of benzyl decanoate, 20 mg benzyl alcohol, decanoic acid (2 equiv.) and 2 mg lipase (in order to respect the substrate: enzyme weight *ratio* of 10:1, the amount of each biocatalyst was determined based on the enzyme loading, see **Table 1**) were added in 5 mL round-bottom flasks. The flasks were stirred (600 rpm) for 90 minutes in an oil bath with magnetic stirring at 30 °C. Subsequently, to remove the resulted water, the flasks were connected to a vacuum line (20 mbar). A similar reaction was set-up using Novozym 435 (33.3 mg) for comparison. After 90 minutes, 1 mL ethanol was added to the reaction mixtures and homogenous samples (10 µL) were withdrawn and diluted with 990 µL ethanol, filtered and injected on HPLC in order to determine the conversion.

Reusability studies

Into magnetically stirred 5 mL round-bottom flasks, 20 mg benzyl alcohol, decanoic acid (2 equiv.) and 2 mg lipase (in order to respect the substrate: enzyme weight *ratio* of 10:1, the amount of each biocatalyst was determined based on the enzyme loading, as described in **Table 1**) were added. The flasks were connected to a vacuum line (20 mbar), stirred (600 rpm) at 30 °C for 90 minutes per reaction cycle. After 90 minutes, 1 mL ethanol was added to the reaction mixtures and homogenous samples (10 µL) were withdrawn and diluted with 990 µL ethanol, filtered and analyzed by HPLC.

After samplings, the flasks were sonicated for 5 minutes and the solvent was taken out. The procedure was repeated three times (adding 3 mL ethanol, sonication and pipetting the solvent out) and the flasks containing the biocatalysts were rotary evaporated to fully remove the solvent. The protocol was repeated seven times for each biocatalyst.

Preparative scale enzymatic synthesis of benzyl decanoate

In a 10 mL round-bottom flask, 1 g of benzyl alcohol, 2 equiv. (18 mmol, 3.18 g) decanoic acid and 100 mg lipase (amount of LS-6 was determined based on the enzyme loading, see **Table 1**) were added. The flask was magnetically stirred (600 rpm) at 30 °C for 90 minutes. In order to efficiently remove the water formed in the system, the flask was connected to a vacuum line (20 mbar). After 90 minutes, 5 mL ethanol was added and a 10 µL homogenous sample was withdrawn, diluted with 990 µL ethanol, filtered and injected on HPLC in order to establish the reaction conversion. The rest of the mass was rotary evaporated in order to completely remove the solvent. 10 mL dichloromethane was added and the organic phase was washed with sodium carbonate (Na_2CO_3, 3 x 20 mL) in order to remove the unreacted decanoic acid. The organic phase was separated and dried with sodium sulfate, evaporated under advanced vacuum, yielding 2.2 g of pure benzyl decanoate (η=91%). The products' purity was verified by ^{1}H-NMR and ^{13}C-NMR.

Benzyl decanoate:

Yield: 91% (2.2 g):^{1}H-NMR (400 MHz, $CDCl_3$): 0.88 (3 H, t), 1.26-1.33 (12 H, m), 1.66 (2 H, m), 2.32 (2 H, m), 5.2 (2 H, s), 7.33 (5 H, s);^{13}C-NMR (100 MHz, $CDCl_3$): 14.1, 22.7, 25.0, 29.0, 29.3 (2 C), 29.6, 31.9, 33.9, 66.4, 127.1-128.9 (4 C), 136.1, 173.1.

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