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Enzyme-catalyzed preparation of chenodeoxycholic esters by an immobilized heterologous *Rhizopus oryzae* lipase

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ABSTRACT

A lipase-catalyzed preparation of ethyl and stearyl esters of chenodeoxycholic acid is described. Stearyl chenodeoxycholate is a new product and both bile acid esters were prepared through an enzymatic approach for the first time. The heterologous *Rhizopus oryzae* lipase, immobilized on two different supports proved to be an efficient catalyst, even more active than *Candida antarctica* lipase, in the esterification reaction using a complex substrate such as a bile acid. The immobilization of the enzyme on Octadecyl Sepabeads at pH 7 and 25 °C was the best choice to catalyze the esterification reaction. The influence of various reaction parameters, such as nature of the alcohol, alcohol:substrate ratio, enzyme:substrate ratio, solvent and temperature, was evaluated. Using the response surface methodology and a central composite rotatable design, the conversion of stearyl chenodeoxycholate was optimized by means of the study of the effect of enzyme:substrate ratio and alcohol:substrate ratio. The value 20 for ratios (E/S) and (A/S) was predicted as the optimal value to reach the maximum conversion. However, including economic aspects these ratios can be reduced up to 15. The well-known advantages of biocatalysis and the activity shown by the immobilized heterologous lipase make the reported procedure a convenient way to prepare chenodeoxycholic esters.

1. Introduction

Bile acids biosynthesis is the main pathway of cholesterol catabolism and consequently plays an important role in its regulation [1]. Bile acids structure exhibits a hydrophobic side (β) and a hydrophilic side (α) which makes them responsible for the amphipathic nature and self-association in water to form multimers above a critical concentration [2]. Their derivatives in C-3, C-7, C-12 or C-24 lacked the supramolecular structure and therefore, have different physicochemical and biological properties.

Among bile acids, chenodeoxycholic acid (CDCA, **1**) derivatives showed to be potential active ingredients in pharmaceuticals exhibiting antitussive and *in vitro* antitumor activity [3,4] and excellent antibacterial activity against *Bacilllus subtilis* and *Staphylococcus aureus* [5].

Moreover, CDCA esters are important as intermediates in the synthesis of related steroid compounds. In the synthesis of 6-ethyl chenodeoxycholic acid, a farnesoid X receptor agonist, it was not possible to achieve the preparation of silylenol ether without the previous protection of the carboxyl function at C-24 as a benzyl ester [6]. It was also necessary to use a methyl ester of CDCA as raw material in the synthesis of 3-bromo and 3-azido CDCA [7] and ethyl chenodeoxycholate was used as intermediate in the synthesis of chenodiol from stigmasterol [8].

So far, CDCA esters were prepared by traditional chemical procedures. Methyl ester was prepared using acetyl chloride and methanol at 0 °C, methanol and *p*-toluenesulfonic acid or methanesulfonic acid [9] and also applying these conditions under microwave irradiation [10]. Benzyl esters were prepared by treatment of CDCA with benzyl bromide and Cs_2CO_3 at 150 °C during 24 h [6]. These chemical methods involve high temperatures and reagents which are not friendly to the environment such as acid chlorides and sulfonic acids.

Biocatalysis proved to be a good alternative to the synthesis of organic compounds [11]. Their chemo-, regio- and stereoselective

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behavior allows carrying out different chemical transformations without the need for tedious protection and deprotection steps in compounds with several functional groups [12–14]. Over the last years, biocatalysis using lipases in non-aqueous media has been widely applied in the synthesis of pharmaceuticals and biologically active compounds, particularly steroids derivatives [15–18].

In the field of bile acids, the acylation of chenodeoxycholic acid methyl ester with trichloroethyl butanoate and fatty acid esters was performed using lipases from *Candida cylindracea* and *Candida antarctica* respectively [19]. As above mentioned, in the chemical acylation of bile acids it was also necessary the previous esterification of carboxyl group in C-24 to carry out the acylation successfully.

The extracellular sn-1,3-regioselective lipase from *Rhizopus oryzae* has been found an interesting catalyst in esterification processes to produce fatty acid esters and enantiomeric resolution [20]. In the last decade, ROL has been cloned and expressed successfully in the cell factory *Pichia pastoris* under different promoters and operational strategies [21].

ROL was immobilized on different supports, particularly resins such as Sepabeads or Lewatit, allowing them to be used in different organic solvents without any swelling effect [22]. The immobilized ROL was used as biocatalyst in a wide range of biotransformations: structured lipids as production of low caloric triacylglycerols and human milk fats substitutes, flavors as pineapple aroma and biodiesel production [23].

Encouraged by these previous results and the possibility of obtaining CDCA esters through a green methodology, in the present work we report the enzymatic esterification of chenodeoxycholic acid (**1**) catalyzed by immobilized commercial *C. antarctica* lipase (CAL B) and recombinant *R. oryzae* lipase (ROL) (Scheme 1). The optimization of the process by using different interfacial activated immobilized preparations of ROL has been performed. Moreover, after fixing some operational parameters, the optimal conditions of enzyme:substrate ratio and alcohol:substrate ratio have been determined by a central composite rotatable experiment design using the response surface methodology, for the best immobilized derivative.

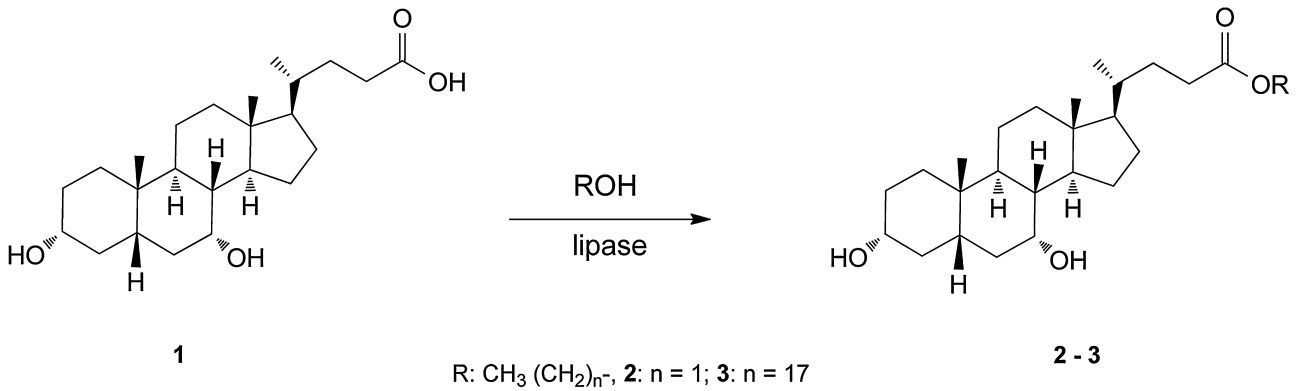
2. Experimental

2.1. General

Chenodeoxycholic acid, *p*-nitrophenylbutyrate (*p*NPB) and solvents used in this work were of analytical grade and purchased from Sigma–Aldrich. *C. antarctica* lipase B (CAL B): Novozym 435 (7400 PLU/g) was a generous gift of Novozymes Spain. Octadecyl Sepabeads was purchased from Resindion rsl and Lewatit VP OC1600 was purchased from Bayer. The immobilized enzymes were dried overnight in vacuum drying oven before use (0.1 kPa, 30 °C). Enzyme:substrate ratio (E/S): enzyme amount (CAL B, ROL1 and ROL2) in mg/substrate amount in mg. Alcohol:substrate ratio (A/S): alcohol amount in mg/substrate amount in mg. Enzyme specific activity (micromoles/mg lipase × h): ROL1: 0.62; ROL2: 0.17; CALB: 0.10. Enzymatic reaction was carried out on Innova 4000 digital incubator shaker, New Brunswick Scientific Co. at 200 rpm. To monitor the reaction progress aliquots were withdrawn and analyzed by TLC performed on commercial 0.2 mm aluminum-coated silica gel plates (F254) and visualized by 254 nm UV or immersion in an aqueous solution of $(NH_4)_6Mo_7O_{24}\cdot 4H_2O$ (0.04 M), $Ce(SO_4)_2$ (0.003 M) in concentrated H_2SO_4 (10%). % Conversion was determined by gas chromatography on a Thermo Focus GC chromatograph equipped with a flame ionization detector and using HP-17 column (10 m × 0.53 mm ID, 0.25 thickness; Agilent Corporation, USA). Nitrogen was the carrier gas. Both injector and detector temperatures were set at 250 and 300 °C respectively. Column temperature was programmed from 100 to 200 °C at a rate of 10 °C/min and stable at 200 °C for 1 min, then at a rate of 15 °C/min up to 290 °C and stable at this temperature for 10 min. Retention times: chenodeoxycholic acid (**1**) 13.5 min; ethyl chenodeoxycholate (**2**): 14.3 min, stearylchenodeoxycholate (**3**): 15.2 min. Melting point was determined on a Fisher Johns apparatus and is uncorrected. Elemental analysis was carried out with a CE-440 Elemental Analyzer. Proton and carbon NMR spectra were carried out on a Bruker AM-500 (500 MHz for ^{1}H and 125.1 for ^{13}C) in $CDCl_3$. Chemical shifts (δ) are reported in ppm downfield from TMS as the internal standard. Coupling constant (J) values are given in Hz. The assignment of the proton signals is based on the chemical shift correlation experiments (COSY) while the carbon nuclei were assigned from the heteronuclear correlation experiments *via* one-bond (HSQC) coupling and long-range (HMBC) coupling. ESI-HR MS was measured in a Bruker microTOF-Q II mass spectrometer.

2.2. Enzyme production

ROL was produced by the Bioprocess Engineering and Applied Biocatalysis group of Universitat Autònoma de Barcelona (UAB). It has been obtained by a fed-batch cultivation of a recombinant *P. pastoris* strain using a methanol non-limiting fed-batch strategy maintaining a set-point of methanol of 3 g L^{-1} by means of a predictive-PI control strategy [21c]. After centrifuged and microfiltered the culture broth, the supernatant was concentrated by ultrafiltration with a Centrasette® Pall Filtron system equipped with an Omega membrane of 10 kDa cut-off, and subsequently



Scheme 1. Enzyme-catalyzed esterification of chenodeoxycholic acid.

dialyzed against 10 mM Tris–HCl buffer pH 7.5 and thereafter lyophilized [21d].

2.3. Enzymatic activity assay

Following the immobilization process, the activities of the supernatant and suspensions were analyzed spectrophotometrically at different times measuring the increment in absorbance at 348 nm produced by the release of *p*-nitrophenol (*p*NP) ($\varepsilon = 5150\,M^{-1}\,cm^{-1}$) in the hydrolysis of 0.4 mM *p*-nitrophenyl butyrate (*p*NPB) in 25 mM sodium phosphate at pH 7 and 25 °C. To initialize the reaction, 0.05–0.2 mL of lipase solution or suspension was added to 2.5 mL of substrate solution. Enzymatic activity is given as micromole of hydrolyzed *p*NPB per minute per milligram of enzyme (IU) under the conditions described above.

2.4. Immobilization of ROL on Octadecyl Sepabeads® (ROL 1) or Lewatit VP OC1600 (ROL 2)

Two grams of dry Octadecyl Sepabeads or Lewatit 1600 was added to 100 mL of a solution water/acetone (50/50, v/v) for 2 h. Then, the solution was filtrated by vacuum and the support was added to 30 mL of sodium dihydrogen phosphate buffer (25 mm, pH = 7) containing 24 mg lipase. The mixture was then gently stirred at 25 °C and 250 rpm overnight on Coulter stirrer. After that, the solution was filtered on sintered glass filter and the supported lipase was washed several times with abundant distilled water. The immobilization yield was more than 99% determined by the enzymatic activity assay described above and Bradford's assay [24]. The prepared catalysts (containing 12 mg lipase per gram of support) were called ROL 1 and ROL 2 respectively. Then, the immobilized biocatalysts (ROL1 and ROL2) were washed several times with acetone to remove the water and dried at 37 °C for 48 h.

2.5. Effect of E/S and A/S ratios on conversion

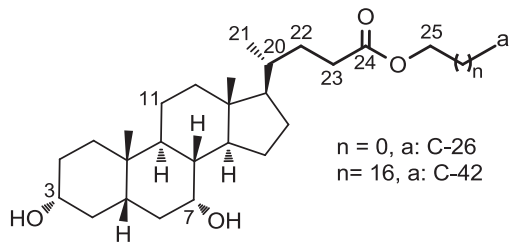
The effect of enzyme/substrate (E/S) and alcohol/substrate (A/S) ratio on the conversion of the chenodeoxycholic esters was studied by means of a central composite design (CCD) with Matlab using ROL1 (ROL immobilized on Octadecyl Sepabeads). The E/S and A/S ratios were selected according with preliminary experiments and were in the range 5–25. The values of both variables were codified from −1.41 to 1.41. The empirical response surfaces were built from the values of conversion after 48 h of conversion.

All the experiments were made under the same conditions of immobilized biocatalyst (dry); temperature and shaking were fixed at 55 °C and 200 rpm, respectively. DIPE was used as solvent, and chenodeoxycholic acid was esterified using stearyl alcohol.

2.6. Enzymatic esterification

2.6.1. General procedure

The heterologous *Rhizopus oryzae* lipase immobilized on Octadecyl Sepabeads (ROL 1) (2 g) was added to a solution of chenodeoxycholic acid (100 mg, 0.25 mmol) in diisopropyl ether (10 mL) and ethanol (0.25 mL, 5 mmol) or stearyl alcohol (1.35 g, 5 mmol). The suspension was shaken (200 rpm) at 55 °C and the progress of the reaction monitored by TLC/GC. When the acid was converted into the alkyl ester, the enzyme was filtered off and the solvent evaporated under reduced pressure. The residue was purified by column chromatography employing mixtures hexane:ethyl acetate as eluent (9:1–7:3). Reuse experiments: the filtered and washed enzyme was used in the next enzymatic esterification under the same reaction conditions.



2.6.2. Ethyl 3α,7α-dihydroxy-5β-cholan-24-oate (**2**)

Colorless oil. Yield: 366 mg (87%). FT-IR (film) $\nu_{max}(cm^{-1}) = 3414, 1754$. ^1H NMR ($CDCl_3$) δ: 3.84 (dd, $J = 3.7$, 2.6 Hz, 1H, H-3), 3.46 (tt, $J =$ 11.1, 4.3 Hz, 1H, H-7), 0.65 (s, 3H, H-18), 0.90 (s, 3H, H-19), 0.92 (d, $J = 6.6$ Hz, 3H, H-21), 4.11 (q, $J = 7.1$ Hz, 2H, H-25), 1.40 (t, $J = 7.2$ Hz, 3H, H-26). ^{13}C NMR ($CDCl_3$): δ: 72.2 (C-3), 68.7 (C-7), 11.9 (C-18), 18.4 (C-19), 17.5 (C-21), 21.9 (C-23), 174.5 (C-24), 60.3 (C-25), 14.4 (C-26). ESI-HR MS *m/z*: calcd. for $C_{26}H_{44}O_4$: $[M+H]^+$ 421.3312, found 621.3317; $[M+Na]^+$ calcd: 443.3132, found 443.3135 [39b]. Analysis for $C_{42}H_{76}O_4$: calcd. C, 74.24; H, 10.54, found: C, 74.22; H, 10.55.

2.6.3. Octadecyl 3α,7α-dihydroxy-5β-cholan-24-oate (**3**)

White solid. Yield: 543 mg (84%). Mp: 61–62 °C. FT-IR (film) $\nu_{max}(cm^{-1}) = 3420, 1761$. ^1H NMR ($CDCl_3$) δ: 3.85 (dd, $J = 3.8$, 2.7 Hz, 1H, H-3), 3.46 (tt, $J = 11.0$, 4.3 Hz, 1H, H-7), 0.65 (s, 3H, H-18), 0.90 (s, 3H, H-19), 0.92 (d, $J = 6.5$ Hz, 3H, H-21), 4.05 (t, $J = 6.7$ Hz, 2H, H-25), 1.62 (m, 2H, H-26), 1.28–1.38 (broad signals, 30H, H-27-41), 0.88 (t, $J = 6.85$ Hz, 3H, H-42). ^{13}C NMR ($CDCl_3$): δ: 72.0 (C-3), 68.6 (C-7), 11.8 (C-18), 18.3 (C-19), 17.6 (C-21), 22.8 (C-23), 174.4 (C-24), 64.5 (C-25), 28.2 (C-26), 14.1 (C-42). ESI-HR MS *m/z*: calcd. for $C_{42}H_{76}O_4$: $[M+H]^+$ 645.5816, found 645.5820; $[M+Na]^+$ calcd. 667.5634 m/z; found 667.5638 m/z. Analysis for $C_{42}H_{76}O_4$: calcd. C, 78.08; H, 12.01, found: C, 78.12; H, 12.05.

3. Results and discussion

3.1. Enzymatic esterification

The enzyme-catalyzed esterification of chenodeoxycholic acid (**1**) allowed us to obtain two ester derivatives: ethyl chenodeoxycholate (**2**) and stearylchenodeoxycholate (**3**) (Scheme 1).

Product **2** was fully identified by ESI-HR MS and ^1H NMR spectroscopy, which was in accordance with reported data [8]. In carbon-13 NMR spectrum, the signals at δ: 60.3 and 14.4, corresponding to carbons in methylene and methyl groups respectively in the ethyl ester were observed as expected. Regarding the signal corresponding to the carbonyl group, it was observed a downfield shift from 178.9 ppm in **1** to 174.5 ppm in **2**.

The identity of stearyl chenodeoxycholate **3**, which is a new compound, was completely determined by spectroscopic methods. Its spectroscopic analysis was consistent with the formation of the stearyl ester of chenodeoxycholic acid. The molecular formula was determined as $C_{42}H_{76}O_4$, on the basis of the elemental analysis and ESI-HR MS spectrum observing the peaks: $[M+H]^+$: 645.5820 and $[M+Na]^+$: 667.5638. The molecular ion was 645 larger from that of chenodeoxycholic acid, which indicated the formation of the stearyl ester.

In the ^1H NMR spectrum, it was observed a triplet at δ 0.88 ppm corresponding to methyl group in the end of the stearyl group, multiplets at 1.28–1.38 ppm corresponding to the methylene groups in the stearyl chain and a triplet at 4.05 ppm assigned to the methylene group bonded to the carboxylic oxygen of the ester. In the ^{13}C NMR spectrum we observed the new signals of the methylene at C-25 at 64.5 ppm and the methyl C-42 at 14.1 ppm from the new stearyl group. The chemical shifts assignments, made on the basis

of the information obtained from ^{1}H NMR, ^{13}C NMR, HSQC, HMBC and COSY (Supplementary data), allowed identifying **3** as octadecyl 3α,7α-dihydroxy-5β-cholan-24-oate.

In order to optimize the reaction conditions we have performed several experiments involving lipase screening and variation of the reaction parameters such as temperature, enzyme:substrate ratio (E/S), alcohol:substrate ratio (A/S) and solvent.

3.1.1. Enzyme screening and solvent effect

To begin, the behavior of CAL B, free ROL, and two immobilized preparations named: ROL1 (immobilized on Octadecyl Sepabeads®) and ROL2 (immobilized on Lewatit® 1600) was evaluated in the esterification of chenodeoxycholic acid with stearyl alcohol.

Previous work on the acetylation reaction catalyzed by ROL [18c] showed that enzyme performance was improved when it was dried before use. The four lipase preparations, CAL B, free ROL, ROL 1 and ROL 2, were evaluated in the esterification of **1** with stearyl alcohol (Table 1).

The solvents used for this screening were acetonitrile, hexane and diisopropyl ether (DIPE). Reactions were carried out at 55 °C using E/S ratio 30 and stearyl alcohol/chenodeoxycholic acid ratio 30. TLC and GC monitoring allowed the identification of the lipase able to promote the esterification of **1.** Control reactions such as using the matrix material without enzyme or without biocatalyst were tested and no conversion was observed in any case.

Among the tested enzymes, ROL1 gave the most satisfactory results followed by CALB (Table 1, entries 1–3 and 7–9 respectively). ROL 2 showed moderate to poor activity (Table 1, entries 10–12) whereas no traces of product were obtained using free ROL.

If we compare the results of the enzyme using DIPE as solvent, even ROL 2 showed higher activity than CAL B, being both immobilized on the same support. Therefore, the best results obtained with ROL 1 in the esterification reaction could be attributed both to the lipase source and the support of immobilization. Moreover, it is interesting to mention that the amount of lipase powder in the immobilized enzyme is lower in ROL1 or ROL 2 (12 mg pure lipase/g of support) than in CAL B (~30 mg/g of support) [22c].

Table 1
Optimization of reaction parameters for ROL-catalyzed esterification of CDCA (**1**).[a]

Entry	Enzyme	Solvent	Temperature (°C)	E/S	A/S	Conversion (%)
Solvent and lipase						
1	ROL 1	Hexane	55	30	30	75.6
2	**ROL 1**	**DIPE**	55	30	30	89.2
3	ROL 1	Acetonitrile	55	30	30	70.4
4	ROL 2	Hexane	55	30	30	51.1
5	ROL 2	DIPE	55	30	30	60.2
6	ROL 2	Acetonitrile	55	30	30	45.8
7	CAL B	Hexane	55	30	30	69.4
8	CAL B	DIPE	55	30	30	52.1
9	CAL B	Acetonitrile	55	30	30	58.3
E/S						
10	ROL 1	DIPE	55	2	20	30.2
11	ROL 1	DIPE	55	5	20	35.6
12	ROL 1	DIPE	55	10	20	71.0
13	ROL 1	DIPE	55	**20**	20	89.6
14	ROL 1	DIPE	55	30	20	89.9
A/S						
15	ROL 1	DIPE	55	20	5	28.8
16	ROL 1	DIPE	55	20	10	58.4
17	ROL 1	DIPE	55	**20**	20	88.9
18	ROL 1	DIPE	55	20	30	90.3
Temperature						
19	ROL 1	DIPE	30	20	20	65.3
20	ROL 1	DIPE	**55**	20	20	90.1

Alcohol: stearyl alcohol. Conversion was determined by GC.
[a] Time: 48 h.
Bold type values mean optimal reaction conditions.

Table 2
Initial rate of esterification of CDCA catalyzed by different lipase immobilized preparations.

Lipase	Conversion (%)	Time (h)	Initial rate (μmol mg^{-1} lipase min) × 10^{-3}
ROL 1	22.3	5	8.3
ROL 2	20.5	11	3.5
CAL B	19.8	16	0.9

CDCA (25 mM), stearyl alcohol (500 mM); DIPE: 10 mL; E/S and A/S: 20; temperature: 55 °C.

Indeed, the specific activity of ROL 1 was 9 fold higher than that of CAL B in the esterification of CDCA with stearyl alcohol, 8.3 × 10^{-3} and 0.9 × 10^{-3} μmol mg^{-1} lipase min^{-1}, respectively. On the other hand the specific activity of ROL 2 was also slightly higher than CAL B (Table 2).

Although specific activity of lipases used in this work is not outstanding, it should be considered that the steroid skeleton is not an easy substrate for enzymatic modification. It was already reported a low enzymatic activity in the lipase-catalyzed acylation of bile acids and other steroids [19a].

These results show how the immobilization of a lipase on different supports may alter its catalytic features. Octadecyl Sepabeads (ROL 1) is a hydrophobic epoxyacrylic matrix with the surface functionalized with octadecyl groups in contrast to Lewatit (ROL 2 and CAL B) which is a divinylbenzene resin. Although the immobilization of ROL was performed following the same procedure in both cases, at low ionic strength after a simple hydrophobic adsorption of the lipase, the esterification results suggest that the immobilization when using Lewatit may involve some other kind of enzyme–support interactions, perhaps by ionic exchange that could be enough to produce changes in the enzyme features.

In previous work, we have also observed large differences in activity and spectacular changes in enantio preference when using these two types of immobilization methods applied to *C. antarctica* lipase catalyzing reactions in aqueous media [22c]. CAL B seems to exhibit good performance in more hydrophobic solvents such as hexane while ROL 1 is more active in a solvent with intermediate polarity as DIPE [18,19].

Considering that free ROL was completely inactive, the immobilization on Sepabeads made ROL 1 a new and excellent biocatalyst for esterification reactions of chenodeoxycholic acid and potentially overall bile acids.

Once the optimal catalyst was selected, we turned our attention to the solvent effect in the ROL 1 catalyzed reaction. Therefore, hexane, diisopropyl ether and acetonitrile were tested.

It is well-known that hydrophobic water-immiscible solvents such as hexane are a good medium for lipase-catalyzed reactions. The organic medium shows interesting advantages, such as the enhancement of solubility of reactants, activity and stability of the enzyme, the shift of the equilibrium toward product formation and easier separation of the enzyme from the reaction medium at the end of the reaction [16]. On the other hand, water-miscible solvents shows a high tendency of stripping off tightly bound water from enzyme and ability to partition deeper into the enzyme active site which in turn causes loss of both structure and activity of enzymes [25]. Due to the presence of the carboxyl and two hydroxyl groups, chenodeoxycholic acid is a polar molecule, insoluble in non-polar solvents (hexane) and soluble in polar solvents (acetonitrile). As acetonitrile proved to be useful as solvent in previous work using a variety of substrates, we decided to test it [26]. However, enzymes performance was poor in this solvent as observed in Table 1 (entries 3, 6 and 9). Only ROL 1 afforded the product **3** in good conversion (70.4%) using acetonitrile as solvent. This result was improved performing the esterification in DIPE, therefore this was the solvent of choice.

3.1.2. Influence of temperature

With the aim of investigating the influence of temperature on the enzymatic esterification we performed it at 30 °C and 55 °C. The other reaction parameters were settled to their optimal values (ROL 1, DIPE, E/S and A/S: 20). The results in Table 1 (entries 19 and 20) show a better performance with the increase in temperature. Therefore we selected 55 °C as the reaction temperature. It is interesting to observe that the stability of the enzyme was kept unaltered with the increase of temperature reaction.

3.1.3. Effect of enzyme:substrate ratio

Then the reaction was analyzed studying the influence of the enzyme:substrate ratio in the enzymatic esterification. This was evaluated at 48 h, using A/S: 20, DIPE as solvent at 55 °C and variable amounts of ROL 1. From the obtained results, E/S: 20 (Table 1, entry 13) resulted as the ratio of choice.

3.1.4. Effect of alcohol and alcohol agent:substrate ratio

In order to test the influence of the alkyl chain length of the alcohol on the enzymatic esterification of chenodeoxycholic acid with the immobilized recombinant lipase ROL 1, we prepared ethyl (**2**) and stearyl (**3**) chenodeoxycholic esters. The results were both very good and much the same, the ethyl chenodeoxycholate (**2**) was obtained in 87% yield while stearyl chenodeoxycholate (**3**) in 84% yield. The alkyl chain length of the alcohol had no significance on yield in the esterification catalyzed by ROL 1.

The influence of alcohol:substrate ratio on reaction yield was evaluated in the esterification of chenodeoxycholic acid **1** with stearyl alcohol in DIPE using ROL 1. As expected, it was observed that a molar excess of alcohol was advantageous for the reaction (Table 1, entries 15–18) with A/S 20 (entry 17) giving very good results (88.9% of conversion). A slightly better conversion was observed using a higher excess of alcohol (A/S: 30 afforded 90.3%).

The optimum reaction time was also studied and conversion was not improved after 48 h of reaction (Supplementary Data Fig. 1S).

E/S and A/S ratios are key parameters in order to establish an optimal relationship not only in conversion but also in economics terms. Thus, a response surface methodology and a central composite experimental design were performed. E/S and A/S selected range is described in Fig. 1, according with the described previous individual experiments.

The constant operational conditions were fixed following the results obtained in the esterification reaction. Therefore, the biocatalyst was previously dry, temperature and shaking were fixed at 55 °C and 200 rpm respectively, stearyl alcohol was used as esterifying agent and DIPE as solvent. The conversions of each reaction are presented in Table 3.

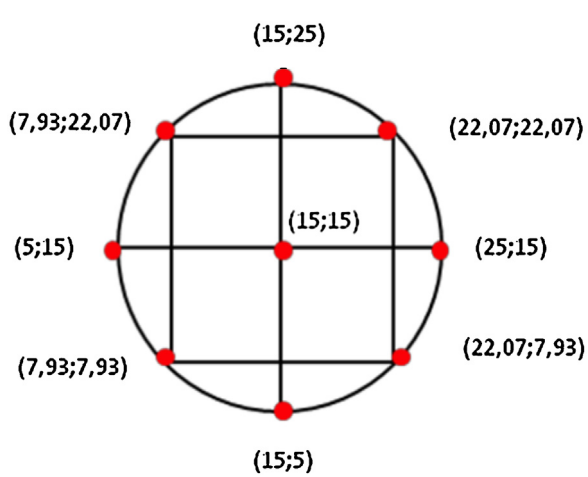


Fig. 1. Diagram of the central composite rotatable design, using the nomenclature (E/S); (A/S).

Table 3
Conversion reached in each reaction at different (E/S) and (A/S) ratios.

Experiment number	Codified factor (E/S)	Codified factor (A/S)	Non codified factor (E/S)	Non codified factor (A/S)	Conversion (%)
1	−1	−1	7.93	7.93	15.2
2	−1	1	7.93	22.07	27.4
3	1	−1	22.07	7.93	35.3
4	1	1	22.07	22.07	88.5
5	−1.4142	0	5	15	12.6
6	1.4142	0	25	15	85.3
7	0	−1.4142	15	5	21.9
8	0	1.4142	15	25	83.2
9	0	0	15	15	84.1
10	0	0	15	15	83.9
11	0	0	15	15	85.3
12	0	0	15	15	87.3
13	0	0	15	15	84.5
14	0	0	15	15	85.0
15	0	0	15	15	81.0
16	0	0	15	15	86.5

The results obtained were fitted to the mathematical modeling expressed in Eq. (1), and the corresponding response surface is plotted in Fig. 2.

$$X = \beta_1 + \beta_2 \cdot ES + \beta_3 \cdot AS + \beta_4 \cdot ES \cdot AS + \beta_5 \cdot ES^2 + \beta_6 \cdot AS^2 \quad (1)$$

The values of each coefficient and the statistical parameter p values are shown in Table 4.

All the terms of Eq. (1) has significant effect on conversion ($p < 0.05$). The term ES·AS has the lowest effect on the estimation. As it has been observed in previous individual experiments about effect of E/S and A/S ratios, the optimum value of (E/S) and (A/S) to obtain practically a total conversion was 20. However from an economical point of view the optimum value can be reduced up to 15 for both ratios.

Thus, optimal conditions from a conversion and economic point of view for the esterification of chenodeoxycholic acid using ROL

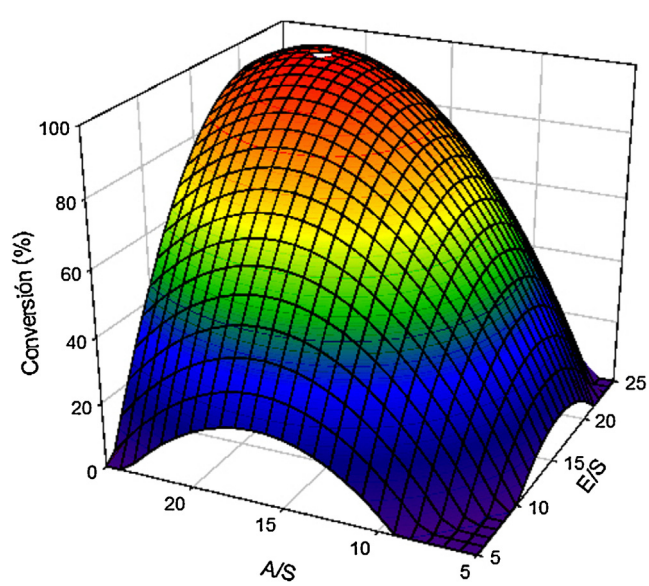


Fig. 2. Response surface of the experimental design, corresponding to the conversion of stearyl deoxycholate (**3**) under different ratios of E/S and A/S. Reactions were carried out at 55 °C and 200 rpm, using dry ROL 1, DIPE as solvent and stearyl alcohol. Reaction time was 48 h.

Table 4
Parameter and statistical *p* values of Eq. (1).

Coefficient	β_1	β_2	β_3	β_4	β_5	β_6
Value	84.700	23.002	19.012	10.250	−20.163	−18.363
p value	1.063×10^{-12}	3.620×10^{-7}	2.111×10^{-6}	0.004	1.232×10^{-6}	2.895×10^{-6}

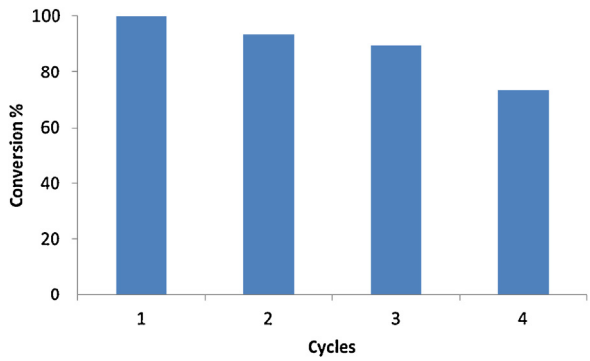


Fig. 3. ROL 1 reuse in the esterification of chenodeoxycholic acid under standard conditions.

1 as biocatalyst are: E/S: 15, solvent: DIPE, temperature: 55 °C and stearyl alcohol/CDCA: 15.

Finally, we studied the possibility of recycling ROL 1. Due to ROL 1 is immobilized, it is insoluble in DIPE. Therefore, it can be easily removed by filtration at the end of the process and can be reused. For the esterification of CDCA with stearyl alcohol ROL 1 kept almost 90% of its original activity after three reaction cycles (Fig. 3).

4. Conclusions

In this report we have studied the performance of CAL B and two immobilized heterologous *Rhizopus oryzae* lipases (ROL1 and ROL 2) as catalysts in the esterification of chenodeoxycholic acid. For the first time two bile acid esters were prepared in very good yield following the biocatalytic approach. One of them, the stearyl chenodeoxycholate is a new product. ROL immobilized on Octadecyl Sepabeads (ROL 1) and using DIPE as solvent, was the best biocatalyst in the preparation of the steroid derivatives, showing an activity nine fold higher than CAL B.

Moreover, ROL 1 performance was not affected by the chain length in the alcohol, affording both esters in similar yield and it kept almost 90% of its original activity after three reaction cycles.

By means of a response surface methodology and a central composite rotatable design, the optimal values of enzyme:substrate ratio and alcohol:substrate ratio were determined in order to maximize conversion and minimize economics of the bioprocess. The optimal values of (E/S) and (A/S) were 15 for both, lower than the values obtained from individual experiments, saving both enzyme and alcohol.

The enzymatic approach provided a simple and mild alternative method for the synthesis of chenodeoxycholic esters and potentially other bile acid derivatives. It proved to be a convenient way to prepare bile acid derivatives in high purity and free of toxic reagents, which is a great advantage in the case of products designed for human consumption such as pharmaceuticals.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.molcatb.2015.05.008

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