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Optimization of reaction parameters for the synthesis of natural aroma esters by factorial design†

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In this study, the synthesis of aroma esters by the direct esterfication of carboxylic acids with aromatic alcohols mediated by lipase B from *Candida antarctica* encapsulated in a sol–gel matrix in a solvent-free system is presented. Vacuum was used in order to remove the resultant water. The reaction parameters were optimized by factorial design experiments considering four factors (acid excess, temperature, vacuum and time) on two levels. As a result, the conversions were significantly increased (for example, from an isolation yield of 49.4% to 94.3% for cinnamyl butyrate). A semi-preparative experiment was further set up for cinnamyl butyrate preparation. The green chemistry metrics, such as the *E*-factor of 4.76 and mass intensity of 6.04, demonstrated that the newly developed enzymatic process is suitable for industrial application based on green chemistry principles.

Introduction

The increasing demand for bio-products has prompted increased efforts by the food and agricultural industries to develop alternative methods for preparing food ingredients with reduced environmental impact.[1,2] Two conventional methods are currently applied at an industrial level to obtain food additives: extraction from natural sources and chemical synthesis; however, both the methods present some disadvantages. Typically, when extracting food additives from natural sources, products with low purity are obtained since the extraction is generally not selective and further purification steps are needed. Another disadvantage of this method is the low yield and the large quantities of starting materials needed. The general public is increasingly hesitant to consume products containing ingredients obtained *via* classical chemical routes, which are characterized by harsh chemical reaction conditions. These concerns led to an increased number of bioprocesses applied at industrial level for obtaining food additives, and research groups all over the world are encouraged to develop more eco-friendly processes.[3]

Aroma compounds can be obtained through enzymatic reactions using mostly lipases, and enzymes can also be employed in the food processing industries, as already reported by several work groups. There are many advantages to employing enzymes to catalyze (*trans*) esterification reactions with flavour esters as end products, such as high productivity and yields, low energy consumption, short reaction times, and the fact that the esters obtained *via* enzymatic synthesis can be considered 'natural'. However, there are still some limitations that reduce the employment of enzymes in industrial processes: mass-transfer resistances, high prices of enzymes, and the presence of water in the reaction system, which can hydrolyze the formed esters.[4] Sarno *et al.* reported the use of lipase from *Thermomyces lanuginosus* (TLL) in order to obtain banana-flavoured esters.[5] Short-chain flavour esters were also obtained by direct esterification mediated by lipase B from *Candida antarctica* (CaL-B).[6] In another study, the immobilized lipase from *Lactobacillus plantarum* was successfully applied in the synthesis of short-chain flavour esters, such as 2,3,4-trihydroxybenzyl acetates; however, the reaction times were quite long (48 h).[7] Short-chain flavour esters have also been synthesized in whole-cell lipase-mediated esterification with *Aspergillus oryzae*, but the conversion values were quite low (~44% after 24 h).[8] To develop a green and economically feasible process, solvent use should be avoided. Indeed, the solvent-free enzymatic synthesis of aroma esters has been previously reported.[6,9–11] Lipases, as versatile biocatalysts, have been reported to have potential in many applications, such as the enzymatic synthesis of a 2-phenethyl esters library,[12] the *O*-acylation of heteroaromatic alcohols,[13] β-lactam antibiotics,[14] biodiesel additives synthesis,[15,16] and biodiesel production.[17–20]

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The underlying need for waste reduction and high (enantio)selectivities has fostered the introduction of enzyme-catalysed reaction cascades as a sustainable technology for the synthesis of active pharmaceutical ingredients and food additives. For this, a certain route should be taken that starts from enzyme selection, reaction development, protein engineering (if required), and enzyme immobilization.[21] Enzyme immobilization is a topic of interest in biochemistry/biotechnology as the use of free enzymes at the industrial level is not recommended for several reasons; for example, a batch of free enzymes can be used only once and therefore the process would not be economically feasible. Another problem that can occur when using free enzymes is the risk of product contamination with proteins and as enzyme inactivation can occur due to it being in direct contact with the reaction medium. These limitations can be solved through enzyme immobilization, which can be achieved by various techniques, including adsorption on a surface, by covalent binding to a surface, by cross-linking enzyme molecules, by encapsulation, or by tag-binding. The most popular form of CaL-B immobilization is by adsorption on a macro-porous resin, which is commercially available under the name Novozym 435. Novozym 435 has been previously used for the efficient synthesis of biodiesel additives[15,16] and food additives,[22,23] as well as for obtaining enantiopure aliphatic secondary alcohols[24] just to name a few applications. However, this biocatalyst also has some limitations, such as mechanical fragility and support dissolution or enzyme desorption under certain conditions.[25] These limitations have encouraged researchers in this domain to try to develop a "perfect" biocatalyst that could be universally applied with satisfactory results. As part of this research effort, a new and innovative co-immobilization method of two or more biocatalysts with different stabilities was reported by Fernandez-Lafuente *et al.*, in which trypsin and chymotrypsin were covalently bonded to agarose beads functionalized with vinyl sulfone groups and β-galactosidase was also immobilized on the beads *via* anion exchange.[26] Of all the above-mentioned immobilization methods, the encapsulation of enzymes using the sol–gel technique has received much attention. Sol–gel-encapsulated lipases have been successfully employed in recent times for the continuous kinetic resolution of aliphatic and aromatic secondary alcohols[27] and 1,5-dihydroxy-1,2,3,4-tetrahydronaphtalene,[28] for the esterification of valeric acid,[29] and for the enzymatic synthesis of short-chain flavour esters.[11] The sol–gel encapsulation of enzymes can be coupled with other supports, for example, magnetic nanoparticles[6] or Celite,[30] to obtain biocatalysts with improved stability or which can be easily removed from the reaction mixture.

The aim of the present study was to optimize the enzymatic synthesis of aroma esters *via* the direct esterification of short-chain fatty acids (*i.e.* propionic, butyric, and hexanoic) with aromatic alcohols (*i.e.* anisyl, cinnamyl, and benzyl) in a solvent-free system (SFS) mediated by lipase B from *Candida antarctica* (CaL-B) immobilized by entrapment in a sol–gel matrix.[11] The novelty of our study is the factorial optimization of the reaction parameters for each flavour ester individually, and the introduction of a stable and active biocatalyst in the system, coupled with an efficient water removal method (by applying a vacuum), resulting in significantly improved conversions and significantly reduced reaction times (maximum of 90 min per reaction cycle) compared to in the previously reported studies.

Results and discussion

Initial screening

An enzymatic reaction system using as a biocatalyst CaL-B immobilized in a sol–gel matrix to obtain seven aroma esters with aromatic moieties is proposed in this study. The initial reaction conditions were taken from our previous study,[11] in which aroma esters with aliphatic structures were successfully synthesized using the same biocatalyst. The biocatalyst showed a synthetic activity of 15.28 mmol min^{-1} g_{enzyme}^{-1} and a recovered activity of 110%.[31] The used conditions proved to be optimal for some esters (anisyl butyrate, benzyl butyrate, and hexanoate), whereas moderate and poor results were obtained in the case of other esters, as shown in Fig. 1.

Using the described conditions, the process yielded excellent conversions (>90%) in the cases of anisyl butyrate, benzyl butyrate, and benzyl hexanoate, moderate conversions in the cases of anisyl propionate and cinnamyl butyrate (<50%) and very low conversions (<5%) in the cases of cinnamyl and benzyl propionate. For cinnamyl and benzyl propionate, the reactions were tested also in a green solvent (2-MeTHF) using molecular sieves for water removal.

The use of a solvent and molecular sieves for water removal did not significantly increase the reaction's conversions to produce cinnamyl propionate and benzyl propionate, with only slightly increased conversions (4% for cinnamyl propionate and 10% for benzyl propionate) obtained. Since poor results were obtained in the case of the four aroma esters, optimization of the reaction system parameters was required. Moreover, the process productivity for anisyl butyrate, benzyl butyrate, and benzyl hexanoate preparation could be improved by reducing the amount of

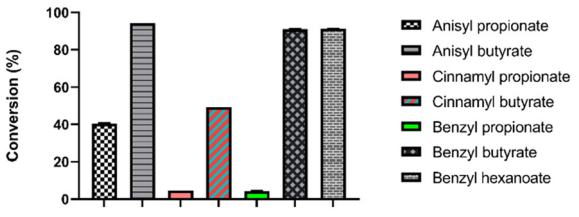

Fig. 1 Synthesis of aroma esters mediated by CaL-B under the initial conditions [0.4 mmol alcohol, 2 equiv. carboxylic acid (propionic, butyric, and hexanoic acid), alcohol : lipase weight ratio of 25 : 1, 20 mbar vacuum, 30 °C, 180 rpm, 1 h].

acid, the temperature, or reaction time. Optimization of the system parameters was performed by the factorial design method. Four parameters were selected to be optimized that were considered to have the most importance over the overall conversion; here: the temperature, vacuum, reaction time, and alcohol : acid molar ratio.

Optimization of the reaction system parameters by factorial design

Optimization of the reaction parameters was performed using a response surface design for the experiments, namely a central composite model. For each aroma ester, four factors were chosen for optimization (alcohol : acid molar ratio, temperature, vacuum, and reaction time) each on two levels (one inferior and one superior). The design of the experiments (DoE) was completed with three additional axial points and one central point (alcohol : acid molar ratio of 1 : 2, temperature of 30 °C, 20 mbar vacuum, and 60 min reaction time).

Anisyl propionate. For anisyl propionate, the optimization of the reaction system parameters showed an increase in the overall conversion from 40.5% under the initial reaction conditions to a maximum conversion of 69.1% (alcohol : acid molar ratio of 1 : 1, 25 °C, 15 mbar vacuum, and 90 min reaction time), as shown in Fig. 2A. Analysing the system, two possible reasons were identified for the low conversions: the 90 min reaction time was not sufficient, or enzyme substrate inhibition occurred (since higher conversions were obtained when lower amounts of propionic acid were used).

Anisyl butyrate. The optimization of reaction system parameters for anisyl butyrate synthesis showed an increase in the conversion from 94.2% under the initial reaction conditions, to a maximum conversion of 98.7% (alcohol : acid molar ratio of 1 : 3, 25 °C, 15 mbar vacuum, and 90 min reaction time), as shown in Fig. 2B. Interestingly enough, higher conversions were obtained in this case when using an excess of butyric acid, suggesting that butyric acid is not an enzyme inhibitor.

Cinnamyl propionate. Cinnamyl propionate synthesis from cinnamyl alcohol and propionic acid yielded the desired compound with a 4.7% conversion under the initial reaction conditions. After optimization of the reaction system's parameters, the desired ester was yielded in a 44% conversion (alcohol : acid molar ratio of 1 : 1, 35 °C, 15 mbar vacuum, 90 min reaction time), as shown in Fig. 3A. Higher conversions were obtained when using an equimolar ratio, while low conversions (<5%) were obtained when using 3 equivalents of propionic acid, similarly to anisyl propionate synthesis, confirming our presumption that at high concentrations propionic acid acts as a biocatalyst inhibitor.

Cinnamyl butyrate. Optimization of the reaction system parameters yielded cinnamyl butyrate with a maximum conversion of 94.3% (alcohol : acid molar ratio of 1 : 3, 25 °C, 25 mbar vacuum, and 90 min reaction time), as described in Fig. 3B. This represented a major improvement since the

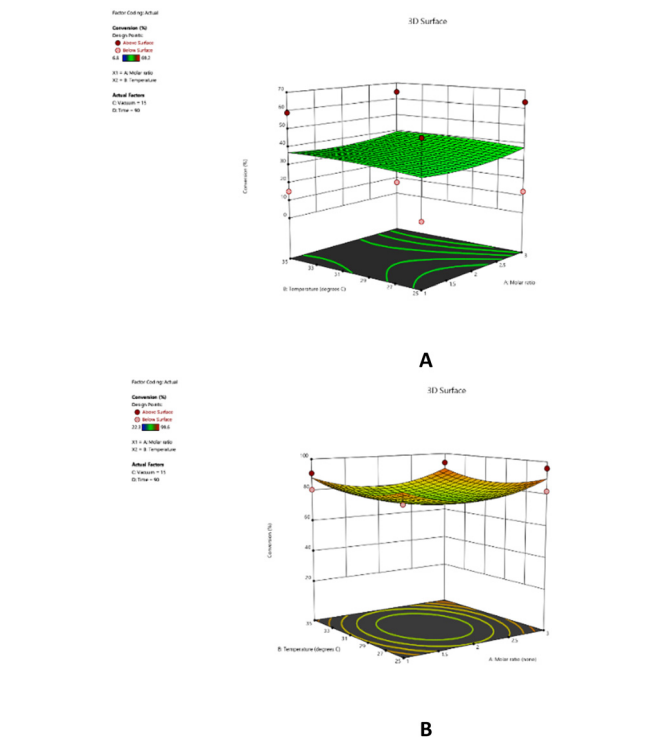

Fig. 2 Surface plot for the optimization of the reaction parameters for (A) anisyl propionate and (B) anisyl butyrate synthesis.

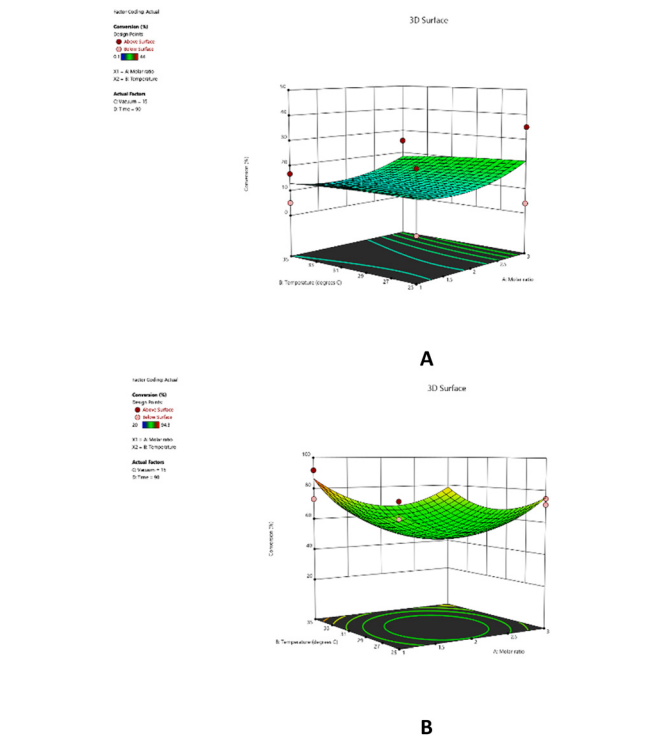

Fig. 3 Surface plot for the optimization of the reaction system parameters for the enzymatic synthesis of (A) cinnamyl propionate and (B) cinnamyl butyrate.

desired ester was obtained with only 49.4% conversion under the initial reaction conditions. The increase in the conversion most likely occurred due to the concomitant increase in butyric acid quantity and in the reaction time.

Benzyl propionate. The same pattern was observed in the case of benzyl propionate: the maximum conversion (38.6%) was recorded when using an equimolar ratio of reactants (alcohol : acid molar ratio of 1 : 1, 25 °C, 15 mbar vacuum, and 90 min reaction time), as described in Fig. 4A. The maximum conversion value obtained after optimization represented an almost ten-fold improvement compared with the initial conditions (4.5%). To confirm the fact that propionic acid acts as an inhibitor of the enzymes' activity, the next step of our study was to monitor the reactions with propionic acid for a longer time.

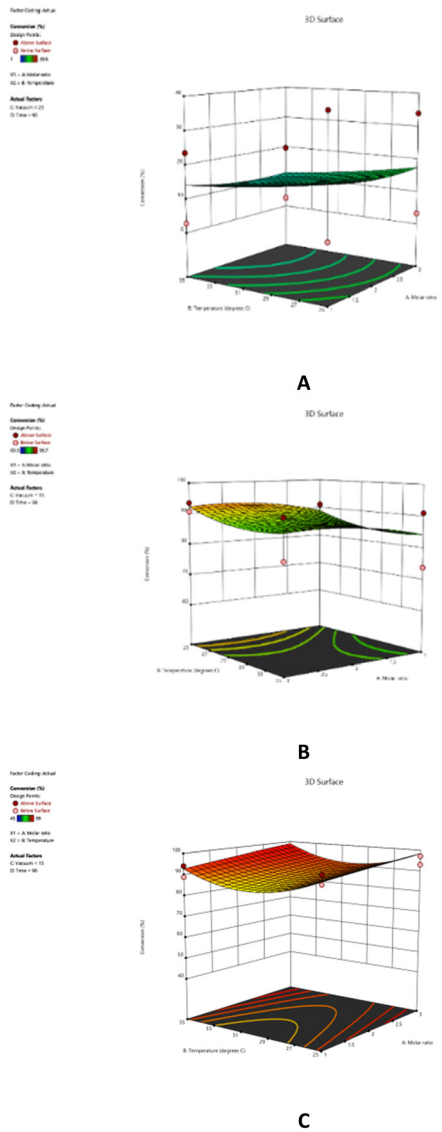

Fig. 4 Surface plot for the optimization of the reaction system parameters for the enzymatic synthesis of benzyl propionate (A), benzyl butyrate (B), and benzyl hexanoate (C).

Benzyl butyrate. Benzyl butyrate was successfully obtained with a 96.2% conversion rate after optimizing the reaction system parameters (alcohol : acid molar ratio of 1 : 3, 35 °C, 15 mbar, and 90 min reaction time), which was a slight increase over the conversion obtained under the initial conditions (91.2%), as described in Fig. 4B. The conversions were higher when an excess of butyric acid was added, which was in concordance with the previously discussed butyrate esters.

Benzyl hexanoate. For benzyl hexanoate, interesting results were obtained, as the maximum conversion value recorded after optimizing the reaction system parameters (alcohol : acid molar ratio of 1 : 3, 25 °C, 15 mbar, 90 min of reaction time) was 97.3%, as shown in Fig. 4C. Since benzyl hexanoate was prepared with a 97.2% conversion in just 30 min simply by increasing the temperature, these conditions must be considered as the best option (alcohol : acid molar ratio of 1 : 3, 35 °C, 15 mbar vacuum, and 30 min reaction time).

Time profiles of the reactions using propionic acid as an acyl donor

As described in the previous section, when using propionic acid, the maximum conversion was 69.1% (for anisyl propionate), which was much lower than when using butyric or hexanoic acid (over 90% in all cases). Two possible reasons were identified for this issue: either propionic acid acted as an enzyme inhibitor, or an insufficient reaction time was chosen. In order to decide which of these reasons was the cause, the enzymatic synthesis under the specific optimum conditions of anisyl, cinnamyl, and benzyl propionate was further monitored for 8 h, withdrawing samples after 2, 4, 6 and 8 h.

As can be seen in Fig. 5, even when increasing the reaction time up to 8 h, the overall conversion was not greatly improved. The highest conversion increase was observed for benzyl propionate: from 32.5% after 2 h to 68.7% after 4 h, then reaching around 75% after 8 h. For cinnamyl and benzyl propionate, the conversions were not significantly improved even after 8 h. Based on these results, we concluded that the enzyme inhibition by propionic acid, a smaller molecule that can accumulate inside the catalytic site of the enzyme or even in-between the pores of the biocatalyst, thereby altering the

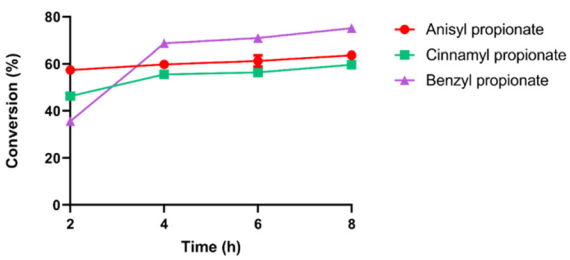

Fig. 5 Time profiles for the anisyl, cinnamyl, and benzyl propionate enzymatic preparation.

Table 1 Comparison of the conversions under the initial reaction conditions (30 °C, 1 : 2 alcohol : acid molar ratio, 20 mbar, 60 min) and after process optimization

	Initial conditions	Optimized conditions				
Desired ester	Conversion [%]	z_1	z_2	z_3	z_4	Conversion [%]
Anisyl propionate	40.4	25	1 : 1	15	90	68.6
Anisyl butyrate	94.2	35	1 : 3	15	90	97.9
Cinnamyl propionate	4.7	25	1 : 3	15	90	42.3
Cinnamyl butyrate	49.4	35	1 : 3	15	90	93.2
Benzyl propionate	4.5	25	1 : 1	15	90	35.5
Benzyl butyrate	91.2	35	1 : 3	15	90	96.1
Benzyl hexanoate	91.3	35	1 : 3	15	90	97.3

diffusion process, was the reason for the lower enzyme activity, as previously noted in the literature.[32,33]

A summary of the overall improvements after the reaction design experiments for the enzymatic processes can be found in Table 1.

Preparative-scale enzymatic esterification of cinnamyl alcohol with butyric acid and evaluation of the sustainability metrics

After demonstrating that DoE optimization is an efficient method for aroma esters preparation with improved conversion, our next aim was to prove the scalability of the newly developed processes. Cinnamyl butyrate was selected as the model, since it showed the best increase in conversion after optimization. The scale-up was set to 1 g cinnamyl alcohol as the starting material. After 90 min, the desired compound, cinnamyl butyrate, was successfully isolated with a 90.5% global yield (1.33 g) as a pure compound.

Cinnamyl butyrate. Yield: 90.5% (1.33 g): ^{1}H-NMR (400 MHz, $CDCl_3$): 0.99 (3 H, d), 1.68 (2 H, m), 2.32 (2 H, d), 4.69 (2 H, d), 6.25 (1 H, m), 6.65 (1 H, d), 7.24–7.33 (5 H, m); ^{13}C-NMR (100 MHz, $CDCl_3$): 13.5, 18.4, 36.2, 65.4, 121.2, 127.9–128.6 (5 C), 133.8, 136.4, 173.1.

The ultimate goal of this study was to evaluate if the newly developed process adheres to the principles of green chemistry. For a process to be considered for industrial scale-up, the amount of waste and overall toxicity must be as low as possible, and the process should be feasible from an economic point of view. Five green metrics (*E*-factor, atom economy, atom efficiency, mass intensity, and reaction mass efficiency) were calculated considering cinnamyl butyrate as the unique product (water formed as a by-product was excluded from the calculations). The results are presented in Table 2 and compared afterwards with the green metrics corresponding to a previously published aqueous enzymatic synthesis of cinnamyl acetate.[34]

From consideration of the *E*-factor, there was a ten-fold difference between the two analysed processes: applying the currently described process would generate ten times less waste due to the process being solvent-free. In terms of atom economy and atom efficiency, the differences were not so significant since both processes used similar reagents (cinnamyl alcohol and butyric acid, or ethyl acetate) and both enzymatic syntheses yielded the desired product with similar conversions. The mass intensity (MI) metric represents the total mass used in the process *versus* the mass of the final product, and it can be seen that the enzymatic esterification of cinnamyl alcohol with butyric acid gave a value of 6.04, which was close to the ideal value of 1 kg kg^{-1}; whereas the enzymatic transesterification of cinnamyl alcohol with ethyl acetate gave an MI value of 35.48 (the process had more purification steps and the total mass used in the process was higher), and the mass of the yielded ester was smaller; therefore, the MI value was higher in the case of cinnamyl acetate. In terms of the reaction mass efficiency, the difference was not significant since this represents the mass of product *versus* the total mass of the reagents used, and the two compared processes were quite similar in these terms. Overall, after optimization, the newly developed enzymatic esterification of butyric acid with cinnamyl alcohol could be considered to be superior in terms of green metrics to the previously described enzymatic synthesis of cinnamyl acetate

Table 2 Sustainability metrics for the enzymatic synthesis of cinnamyl butyrate compared to those for cinnamyl acetate production

	Product	
Sustainability metric	Cinnamyl butyrate[a]	Cinnamyl acetate[b]
E-Factor	4.76	48.07
Atom economy (AE)	89.00	79.27
Atom efficiency (AEf)	80.00	73.72
Mass intensity (MI)	6.04	35.48
Reaction mass efficiency (RME)	44.78	34.17

[a] Enzymatic esterification of butyric acid with cinnamyl alcohol in a solvent-free system. [b] Enzymatic transesterification of cinnamyl alcohol with ethyl acetate in phosphate buffer.[34]

and it adhered more closely to the principles of green chemistry, which brings it one step closer to potential industrial scale-up.

Materials and methods

Materials

Lipase B from *Candida antarctica* was purchased in solution from Novozymes (Copenhagen, Denmark), while polyvinyl alcohol (M = 130 000) was obtained from Fluka (Switzerland), and methanol, dichloromethane, sodium fluoride (NaF), octyltriethoxysilane (OTEOS), *n*-propyltrimethoxysilane (*n*-PTMOS), tetramethoxy-silane (TMOS), hexanoic acid, molecular sieves (0.3 nm, beads about 2 mm), cinnamaldehyde, benzaldehyde, 4-methoxybenzaldehyde (anisaldehyde), sodium carbonate (Na_2CO_3), chlorohydric acid (HCl), sodium sulphate (Na_2SO_4), sodium borohydride ($NaBH_4$), HPLC-grade *n*-hexane, iso-propyl alcohol, 2-methyltetrahydrofuran (2-MeTHF) and propionic acid were purchased from Merck (Germany). Butyric acid was purchased from Alfa Aesar (USA). Triethylamine (TEA), *N*,*N*′-dimethylaminopyridine polymer bound (DMAP) and hexanoic anhydride were acquired from Sigma-Aldrich (USA). All the solvents and reagents were freshly distilled and dried by standard methods before use. For the quantitative spectrophotometric determinations of the enzyme load through the BCA method using the Pierce™ BCA protein assay kit (Thermo Fisher Scientific Inc., USA), an Agilent 8453 UV-vis spectrophotometer equipped with a thermostat was used. ^{1}H-NMR and ^{13}C-NMR spectra were recorded in $CDCl_3$ at 20 °C on a Bruker Avance 400 NMR spectrometer, operating at 400 and 100 MHz, respectively.

Cinnamyl alcohol, benzyl alcohol, and anisyl alcohol were synthesized by the classic reduction with $NaBH_4$ of the corresponding aldehydes dissolved in methanol under continuous stirring at 0 °C with adding small portions of $NaBH_4$. The reaction was periodically verified by thin-layer chromatography (TLC) and the reaction was stopped by the addition of water when complete transformation of the substrate was observed. The methanol was evaporated, and dichloromethane was added for extraction. The organic phase was washed with HCl (2 × 10 mL) and Na_2CO_3 (2 × 10 mL), dried over Na_2SO_4 and the solvent evaporated off, resulting in obtaining the pure corresponding alcohol, as confirmed by the ^{1}H-NMR and ^{13}C-NMR spectra.

The esters were chemically synthesized by mixing the corresponding alcohols with 2 equivalents of propionic, butyric or hexanoic anhydride, 2.5 equivalents of TEA, and a catalytic amount of polymer bound-DMAP in a solvent-free media. After 2 h, water and dichloromethane were added and the reactions were vigorously stirred, and the organic layer was extracted. The organic phase was washed with HCl 10% (3 × 10 mL) and Na_2CO_3 1 M solution (3 × 10 mL), dried over anhydrous Na_2SO_4 and the solvent evaporated off to obtain the pure esters, as confirmed by the ^{1}H-NMR and ^{13}C-NMR spectra.

Chromatographic analysis of the esterification products

At the end of the esterification, the reaction mixture was analysed on an Agilent 7890A gas chromatograph equipped with a flame ionization detector using a 30 m × 0.25 mm Astec CHIRALDEX® B-DM capillary column with a 0.12 μm film thickness. The analysis conditions were: 2 μL injection volume, 50 : 1 split ratio, and injection temperature of 250 °C. The temperature conditions are described in detail for each compound in Table S1.† Conversions were determined using the relative response factor method, by injecting mixtures of pure alcohols and corresponding esters of known concentration and then calculating the relative response factors (Table S1†).

Lipase immobilization by sol–gel entrapment

The sol–gel entrapment of CaL-B was performed as previously reported by us:[11] a mixture of 0.5 mL CaL-B solution (34 mg protein per mL), 100 μL NaF 1 M, 200 μL iso-propyl alcohol, and 200 μL PVA 4% aqueous solution was shaken (500 rpm) in a 4 mL glass vial for 30 min at room temperature. After completing the homogenization, 3 mmol of silane precursors was added (OTEOS : *n*-PTMOS : TMOS, in a 1.6 : 0.4 : 1 molar ratio). The resulting gels were matured for 24 h at room temperature and then washed with 7 mL iso-propyl alcohol, 5 mL distilled water, 5 mL iso-propyl alcohol and 2.5 mL *n*-hexane. The unified washing waters were collected and used to determine the amount of unbound enzyme (through BCA protein assay[35]). The obtained gel was dried for 24 h at room temperature, crushed in a mortar, and stored at 4 °C in glass vials. The synthetic activity of the biocatalyst was determined[36] and it showed a value of 15.28 mmol min^{-1} g_{enzyme}^{-1} and a recovered synthetic activity[37] of 110%.

Initial screening

The best conditions determined in the previous study published by our research group[11] were chosen as the initial conditions. Here, 0.4 mmol alcohol (anisyl alcohol, benzyl alcohol, cinnamyl alcohol), 0.8 mmol acid (propionic, butyric, hexanoic), and the weighted biocatalyst containing lipase in a 25 : 1 substrate : enzyme weight ratio (10.2 mg biocatalyst for anisyl alcohol, 8 mg biocatalyst for benzyl alcohol and 9.9 mg of biocatalyst in the case of cinnamyl alcohol) were added in a magnetically stirred (180 rpm) 5 mL round-bottom flask, maintained at 30 °C (on a temperature-controlled oil bath) and connected to a vacuum line (20 mbar vacuum) for controlled water removal. After 1 h, methanol (1 mL) was added to the reaction mixture and homogenous samples (25 μL) were withdrawn and diluted with methanol (975 μL), filtered, and transferred into vials to be analysed by gas chromatography.

Due to the unsatisfactory results achieved in the solvent-free system for the esterification of cinnamyl and benzyl alcohols with propionic acid, some initial screenings were performed in a solvent. For this, in 1.5 mL glass vials, 0.1 mmol alcohol (cinnamyl and benzyl) and 2 equivalents

propionic acid (0.2 mmol, 14.8 mg) were dissolved in 1 mL 2-MeTHF, which is known as a green solvent. Lipase was added in an amount to respect the 25 : 1 alcohol : lipase weight ratio (2.5 mg biocatalyst for cinnamyl alcohol and 2 mg biocatalyst in the case of benzyl alcohol) and 100 mg of molecular sieves were added to capture the resulting water. A magnetic stirrer equipped with a temperature-controlled oil bath was used to keep the reaction mixture at 30 °C under shaking at 180 rpm. After 1 h, a homogenous sample of 50 μL was withdrawn and the solvent evaporated. The dry sample was reconstituted with 1 mL methanol, filtered, and transferred into vials to be analysed by gas chromatography.

Optimization of the reaction system parameters by factorial design

To optimize the reaction system parameters for each targeted compound, the factorial design method based on the response surface (central composite) was chosen in this study, considering the four parameters with the highest influence over the process efficiency, namely the alcohol : acid molar ratio (z_1), temperature (z_2), vacuum (z_3), and reaction time (z_4). All the experiments were performed in duplicate, and the mean results and standard deviations are presented herein. The factorial design experiments were therefore constructed as 2^4 optimization matrices, meaning 16 experiments for factorial points per desired compound, three axial points, and one central point. The design of experiments was performed using the DesignExpert software. Each tested reaction parameter was evaluated at 2 levels, one inferior and one superior. The base level for each parameter was selected as the initial value previously tested. The step for each parameter was selected as follows: alcohol : acid molar ratio Δz_1 = 1 equiv., temperature Δz_2 = 5 °C, vacuum Δz_3 = 5 mbar and reaction time Δz_4 = 30 min. The detailed reaction optimization matrices can be found in Table S2.† The coded reaction optimization matrix used for the optimization of all the desired products is presented in Table 3.

Table 3 Coded matrix for optimization of the reaction system parameters by factorial design

Reaction no.	z_1	z_2	z_3	z_4
1	+1	+1	+1	+1
2	−1	+1	+1	+1
3	+1	−1	+1	+1
4	−1	−1	+1	+1
5	+1	+1	−1	+1
6	−1	+1	−1	+1
7	+1	−1	−1	+1
8	−1	−1	−1	+1
9	+1	+1	+1	−1
10	−1	+1	+1	−1
11	+1	−1	+1	−1
12	−1	−1	+1	−1
13	+1	+1	−1	−1
14	−1	+1	−1	−1
15	+1	−1	−1	−1
16	−1	−1	−1	−1

In 5 mL round-bottom flasks, the required amounts of alcohol, acid and biocatalyst (5.1 mg biocatalyst for anisyl alcohol, 4.9 mg biocatalyst in the case of cinnamyl alcohol, and 4 mg biocatalyst for benzyl alcohol) were added. A magnetic stirrer equipped with a temperature-controlled oil bath was used to keep the reaction mixtures' temperature at the required temperature (25 °C or 35 °C) under shaking at 180 rpm. The flasks were connected to a vacuum line (15 or 25 mbar vacuum) for controlled water removal. After the reactions were complete, methanol (1 mL) was added to the mixtures and homogenous samples (25 μL) were withdrawn and diluted with methanol (975 μL), filtered, and transferred into vials to be analysed by gas chromatography.

Time profile of the esterifications with propionic acid

In 5 mL round-bottom flasks, 0.2 mmol of anisyl, cinnamyl, and benzyl alcohol, 1 equiv. of propionic acid and 5.1 mg (for anisyl alcohol), 4.9 mg (for cinnamyl alcohol), 4 mg (for benzyl alcohol) of biocatalyst were added. A magnetic stirrer equipped with a temperature-controlled oil bath was used to keep the reaction mixtures' temperature at 25 °C and shaken at 180 rpm. The flask was connected to a vacuum line (15 mbar) for controlled water removal. For each substrate, four identical reactions were set up and they were stopped after 2, 4, 6 and 8 h, respectively, by adding methanol (1 mL) to the reaction mixtures. Homogenous samples (25 μL) were withdrawn and diluted with methanol (975 μL), filtered, and transferred into vials to be analyzed by gas chromatography.

Preparative-scale enzymatic esterification of cinnamyl alcohol with butyric acid and evaluation of the sustainability metrics

Here, 1 g cinnamyl alcohol (7.45 mmol), 1.97 g butyric acid (22.35 mmol), and 40 mg of lipase (184.33 mg biocatalyst, based on the enzyme loading) were added into a magnetically stirred 10 mL round-bottom flask at 35 °C and shaken at 180 rpm. The flask was connected to a vacuum line (15 mbar) for efficient water removal. Methanol (5 mL) was added in the reaction mixture after 90 min and homogenous samples (10 μL) were withdrawn and diluted with methanol (990 μL), filtered, and transferred into vials to be analyzed by gas chromatography to determine the conversion value. The remaining mixture was filtered for biocatalyst recovery. The liquid mixture was washed with 1 M Na_2CO_3 solution (2 × 5 mL) and dried over Na_2SO_4. Drying at 42 °C under advanced vacuum afforded 1.33 g pure cinnamyl butyrate (90.5% isolation yield). The product's purity was confirmed by ^{1}H-NMR and ^{13}C-NMR.

To evaluate the newly developed enzymatic method for obtaining cinnamyl butyrate by a green and sustainable method, some sustainability metrics were determined. Specifically, the sustainability metrics *E*-factor, atom economy, atom efficiency, mass intensity, and reaction mass efficiency were calculated using the reactants/products and

their masses in accordance with the literature.[38–40] The formed water, considered as the only by-product, was excluded from the waste mass, as calculations for each metric were performed under the assumption that cinnamyl butyrate was the unique resultant product. The newly proposed enzymatic method was finally compared from a sustainability metrics point of view with a previously published enzymatic method for the synthesis of cinnamyl acetate.[34]

Conclusions

In this study seven natural aroma esters were efficiently synthesized by the enzymatic esterification of aromatic alcohols (anisyl, cinnamyl, and benzyl) with short-chain acids (propionic, butyric, and hexanoic) mediated by CaL-B immobilized in a sol–gel matrix in the presence of PVA as an additive. Using response surface methodology (central composite model), the processes were individually optimized with an aim to maximize the conversions and to increase the productivity. The most significant conversions' increase was registered in the case of cinnamyl butyrate, with the desired ester being obtained with 94.3% conversion after optimization of the reaction parameters (alcohol : acid molar ratio, temperature, vacuum, and reaction time), as compared to 49.4% in the initial conditions. Furthermore, this study showed that by using low pressure, water could be efficiently removed from the reaction system without the need for other components in the medium (molecular sieves or other desiccants), as no decreased conversions were registered due to the substrates regeneration.

The preparative-scale enzymatic synthesis of cinnamyl butyrate (starting from 1 g of cinnamyl alcohol) yielded the desired ester in a 90.5% global yield. The tested green metrics (*E*-factor, atom economy, atom efficiency, mass intensity, and reaction mass efficiency) proved that the proposed process is a good candidate for potential industrial scale-up. Furthermore, the aroma compounds synthesized by this newly proposed method can be classified as "natural" since they were obtained from substrates of natural origin using an enzyme as a natural catalyst (as per Regulation No. 1334/2008 of the European Parliament and of the Council).

Data availability

The data supporting this article have been included as part of the ESI.†

Author contributions

Conceptualization: M. I. T. and A. I. D.; data curation: A. I. D.; investigation: A. I. D.; methodology: M. I. T. and C. P.; project administration: M. I. T. supervision: M. I. T.; writing – original draft: A. I. D.; writing – review & editing: M. I. T. and C. P.

Conflicts of interest

There are no conflicts to declare.

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References

1 M. A. Longo and M. A. Sanromán, *Food Technol. Biotechnol.*, 2006, **44**, 335–353.
2 S. A. Dubal, Y. P. Tilkari, S. A. Momin and I. V. Borkar, *Adv. Biotechnol.*, 2008, **14**, 20–31.
3 A. Sales, B. N. Paulino, G. M. Pastore and J. L. Bicas, *Curr. Opin. Food Sci.*, 2018, **19**, 77–84.
4 K. S. Jaiswal and V. K. Rathod, *Chem. Rec.*, 2022, **22**(3), e202100213.
5 M. Sarno, M. Iuliano, M. Polichetti and P. Ciambelli, *Process Biochem.*, 2017, **56**, 98–108.
6 C. Vasilescu, A. Todea, A. Nan, M. Circu, R. Turcu, I. C. Benea and F. Peter, *Food Chem.*, 2019, **296**, 1–8.
7 S. R. Uppada, M. Akula, A. Bhattacharya and J. R. Dutta, *J. Genet. Eng. Biotechnol.*, 2017, **15**, 331–334.
8 H. De Yan, Q. Zhang and Z. Wang, *Catal. Commun.*, 2014, **45**, 59–62.
9 A. C. de Meneses, A. G. Almeida Sá, L. A. Lerin, M. L. Corazza, P. H. H. de Araújo, C. Sayer and D. de Oliveira, *Process Biochem.*, 2019, **78**, 50–57.
10 K. S. Jaiswal and V. K. Rathod, *Catal. Today*, 2021, **375**, 120–131.
11 A. I. Dudu, M. A. Lăcătuș, L. C. Bencze, C. Paizs and M. I. Toșa, *ACS Sustainable Chem. Eng.*, 2021, **9**(15), 5461–5469.
12 C. Li, J. Sun, T. Li, S. Q. Liu and D. Huang, *Food Chem.*, 2014, **154**, 205–210.
13 M. E. Moisă, C. G. Spelmezan, C. Paul, J. H. Bartha-Vári, L. C. Bencze, F. D. Irimie, C. Paizs, F. Péter and M. I. Toșa, *RSC Adv.*, 2017, **7**, 52977–52987.
14 V. Kasche, *Enzyme Microb. Technol.*, 1986, **8**, 4–16.
15 M. A. Lăcătuș, A. I. Dudu, L. C. Bencze, G. Katona, F. D. Irimie, C. Paizs and M. I. Toșa, *ACS Sustainable Chem. Eng.*, 2020, **8**, 1611–1617.
16 M. A. Lăcătuș, L. C. Bencze, M. I. Toșa, C. Paizs and F. Irimie, *ACS Sustainable Chem. Eng.*, 2018, **6**(9), 11353–11359.
17 M. R. Mehrasbi, J. Mohammadi, M. Peyda and M. Mohammadi, *Renewable Energy*, 2017, **101**, 593–602.
18 O. H. Giraldo, *Fuel*, 2017, **196**, 481–487.
19 S. Gihaz, D. Weiser, A. Dror, P. Sátorhelyi, M. Jerabek-Willemsen, L. Poppe and A. Fishman, *ChemSusChem*, 2016, **9**, 3161–3170.
20 V. Dossat, D. Combes and A. Marty, *Enzyme Microb. Technol.*, 1999, **25**, 194–200.
21 R. A. Sheldon and D. Brady, *ACS Sustainable Chem. Eng.*, 2021, **9**, 8032–8052.

22 I. N. A. Rahman and R. A. Wahab, *eProceedings Chemistry*, 2017, **1**, 21–28.
23 D. M. Scrob, M. A. Lăcătuș and A. I. Dudu, *Stud. Univ. Babes-Bolyai, Chem.*, 2021, **66**, 277–286.
24 H. V. Ferreira, L. C. Rocha, R. P. Severino and A. L. M. Porto, *Molecules*, 2012, **17**, 8955–8967.
25 C. Ortiz, M. L. Ferreira, O. Barbosa, J. C. S. Dos Santos, R. C. Rodrigues, Á. Berenguer-Murcia, L. E. Briand and R. Fernandez-Lafuente, *Catal. Sci. Technol.*, 2019, **9**, 2380–2420.
26 R. Morellon-Sterling, D. Carballares, S. Arana-Peña, E. H. Siar, S. A. Braham and R. Fernandez-Lafuente, *ACS Sustainable Chem. Eng.*, 2021, **9**, 7508–7518.
27 A. Todea, P. Borza, A. Cimporescu, C. Paul and F. Peter, *Catal. Today*, 2018, **306**, 223–232.
28 A. Cimporescu, A. Todea, V. Badea, C. Paul and F. Peter, *Process Biochem.*, 2016, **51**, 2076–2083.
29 S. Cebrián-García, A. M. Balu, A. García and R. Luque, *Molecules*, 2018, **23**(9), 2283.
30 C. Zarcula, C. Kiss, L. Corici, R. Croitoru, C. Csunderlik and F. Peter, *Rev. Chim.*, 2009, **60**(9), 922–927.
31 A. I. Dudu, M. A. Lacatus, L. C. Bencze, C. Paizs and M. I. Tosa, *ACS Sustainable Chem. Eng.*, 2021, **9**, 5461–5469.
32 E. Alvarez, J. Rodriguez, R. Villa, C. Gomez, S. Nieto, A. Donaire and P. Lozano, *ACS Sustainable Chem. Eng.*, 2019, **7**, 13307–13314.
33 A. G. A. SÁ, A. C. de Meneses, P. H. H. de Araújo and D. de Oliveira, *Trends Food Sci. Technol.*, 2017, **69**, 95–105.
34 I. C. Perdomo, S. Gianolio, A. Pinto, D. Romano, M. L. Contente, F. Paradisi and F. Molinari, *J. Agric. Food Chem.*, 2019, **67**, 6517–6522.
35 P. K. Smith, R. I. Krohn, G. T. Hermanson, A. K. Mallia, F. H. Gartner, M. D. Provenzano, E. K. Fujimoto, N. M. Goeke, B. J. Olson and D. C. Klenk, *Anal. Biochem.*, 1985, **150**, 76–85.
36 J. Zheng, X. Fu, X. Ying, Y. Zhang and Z. Wang, *Anal. Biochem.*, 2014, **452**, 13–15.
37 J. Boudrant, J. M. Woodley and R. Fernandez-Lafuente, *Process Biochem.*, 2020, **90**, 66–80.
38 R. A. Sheldon, *ACS Sustainable Chem. Eng.*, 2018, **6**, 32–48.
39 R. A. Sheldon, *Green Chem.*, 2017, **19**, 18–43.
40 D. J. C. Constable, A. D. Curzons and V. L. Cunningham, *Green Chem.*, 2002, **4**, 521–527.