BABEȘ-BOLYAI UNIVERSITY OF CLUJ NAPOCA
FACULTY OF CHEMISTRY AND CHEMICAL ENGINEERING
DOCTORAL SCHOOL OF CHEMISTRY

Encapsulation of lipase B from *Candida antarctica* in tailored sol-gel matrices with biocatalytic applications

PhD Thesis Abstract

PhD candidate: **Eng. Adrian-Ioan Dudu**

Jury:

President: Acad. Prof. Dr. Cristian Silvestru

Scientific advisor: **Prof. Habil. Dr. Eng. Monica-Ioana Toșa**

Reviewers: Prof. Dr. Eng. Francisc Peter, Politechnical University of Timișoara

Prof. Dr. Francisc Dulf, Agronomy and Veterinary Medicine University of Cluj Napoca

Asoc. Prof. Anamaria-Elena Terec, Babeș-Bolyai University of Cluj Napoca

Public defense: 14th June 2022

Cluj Napoca

2022

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Key words: biocatalysis, enzymatic kinetic resolution, enzymatic esterification, enzymatic transesterification, lipase, sol-gel immobilization, solvent-free synthesis, green chemistry, chromatographic separation

Chapter 1. General introduction

Biocatalysis is a very important area of research in today's scientific life. The consumers have increased reservations in using products obtained *via* classic chemical synthesis, especially those used in food, cosmetic and pharmaceutical industries. The most widely applied method for obtaining aroma compounds is the extraction from natural sources, however this method presents low extraction yields. In order to solve the limitations of the two previously mentioned methods used in the food industry, an increased interest in alternative biocatalytic routes is remarked, as shown by the increased number of scientific publications on this subject.

Biocatalytic processes use as catalysts either isolated enzymes or whole cells. The application of enzymes in industrial processes depends on the catalytic activity and selectivity of the enzyme and it depends on the enzyme stability and recyclability. The stability and recyclability of an enzyme directly influences the productivity of the process, therefore the economic feasibility of the process is strongly related to these two factors. To increase the enzyme's stability and recyclability, important efforts were made to find the optimum immobilization method. By immobilization, the activity of the enzyme can also be altered. In order to preserve the enzyme stability, some additives might be added during the immobilization step.

Short-chain esters with aliphatic or aromatic structures are a class of compounds used particularly as aroma compounds in the food industry. They can be obtained *via* the direct esterification of a short-chain fatty acid with an alcohol. The by-product of the esterification is water, which shifts the reaction equilibrium towards substrate regeneration, if not properly removed from the reaction system. Several means to remove the formed water can be employed, such as the use of molecular sieves, or by applying vacuum (if the process is solvent-free).

The two enantiomers of a chiral compound can have different biological activities when discussing about the pharmaceutical industry, or different organoleptic properties when the food industry is in question. Enzymes can be employed in processes to obtain optically pure compounds, *via* enzymatic kinetic resolutions, or dynamic kinetic resolutions. The selectivity of the enzyme is a very important factor in this case, as the enantio-preference of the enzyme directly influences the optical purity of the obtained compound. Another important aspect that is worth mentioning is the method used for product(s) extraction from the reaction, as it needs to be carefully chosen in order to enable high-yield product(s) recovery.

The present thesis addresses several aspects regarding the use of CaL-B to obtain short-chain flavor esters used as additives in the food industry in a solvent-free system and also the use of DES as additives in the encapsulation of CaL-B *via* the sol-gel technique and its applicability in EKR processes to obtain enantiopure chiral building-blocks used in the pharmaceutical industry.

The first part of Chapter 4 (Personal contribution), **Biocatalytic route towards aroma compounds using lipase B from *Candida antarctica***, describes the use of two different immobilized forms of CaL-B, one obtained by adsorption on a macroporous resin (commercially available as Novozym 435) and the second obtained by encapsulation of CaL-B in a sol-gel matrix to obtain aroma compounds with aromatic and aliphatic structures. A green and sustainable process used to synthesize these compounds is described in this part of the chapter.

The second part of Chapter 4, **Deep eutectic solvents as additives in the encapsulation of lipase B from *Candida antarctica***, describes the novel use of DESs in the encapsulation of CaL-B *via* the sol-gel technique in comparison with the more traditional ILs. The novel biocatalysts were

successfully applied in the transesterification of 1-phenylethanol, which is a compound of great interest for the pharmaceutical industry.

Chapter 2. Literature overview (literature data)

Chapter 3. Aim of the thesis

- to develop robust and efficient biocatalysts by entrapping lipase B from *Candida antarctica* in tailored silica matrices *via* the sol-gel method;

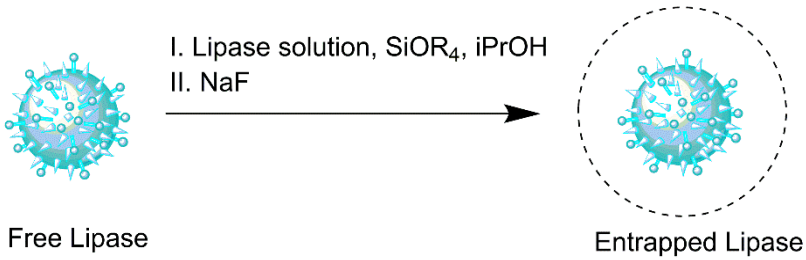


Figure 1. Entrapment of a lipase *via* the sol-gel method.

- to develop a green and sustainable process for the synthesis of aroma esters with both aliphatic and aromatic moieties by direct esterification of various alcohols with different acids, mediated by CaL-B entrapped in tailored silica matrix;

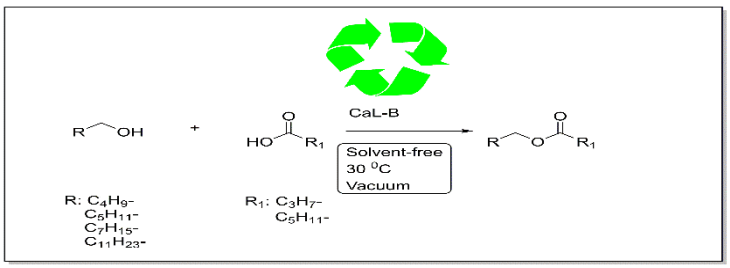


Figure 2. Green process for the synthesis of aroma esters with aliphatic structure.

- to prove the efficient use of biocatalysts obtained *via* the sol-gel encapsulation of CaL-B using DESs as additives during the immobilization in the transesterification of 1-phenylethanol, an important chiral building block in the pharmaceutical industry.

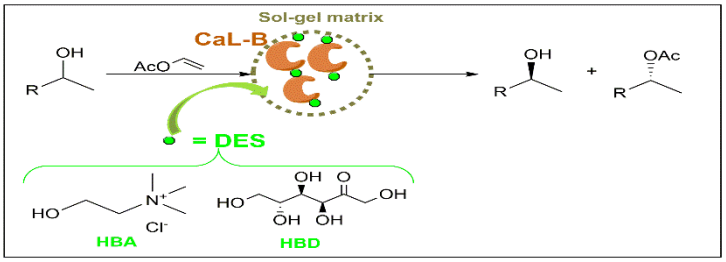


Figure 3. Transesterification of 1-phenylethanol mediated by CaL-B encapsulated in a sol-gel matrix using DESs as additives.

Non-chiral aroma esters were chosen as compounds of interest due to their use in the food industry as flavors. Some of the obtained aroma esters are also used in the cosmetics industry as fragrances. The transesterification of 1-phenylethanol was chosen as model reaction for testing the novel obtained biocatalysts due to the compound importance in the pharmaceutical industry as a chiral building block and also due to its use as fragrance in the cosmetics industry.

Chapter 4. Personal contribution

4.1. Biocatalytic route towards aroma compounds using lipase B from *Candida antarctica*

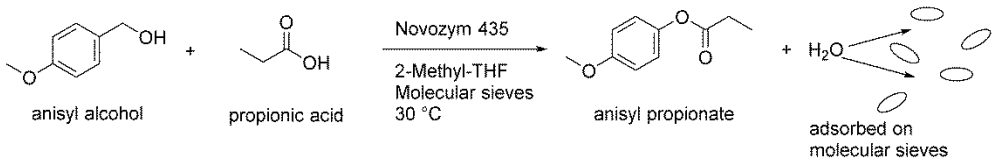
4.1.1. Enzymatic synthesis of anisyl propionate mediated by lipase B from *Candida antarctica*

This chapter of the thesis describes the use of a commercially available biocatalyst (Novozym 435) in a bioprocess for the synthesis of anisyl propionate which is a flavor and fragrant agent used in the food and cosmetic industries.

4.1.1.1. **Introduction** (literature data)

4.1.1.2. Results and discussion

Analytical scale experiments were carried out in order to optimize the parameters for the esterification of benzyl alcohol with propionic acid. Based on the obtained results further experiments were performed in order to maximize the productivity of the process. In order to achieve total conversion of anisyl alcohol into the desired product (anisyl propionate) the reaction equilibrium had to be shifted towards product formation and in order to do that, molecular sieves were used for the efficient removal of the water formed in the system, as seen in **Scheme 1**.



Scheme 1. Enzymatic synthesis of anisyl propionate *via* direct esterification of anisyl alcohol with propionic acid in 2-Methyl-THF mediated by Novozym 435 in the presence of molecular sieves.

4.1.1.2.1. Solvent screening

In order to find the optimal solvent for anisyl propionate synthesis five solvents with different polarities were tested, as seen in **Figure 4**.

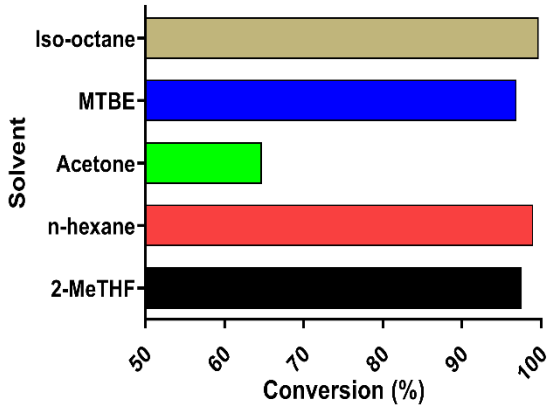


Figure 4. Solvent screening for anisyl propionate synthesis. Reaction conditions: 10 mM anisyl alcohol, 2 equiv. propionic acid, 25 mg Novozym 435, 1 mL solvent, 50 mg molecular sieves, 800 rpm, 30 °C, 12 hours.

High enzymatic activity was found in *n*-hexane and *iso*-octane (over 98%) as well as in etheric solvents (MTBE and 2-Methyl-THF, ~96%). When using acetone as solvent the conversion reached a much lower value (~65%). Considering its classification as a green solvent and the fact that high conversion value was obtained when using it as solvent for anisyl propionate production 2-Methyl-THF was chosen as optimal solvent and was used for further optimizations.

4.1.1.2.2. Substrate concentration screening

The productivity of a process is directly dependent on the substrate concentration and for this the substrate concentration was varied (10-50 mM) and its influence over the reaction conversion was evaluated. Other reaction parameters such as alcohol: acid molar *ratio* (1:2), Novozym 435 (25 mg/mL) and molecular sieves (50 mg/mL) were kept constant.

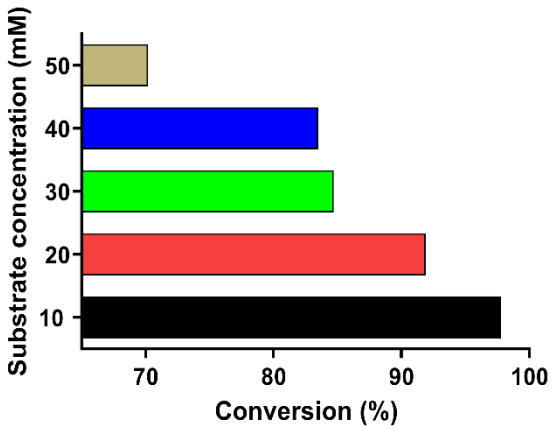


Figure 5. Influence of the anisyl alcohol concentration on the overall conversion. Reaction conditions: different concentrations of anisyl alcohol (10-50 mM), 2 equiv. propionic acid, 25 mg biocatalyst, 1 mL 2-Methyl-THF, 50 mg molecular sieves, 800 rpm, 30 °C, 12 hours.

As shown in **Figure 5** the increase in substrate concentration leads to a decrease in conversion as a 97.8% conversion was observed at 10 mM anisyl alcohol and a conversion value of only 70.2% when using 50 mM substrate. Starting from a larger quantity of substrate leads to the formation of a larger quantity of water and it can be emphasized that the decrease in conversion occurs due to the inadequate quantity of molecular sieves (50 mg/mL) used in the reaction medium.

4.1.1.2.3. Influence of the quantity of molecular sieves on anisyl propionate production

The influence of the quantity of molecular sieves upon anisyl propionate production was evaluated using a constant anisyl alcohol concentration of 50 mM and 100 mM propionic acid, as described in **Figure 6**.

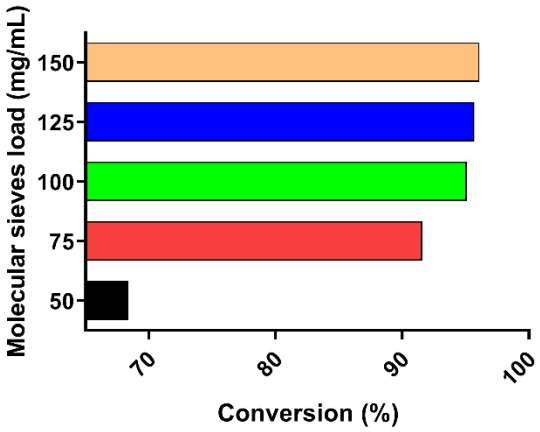


Figure 6. The influence of the quantity of molecular sieves on the formation of anisyl propionate. Reaction conditions: 1 mL 2-Methyl-THF, 50 mM anisyl alcohol, 2 equiv. propionic acid, 25 mg Novozym 435, 800 rpm, 30 °C, 12 hours.

By adding more drying agent, the reaction conversion was considerably improved up to 95.1% when adding 100 mg/mL molecular sieves from 68.4% when using only 50 mg/mL drying agent. Adding more molecular sieves (125 and 150 mg/mL) only increased by 1% the reaction conversion, therefore the optimum molecular sieves load used for further experiments was selected as 100 mg/mL.

4.1.1.2.4. Temperature screening

The kinetics of any reaction is greatly influenced by the reaction medium temperature. In order to establish the influence of the temperature on the activity and stability of Novozym 435 reactions were set-up using the previously determined optimal conditions at different temperatures (30, 40, 50 and 60 °C) and samples were withdrawn at 2 hours intervals.

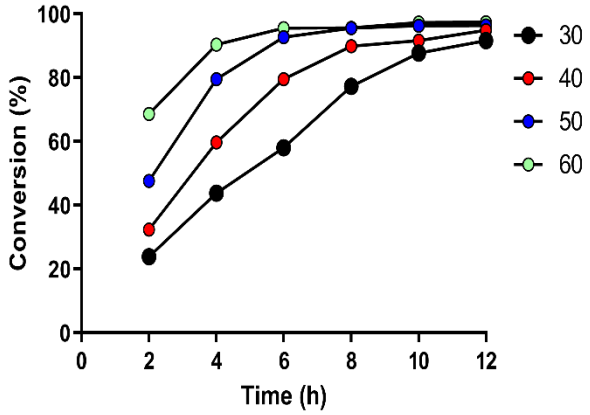


Figure 7. Evolution of the reaction conversion in time for the enzymatic production of anisyl propionate. Reaction conditions: 50 mM anisyl alcohol, 2 equiv. propionic acid, 1 mL 2-Methyl-THF, 25 mg Novozym 435, 100 mg molecular sieves, 800 rpm.

Novozym 435 has proven to be an active and stable biocatalyst even at 60 °C. As it can be seen in **Figure 7**, at 60 °C the reaction conversion reached a maximum of ~96% after only 6 hours, which represents half the time which was needed to reach this conversion at 30 °C. Based on this, the optimum temperature which was used for scaling-up experiments was 60 °C, as a short reaction time is needed for almost complete substrate conversion.

4.1.1.2.5. Process scale-up in optimum conditions

After the optimization of reaction parameters at analytical scale was performed, the next step in this study was the scaling-up of the process. Using the optimal parameters (25 mg/mL biocatalyst, 100 mg/mL molecular sieves, 2 equiv. excess of propionic acid, 6 hours reaction time and 2-Methyl-THF as solvent) in order to increase the productivity of the process the substrate (anisyl alcohol) concentration was incrementally increased with 10 units in the 60-100 mM domain. The obtained results were encouraging, as even at 100 mM anisyl alcohol concentration the performance of the developed reaction system remained unaltered, as the conversion value increased from 88% at 60

mM substrate to 95% at 100 mM anisyl alcohol, further reactions were performed at a much higher substrate concentration (200, 300, 500 and 1000 mM), as shown in **Figure 8**.

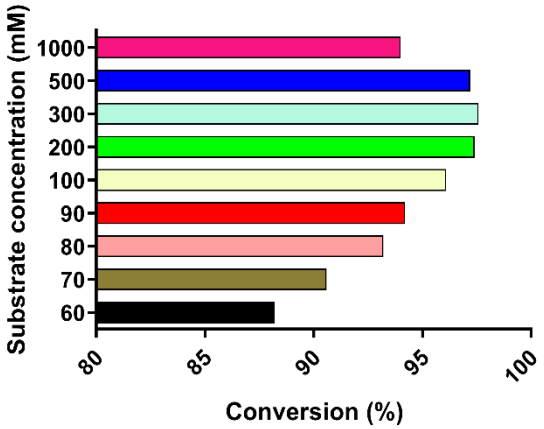


Figure 8. Scaling-up for anisyl propionate production. Reaction conditions: 60-1000 mM anisyl alcohol, 2 equiv. propionic acid, 1 mL 2-Methyl-THF, 25 mg Novozym 435, 100 mg molecular sieves, 800 rpm, 60 °C, 6 hours.

The results shown graphically in **Figure 8** can be explained by the dependence of the reaction's rate on the reagents concentration and also based on these results it can be said that no substrate or product inhibition occurs in the developed process. Excellent conversion value was observed at 1000 mM substrate concentration as after only 6 hours 95% of the initial substrate quantity was transformed. The reaction that started from 1000 mM anisyl alcohol was worked-up and the ester (anisyl propionate) was isolated. After purification, anisyl propionate was obtained with 92% yield and its structure was confirmed by NMR spectroscopy (^{1}H-NMR and ^{13}C-NMR).

4.1.1.3. Conclusions

Anisyl propionate was successfully synthesized by the direct esterification of anisyl alcohol with propionic acid in a green solvent (2-Methyl-THF) mediated by Novozym 435 using molecular sieves as an efficient water

removal tool. After process optimization, anisyl propionate was isolated with 92% yield starting from a substrate concentration of 1000 mM. Considering these aspects, the newly developed enzymatic process can be considered a strong candidate for an industrial scale-up.

4.1.2. Solvent-free synthesis of short-chain aroma esters

In this part of the thesis a solvent-free procedure was developed for the efficient synthesis of short-chain esters *via* direct esterification mediated by CaL-B included in a tailored sol-gel matrix.

4.1.2.1. Introduction (literature data)

4.1.2.2. Results and discussion

4.1.2.2.1. Lipase immobilization by sol-gel entrapment

The vast library of available silane precursors makes the entrapment of enzymes *via* the sol-gel technique a very versatile immobilization method which allows the fine tuning of matrix porosity and hydrophobicity/ hydrophilicity. Moreover, during the gelifiation step some additives might be added that can alter the enzyme's stability (by offering a layer of protection) and activity (modifying the biomolecule's conformation). In this study a novel silica-based support was developed by mixing an alcoxy-silane containing ethoxy groups (OTEOS) with two alcoxy-silanes containing methoxy moieties (*n*-PTMOS and TMOS). Some additives were added during the immobilization procedure and the synthetic activity of the novel biocatalysts was evaluated as well as the recovered activity with the results being presented in **Table 1**.

Table 1. Biocatalysts obtained using a ternary mixture of OTEOS: *n*-PTMOS: TMOS in 1.6:0.4:1 molar *ratio*.

Entry	Sol-gel code	Additive[a]	Enzyme load [$\mu g_{enzyme}/mg_{biocatalyst}$][b]	Synthetic activity [$mmol/min*g_{enzyme}$]	Recovered activity [%][c]
1	SG-1	β-cyclodextrin	6	13.89 ± 0.01	100.12
2	SG-2	PVA	7.6	15.28 ± 0.12	110.04
3	SG-3	Glycerol	7.7	10.63 ± 0.06	76.72
4	SG-4	-	8.4	13.77 ± 0.07	69.16

[a]200 µL of 4% aqueous solutions; [b]Enzyme load was determined by reporting the amount of lipase in the biocatalyst (the initial amount of enzyme that was encapsulated as no traces of unbound enzyme were found in the washing waters) *versus* the amount of obtained biocatalyst; [c]Recovered activity was calculated by reporting the synthetic activity of each biocatalyst to the synthetic activity of the free lipase[1].

The three additives used for this study were chosen due to their structure, since the hydroxyl moieties are able to modify the enzyme's catalytic site by replacing some water molecules which are smaller in comparison with the selected polyhydroxy compounds, therefore altering the enzyme's activity. The synthetic activity of the novel biocatalysts was evaluated in comparison with the synthetic activity of the free lipase solution (which was encapsulated *via* the sol-gel technique, 13.77 $mmol/min*g_{enzyme}$). As shown in **Table 1** SG-4 was prepared without any additive and a recovered activity of just 69.16% was observed which means a loss of enzyme active occurred due to immobilization, an activity drop that can also be observed in the case of SG-3 when using glycerol as additive (76.72% recovered activity, entry 3). When using PVA as additive (SG-2, entry 2) the recorded recovered activity of 110.04% shows an increase in enzyme activity through immobilization, therefore it can be said that PVA added during the immobilization procedure helped the enzyme adopt a more active conformation. When using β-

cyclodextrin as additive (SG-1, entry 1) the enzyme's synthetic activity remains unaltered through immobilization.

4.1.2.2.2. Initial screenings

The model reaction chosen for this study was the direct esterification of butyric acid with 1-hexanol in a SFS. The preliminary results were quite modest as after 4 hours low conversion in hexyl butyrate was obtained when using each of the 3 novel biocatalysts (20.9% for SG-1, 18.2% for SG-2 and 21.3% for SG-3). A commercially available form of CaL-B (Novozym 435) was also tested on the same substrate in the same reaction conditions and hexyl butyrate was obtained with 91.2% conversion. In order to improve the conversion in the desired ester the reaction system was optimized by altering 3 major parameters influencing the biotransformation: alcohol: lipase weight *ratio*, alcohol: acid molar *ratio* and temperature.

4.1.2.2.3. Optimization of the reaction system

4.1.2.2.3.1. The effect of alcohol: lipase weight ratio

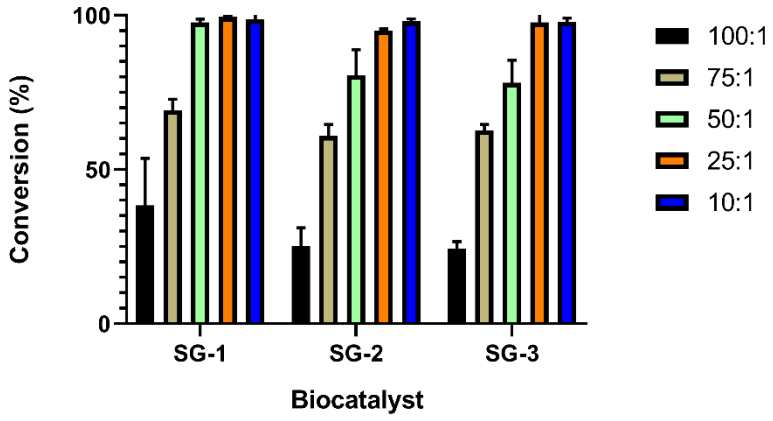


Figure 9. Influence of alcohol: lipase weight *ratio* on the overall reaction conversion. Solvent-free conditions: 0.2 mmol hexan-1-ol, 4 equiv. of butyric acid, alcohol: lipase weight *ratio*s of 100:1, 75:1, 50:1, 25:1 and 10:1, 50 °C, 20 mbar vacuum, 4 hours.

As expected, the alcohol: lipase *ratio* has major influence on the formation of hexyl butyrate as by lowering the *ratio* (increasing the enzyme quantity) the conversion of the reaction increases its value. Although the alcohol: lipase *ratio* of 50:1 delivers excellent results in the case of the reaction mediated by SG-1 the optimal value for the *ratio* was chosen to be 25:1, as high conversions were obtained for all three novel biocatalysts (>94%). Process sustainability was also taken into consideration as the alcohol: lipase *ratio* of 10:1 didn't significantly improve the conversion of the reaction so the increased amount of biocatalyst wouldn't be justified.

4.1.2.2.3.2. The effect of alcohol: acid molar ratio

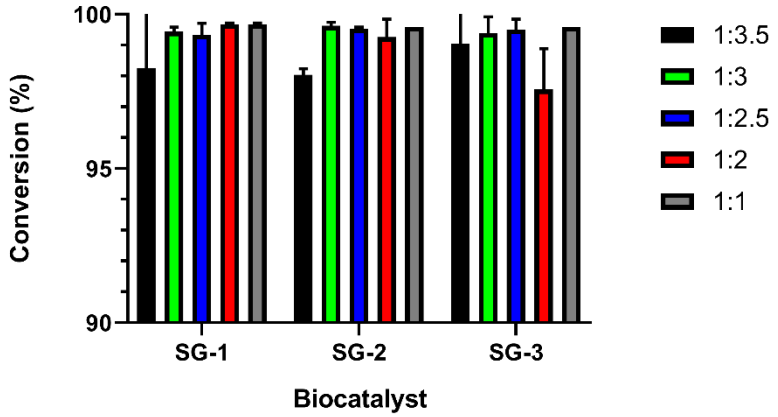


Figure 10. Influence of the alcohol: acid molar *ratio* on the overall reaction conversion. Solvent-free conditions: alcohol: acid molar *ratio*s of 1:3.5, 1:3, 1:2.5, 1:2, 1:1, alcohol: lipase weight *ratio* of 25:1, 50 °C, 20 mbar vacuum, 4 hours.

A very important reaction parameter is the reactants *ratio*, an optimum value for this parameter should be somewhere close to stoichiometric. For this, the alcohol: acid *ratio* was gradually decreased by increasing the initial amount of alcohol and for the 25:1 alcohol: lipase weight *ratio* be preserved the amount of added enzyme was gradually increased, correspondingly. As it can be seen in **Figure 10**, by decreasing the molar alcohol: acid *ratio* a slight increase in

the conversion was observed for SG-1 and SG-2, whilst a slight decrease in the value of the conversion (~2%) was noticed in case of SG-3 for a 1:2 alcohol: acid molar *ratio*. When selecting the ideal value for a reaction system parameter all aspects should be taken in consideration, in this case the possibility to recover and reuse any of unreacted compounds (butyric acid in this case) should be considered an important aspect. Untransformed acid can be treated with a base and recovered in its salt form (easier to isolate from the reaction medium) and then it can be reused in another batch which is really important for a developed process to be considered a green and sustainable one. Another important aspect is the price of the reactants. To correctly determine the optimal value for the alcohol: acid molar *ratio* a time profile was determined for all three novel biocatalysts using 1:2 and 1:1 alcohol: acid molar *ratio*s.

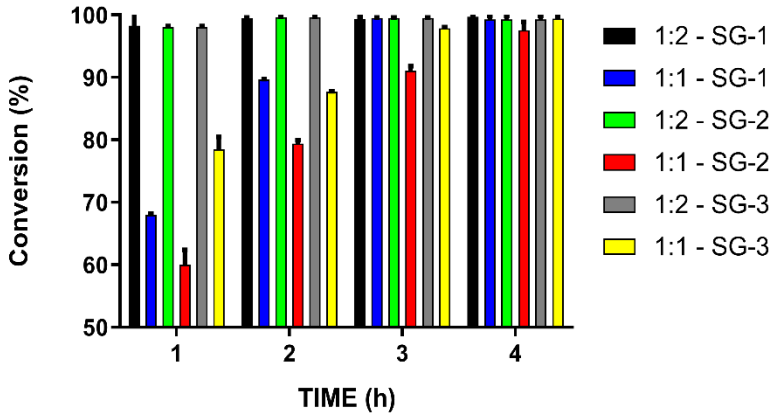


Figure 11. Influence of the alcohol: acid molar *ratio* on the initial reaction rate. Solvent-free conditions: alcohol: acid molar *ratio*s of 1:2, 1:1, alcohol: lipase weight *ratio* of 25:1, 50 °C, 20 mbar vacuum, 1, 2, 3 and 4 hours.

A deeper look into the influence of the alcohol: acid molar *ratio*'s influence over the formation of hexyl butyrate revealed that in the case of using a 1:2 molar *ratio* maximum conversion is obtained after 1 hour for all three

biocatalysts. However, when using an equimolar *ratio* ideal conversion is reached after 3 hours (in the case of SG-1 and SG-3) or 4 hours (for SG-2). In consequence, process optimization was further performed using a 1:2 alcohol: acid molar *ratio* as it yields excellent conversions (>95%) for all three novel biocatalysts.

4.1.2.2.3.3. The effect of the temperature

In order to establish the influence of the reaction medium temperature over the conversion, similar reactions were setup at different temperatures (30, 40, 50 °C) using the previously determined ideal reaction conditions obtaining similar conversion values (>98.5%) for all biocatalysts at all temperatures. Further analysis of the conversion at different time points almost identical values were observed for the novel biocatalysts at different temperatures (~93% after 1 hour, ~95% after 2 hours and ~98% after 3 hours) proving that the temperature doesn't present a significant influence over the process. Since the yield of esterification slowly increased with the increase of temperature it can be said that the developed process is an endothermic thermodynamically controlled process[2]. Considering the fact that when the reaction was performed at 30 °C excellent conversion value was obtained one might be encouraged to further decrease the temperature, however some aspects must be taken into consideration. The efficiency of water removal is directly influenced by the temperature of the reaction system. By using the same applied vacuum at a lower temperature the efficiency of water removal is decreased and as a consequence the equilibrium would be shifted towards substrate regeneration. On the other hand, if the temperature was lowered and concomitantly the applied vacuum was increased the risk of reactant or product distillation would have increased leading to erroneous results as the vacuum was carefully chosen from the start of this study as a compromise between water removal while avoiding reactants or products distillation. In a green and sustainable

process energy consumption should be kept at a minimum, as a result the optimal reaction temperature was chosen at 30 °C.

4.1.2.2.4. Enzymatic synthesis of natural flavor esters in a solvent-free system

Various short-chain primary alcohols (1-pentanol, 1-hexanol, 1-octanol, 1-dodecanol) were used in esterification reactions of natural acids (butyric and hexanoic acid) in order to establish the applicability of the novel biocatalysts in the previously determined ideal reaction conditions (**Table 2**).

Table 2. Conversion values for the esterification reactions catalyzed by three novel biocatalysts in optimal conditions.

Entry	Biocatalyst	Alcohol	Acid	Product	Conversion [%]
1	SG-1	1-Pentanol	Butyric	Pentyl butyrate	98.0 ± 3.4
2		1-Octanol		Octyl butyrate	99.3 ± 0.4
3		1-Dodecanol		Dodecyl butyrate	99.6 ± 0.2
4		1-Pentanol	Hexanoic	Pentyl hexanoate	99.9 ± 0.0
5		1-Hexanol		Hexyl hexanoate	99.8 ± 0.2
6		1-Octanol		Octyl hexanoate	99.9 ± 0.0
7	SG-2	1-Pentanol	Butyric	Pentyl butyrate	99.8 ± 0.2
8		1-Octanol		Octyl butyrate	99.3 ± 0.1
9		1-Dodecanol		Dodecyl butyrate	99.6 ± 0.2
10		1-Pentanol	Hexanoic	Pentyl hexanoate	99.9 ± 0.0
11		1-Hexanol		Hexyl hexanoate	98.9 ± 0.1
12		1-Octanol		Octyl hexanoate	99.5 ± 0.2
13	SG-3	1-Pentanol	Butyric	Pentyl butyrate	99.2 ± 1.0
14		1-Octanol		Octyl butyrate	99.2 ± 0.3
15		1-Dodecanol		Dodecyl butyrate	99.5 ± 0.2
16	SG-3	1-Pentanol	Hexanoic	Pentyl hexanoate	99.9 ± 0.1
17		1-Hexanol		Hexyl hexanoate	99.7 ± 0.1
18		1-Octanol		Octyl hexanoate	99.7 ± 0.1

* Reaction conditions: 25:1 alcohol: lipase weight *ratio*; 1:2 alcohol: acid molar *ratio*; 30 °C reaction medium temperature; 20 mbar vacuum; 4 hours reaction time.

The excellent obtained results recommend the newly developed enzymatic method as a replacement for short-chain aliphatic aroma esters synthesis at

industrial scale instead of classic isolation from natural sources. The strong points of this methodology are: high product yield in short reaction time, easy product isolation and low energy consumption.

4.1.2.2.5. Time course of the esterification of butyric acid with 1-hexanol

In order to establish the ideal reaction time for the reusability study the direct esterification of butyric acid with hexan-1-ol mediated by the three novel biocatalysts was monitored in time.

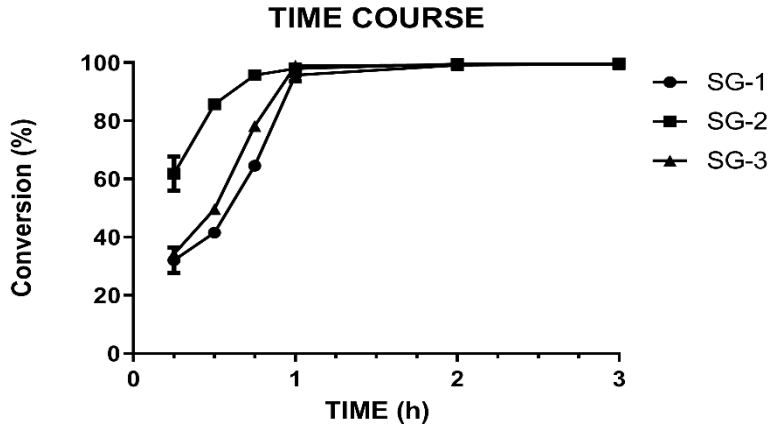


Figure 12. Time course of the esterification of butyric acid with 1-hexanol mediated by SG-1, SG-2 and SG-3. Solvent-free conditions: 0.4 mmol 1-hexanol, 2 equiv. butyric acid, 1.6 mg lipase, 30 °C, 20 mbar vacuum.

As it can be seen in **Figure 12** excellent conversion value was obtained for all biocatalysts after 1 hour of reaction time (>98%), therefore the reaction time for the reusability study was chosen to be 1 hour.

4.1.2.2.6. Reusability studies

The reusability of a biocatalyst is a significant feature of applied biocatalysis as it directly influences the production costs. The recyclability of the three novel biocatalysts was evaluated in comparison with a commercially available biocatalyst (Novozym 435) in the reaction between butyric acid and 1-

hexanol. As shown in **Figure 13**, all three novel biocatalysts present a high operational stability, as even after eight reaction cycles the enzymatic activity remains intact in the case of SG-2 and SG-3 whilst a small decrease in activity can be observed for SG-1 which might be caused by a degradation of β-cyclodextrin due to slightly acidic conditions (pH values of 4.5-6)[3].

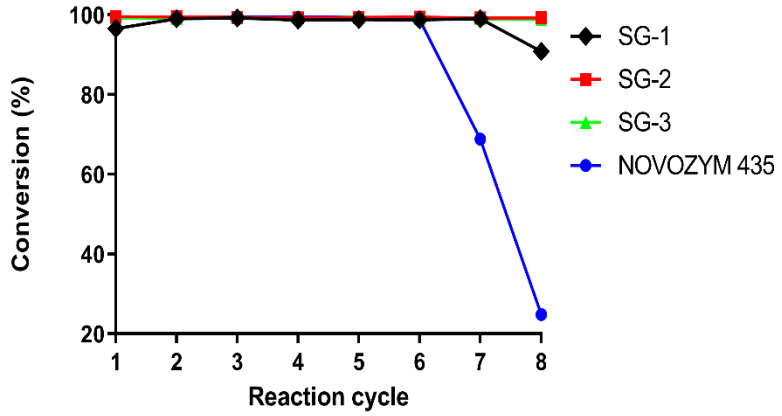


Figure 13. Reusability study for the three novel biocatalysts in comparison with Novozym 435 in the esterification of butyric acid with 1-hexanol. Solvent-free conditions: 0.4 mmol 1-hexanol, 2 equiv. butyric acid, 1.6 mg lipase, 30 °C, 20 mbar vacuum, 1 hour.

Novozym 435 showed a sudden drop in activity after the sixth reaction cycle, losing 25% of its initial activity after the seventh reaction cycle and almost all its initial activity after the eight reaction cycle. The most probable cause for the activity drop is due to the repeated sonications (for washing the biocatalyst in-between reaction cycles) affecting the resin. Another reason for the sudden activity drop might be due to the hydrophilicity of the resin which might lead to enzyme inactivation due to the formation of a water layer on the surface of the support.[4] Based on the results obtained in the reusability study it can be said that biocatalysts SG-2 and SG-3 are robust biocatalysts that maintain 100% of their initial activity even after eight reaction cycles, fact that makes them recommendable for a potential industrial scale-up.

4.1.2.2.7. Preparative scale enzymatic esterification of 1-hexanol with butyric and hexanoic acid and sustainability metrics evaluation

The previously determined optimum reaction conditions on small-scale reactions were successfully applied in two preparative scale direct esterifications of 1 g of 1-hexanol (9.8 mmol) with butyric and hexanoic acid, respectively. The preparative scale reactions were used for the quantitative evaluation of the sustainability metrics for the two procedures. In terms of atom economy both reactions exhibit similar values for this metric, as seen in **Table 3**, but a slight difference can be observed in the value of the atom efficiency values; the higher value that occurs in the case of hexyl hexanoate is due to a slightly higher product yield (98.5% in hexyl hexanoate compared to 97.6% in hexyl butyrate). The process that targets hexyl hexanoate as desired product generates less waste/ kg of product, therefore the environmental factor (*E*-factor) value is slightly smaller compared with the process in which the desired product is hexyl butyrate. However, the *E*-factor values are quite high for both processes and that is due to the removal of the unreacted acids with sodium carbonate. Going further, the use of sodium carbonate for acid neutralization and the use of 2 equivalents excess of acid are also reflected in the high values of mass intensity metric (18.58 kg/kg and 16.07 kg/kg, respectively) with the process in which the desired product is hexyl hexanoate having a closer value for this metric to the ideal case (1 kg/kg). The values for the reaction mass efficiency metric are very similar in both cases again due to using 2 equivalents excess of acid. Based on these results is safe to say, in terms of sustainability, that the newly developed enzymatic procedure for the synthesis of both hexyl butyrate and hexyl hexanoate, respectively, in a SFS is far superior than the previously reported aqueous enzymatic approach for the synthesis of cinnamyl acetate[5], which is also a short-chain flavor ester.

Table 3. The sustainability metrics for the enzymatic synthesis of hexyl butyrate and hexyl hexanoate *versus* the aqueous cinnamyl acetate production.

Sustainability metric	Product		
	Hexyl butyrate[a]	Hexyl hexanoate[b]	Cinnamyl acetate[c]
***E*-factor**	17.22	14.77	48.07
Atom Economy (AE)	90.53	91.76	79.27
Atom Efficiency	88.36	90.38	73.72
Mass intensity	18.58	16.07	35.48
Reaction mass efficiency	60.07	58.84	34.17

[a]Enzymatic esterification of butyric acid with 1-hexanol in a SFS; [b]Enzymatic esterification of hexanoic acid with 1-hexanol in a SFS; [c]Enzymatic transesterification of cinnamic alcohol with ethyl acetate in aqueous media (phosphate buffer)[5].

4.1.2.3. Conclusions

Seven natural short-chain aroma esters were successfully prepared through a novel enzymatic solvent-free direct esterification catalyzed by three novel biocatalysts containing lipase B from *Candida antarctica* included in a sol-gel matrix which have proven to be active and robust biocatalysts. According to Regulation No. 1334/2008 of the European Parliament and of the Council the flavors obtained using this enzymatic procedure can be classified as "natural" as they are obtained from substrates of natural origin and the process uses an enzyme as catalyst. The superiority of this proposed method is also supported by the evaluation of sustainability metrics in comparison with a previously described enzymatic procedure. To conclude, based on excellent product yields, short reaction time, efficient water removal and simple product isolation this proposed enzymatic approach can be classified as a green and sustainable process- starting materials being available from renewable resources.

4.2. Deep eutectic solvents as additives in the encapsulation of lipase B from *Candida antarctica*

4.2.1. Introduction (literature data)

4.2.2. Results and discussion

4.2.2.1. Lipase immobilization by sol-gel entrapment

Seven biocatalysts were prepared: one without the use of any additive, two using ILs as additives and four biocatalysts using DESs as additives for CaL-B entrapment *via* sol-gel technique. In terms of enzyme loading, the best result was obtained for SG-6 (**Table 4**, entry 2), however in terms of hydrolytic and synthetic activities the best biocatalysts seem to be SG-10 and SG-11 which were prepared by adding ILs (1-methyl-3-octyl-imidazolium-tetrafluoroborate- OMIMBF$_4$ or 1-ethyl-3-methylimidazolium chloride-EMIC, respectively). SG-7, SG-8 and SG-9 shown promising results (**Table 4**, entries 3-5). Since the aim of this study was to offer alternative additives to ILs added during sol-gel formation, optimization experiments were performed for all prepared biocatalysts, using the acylation of racemic 1-phenylethanol as model reaction.

Table 4. Biocatalysts prepared using a ternary mixture of OTEOS/*n*-PTMOS/TMOS silane precursors. Additives used, enzyme loadings, hydrolytic and synthetic activities are presented.

Sol-gel code	Additive	Enzyme loading [μg_{enzyme}/mg biocatalyst]	Hydrolytic activity [U]	Synthetic activity [mmol/min/g_{enzyme}]
SG-5	-	11.3 ± 2.1	2.76 ± 0.04	11.72 ± 0.07
SG-6	ChCl:Fructose 1:1 20% H_2O	16.0 ± 1.7	5.10 ± 0.07	13.06 ± 0.12
SG-7	ChCl:Fructose 1:1 30% H_2O	12.3 ± 0.7	6.49 ± 0.05	14.42 ± 0.15
SG-8	ChCl:Glycerol 1:2	15.1 ± 1.1	5.66 ± 0.09	13.34 ± 0.02
SG-9	ChCl:Acetic acid 1:2	7.1 ± 0.3	1.23 ± 0.11	13.26 ± 0.08
SG-10	OMIMBF$_4$	12.3 ± 1.3	6.29 ± 0.08	14.62 ± 0.12
SG-11	EMIC	8.4 ± 0.5	9.42 ± 0.14	15.84 ± 0.17

4.2.2.2. Optimization of reaction parameters

4.2.2.2.1. Solvent screening

Four solvents with different polarities (*n*-hexane, MTBE, DIPE and 2-Methyl-THF) were tested in order to find the optimum solvent for the *O*-acylation of 1-phenylethanol (**Figure 14**). Solvents were chosen based on their high volatility, low boiling points and also the reactants (1-phenylethanol and vinyl acetate) solubility in these solvents. The importance of using an adequate additive during encapsulation is proven in this study as poor conversion values (under 20%) were obtained for SG-5 and SG-8 in *n*-hexane (SG-5 was prepared without an additive and SG-8 contains a DES formed by mixing ChCl with glycerol which may affect the enzyme's activity in organic solvents), whereas for the other biocatalysts excellent results were obtained

(conversion values of over 40%). Good conversion values were also obtained for the reactions in which MTBE and DIPE were used as solvents, however almost no enzyme activity was detected when 2-Methyl-THF was used as solvent. Based on the obtained results, *n*-hexane was chosen as optimal solvent and biocatalysts SG-1 and SG-4 were dropped from further optimizations.

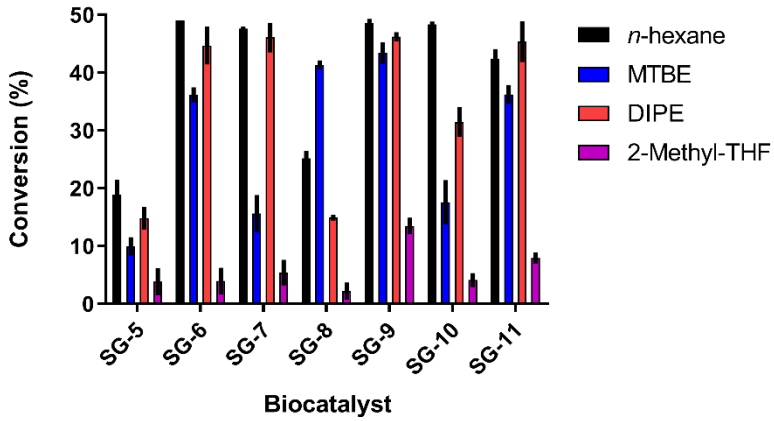


Figure 14. Solvent screening for the transesterification of racemic 1-phenylethanol. Reaction conditions: 1.5 mL glass vial, 8 µmol racemic 1-phenylethanol, 2 equiv. vinyl acetate, 25 µg lipase, 500 µL solvent, 3 hours, 40 °C, 1000 rpm.

4.2.2.2.2. Substrate: enzyme weight ratio

The next step in process optimization consisted in evaluating the influence of the substrate: enzyme *ratio* (w/w %). The enzyme load of a process is a very important parameter as a higher enzyme loading leads to a greater number of enzyme molecules being present to transform the substrate and it directly influences the overall conversion of the reaction. As shown in **Figure 15**, the highest substrate conversion was observed when working with a 20:1 substrate: enzyme *ratio* (w/w %), whilst lower conversion values were obtained with a substrate: enzyme weight *ratio* of 100:1, therefore the optimum substrate: enzyme *ratio* (w/w %) was chosen to be 20:1.

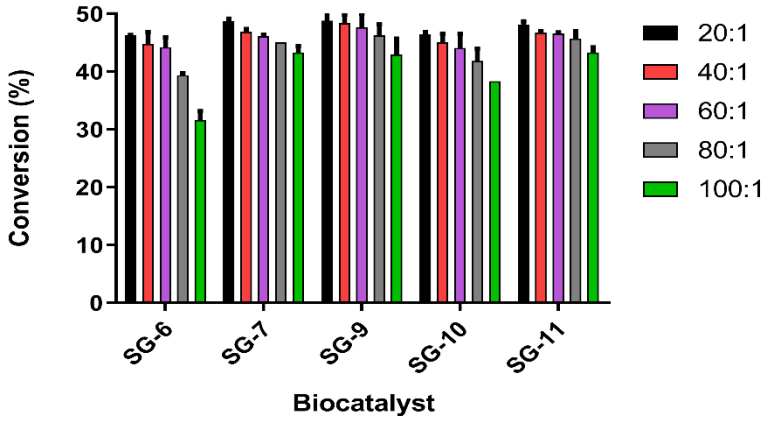


Figure 15. Substrate: enzyme *ratio* (w/w %) screening. Reaction conditions: 1.5 mL glass vial, 16 µmol racemic 1-phenylethanol, 2 equiv. vinyl acetate, 1 mL *n*-hexane, 40 °C, 1000 rpm, 3 hours.

4.2.2.2.3. Substrate: acyl donor molar ratio screening

Substrate: acyl donor molar *ratio* is an important parameter of a bioprocess that can significantly influence the rate of a reaction. In order to establish the influence of this parameter over the transformation of 1-phenylethanol four molar *ratio*s were tested (1:1, 1:2, 1:3 and 1:4, as seen in **Figure 16**).

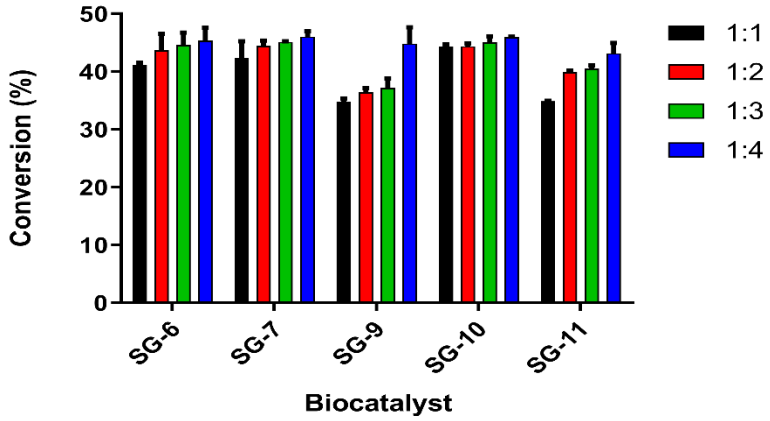


Figure 16. Influence of different substrate: acyl donor *ratio*s (mol/mol %) over the transesterification of racemic 1-phenylethanol. Reaction conditions: 1.5 mL glass vial, 16 μmol racemic 1-phenylethanol, 1 mL *n*-hexane, 100 μg lipase, 40 °C, 1000 rpm, 3 hours.

The best results were obtained when using a substrate: acyl donor *ratio* (mol/mol %) of 1:2 (~40 % conversion) and a further increase in acyl donor quantity (up to 4 equiv.) didn't lead to a significant improvement of the conversion values for the reactions mediated by SG-7 and SG-10, who were the most active in these conditions. Due to being less active in these conditions, SG-6, SG-9 and SG-11 were dropped from further optimizations and a substrate: acyl donor molar *ratio* of 1:2 was chosen as optimal for the two remaining biocatalysts (SG-7 and SG-10).

4.2.2.2.4. Temperature screening

SG-7 and SG-10 were tested at different temperatures using the previously determined optimal conditions in order to establish the influence of the reaction medium temperature over the reaction conversion.

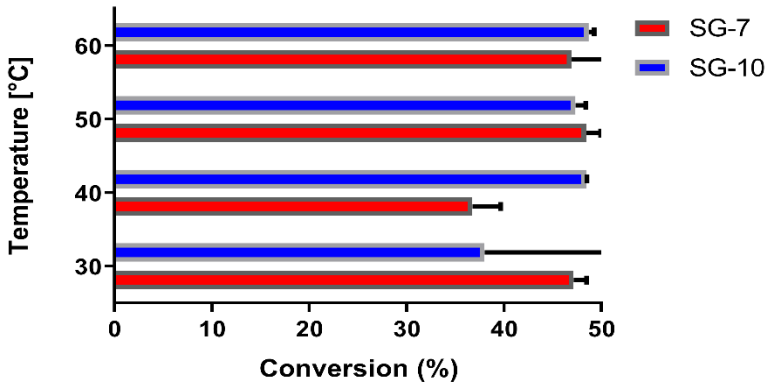


Figure 17. Temperature screening in the acylation of racemic 1-phenylethanol with vinyl acetate at different temperatures (30, 40, 50 and 60 °C). Reaction conditions: 1.5 mL glass vial, 16 µmol racemic 1-phenylethanol, 2 equiv. vinyl acetate, 100 µg CaL-B, 1 mL *n*-hexane, 1000 rpm, 3 hours.

As shown in **Figure 17** excellent conversion value (~50%) was obtained after 3 hours in the reaction catalyzed by SG-7 at 30 °C. In the meantime, the reaction catalyzed by SG-10 yielded a lower conversion value (~40%) after 3 hours at 30 °C and also a high standard deviation can be observed. The lower conversion value and low reproducibility for SG-10 at 30 °C is likely a consequence of the higher viscosity of the used additive (OMIMBF$_4$) at lower temperatures, since when tested at higher temperatures SG-10 reaches higher product yields in comparison with SG-7 in the same conditions. Considering the economic feasibility, materials price and energy consumption, SG-7 was chosen as the optimum biocatalyst for the optimized process and SG-10 was dropped from further optimization.

4.2.2.2.5. Acyl donor screening

The best performing biocatalyst (SG-7) was further tested in the transesterification of racemic 1-phenylethanol with different activated esters with increased alkyl chain length acting as acyl donors (vinyl acetate, vinyl

propionate and vinyl decanoate) in the optimal conditions and the evolution of the reactions conversion was monitored for 4 hours, with the results presented in **Figure 18**.

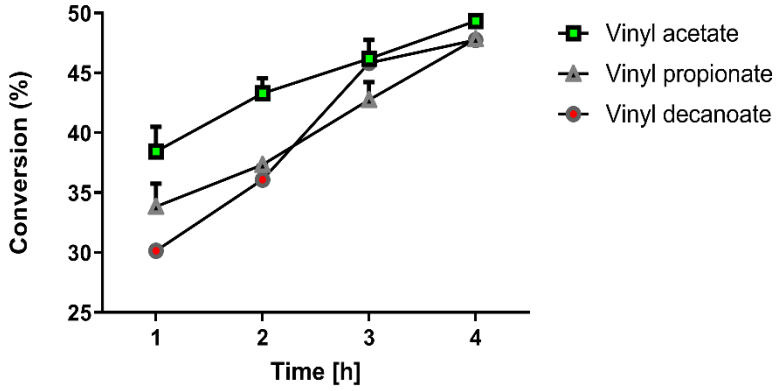


Figure 18. Time course of the transesterification of racemic 1-phenylethanol mediated by SG-7 using different acyl donors. Reaction conditions: 1.5 mL glass vial, 16 μmol racemic 1-phenylethanol, 2 equiv. vinyl ester, 100 μg lipase (SG-7), 1000 rpm, 30 °C.

The time profile of the transesterification of racemic 1-phenylethanol with different vinyl esters mediated by SG-7 shows that although the difference is not significant the reaction in which vinyl acetate acted as acyl donor delivered the best conversion values at all time-points, which shows that the reaction rate is dependent on the carbon-chain length of the vinyl ester. As result, vinyl acetate was selected as optimal acyl donor for further optimization steps.

4.2.2.3. Reusability studies

The operational stability of the biocatalyst is a very important parameter of a newly developed bioprocess as it is necessary for the biocatalyst to present high operational stability for the novel bioprocess to even be considered as a suitable replacement for a classic synthesis method applied at industrial level. For this, the recyclability of SG-7 was evaluated as presented in **Figure 19**.

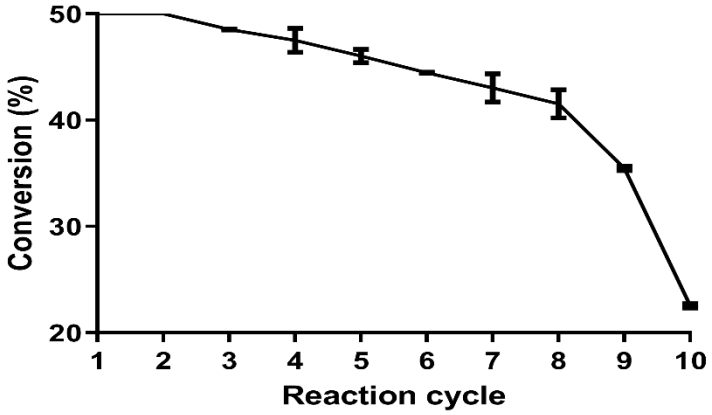


Figure 19. Evaluation of the recyclability of SG-7 in optimum conditions. Reaction conditions: 1.5 mL glass vial, 16 μmol racemic 1-phenylethanol, 2 equiv. vinyl acetate, 1 mL *n*-hexane, 100 μg CaL-B, 1000 rpm, 30 °C, 4 hours/ reaction cycle.

The novel prepared biocatalyst using a DES as additive (SG-7) presents high operational stability as it maintains ~85% of its initial activity even after eight reaction cycles. Based on the obtained results up to this point, SG-7 should be considered a viable candidate for the resolution of racemic 1-phenylethanol or other structurally similar compounds at industrial level.

4.2.2.4. Substrate concentration screening in optimum conditions

In order to increase the productivity of the developed process the substrate concentration was continuously increased until a drop in enzyme activity was observed, as presented in **Figure 20**.

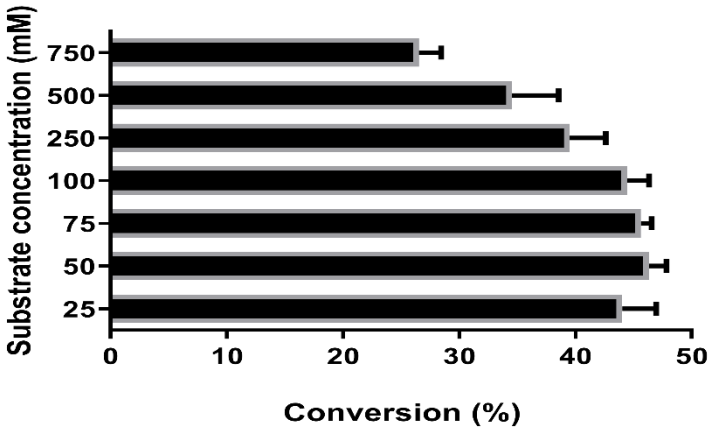


Figure 20. Substrate concentration screening for the transesterification of racemic 1-phenylethanol. Reaction conditions: 1.5 mL glass vial, 25-750 mM racemic 1-phenylethanol, 2 equiv. vinyl acetate, 1 mL *n*-hexane, 150-4500 μg CaL-B, 30 °C, 1000 rpm, 4 hours.

The obtained results show that a substrate concentration of 100 mM delivered excellent results (conversion of ~47%) and that a further increase of substrate concentration (250-750 mM) leads to a decrease in conversion (from ~47% at 100 mM to <30% at 750 mM initial 1-phenylethanol). Based on the obtained results the optimum substrate concentration was chosen as 100 mM in order to achieve maximum productivity of the developed process.

4.2.2.5. Synergic effect of additives in case of the optimum biocatalyst

The DES composed of ChCl and fructose has proven to be an efficient additive as SG-7 delivered excellent results in the transesterification of racemic 1-phenylethanol with vinyl acetate. In order to check if the high activity of the obtained biocatalyst is due to the DES's effect or is due to the action of a single component of said DES 3 additional biocatalysts containing CaL-B were prepared as follows: SG-71 with an aqueous solution of ChCl, SG-72 with an aqueous solution of fructose and SG-73 was prepared by adding an aqueous solution of ChCl and an aqueous solution of fructose (in a volumetric *ratio* of 1:1). The obtained biocatalysts were tested in the transesterification of racemic

1-phenylethanol with vinyl acetate using the previously determined optimal conditions in comparison with SG-7.

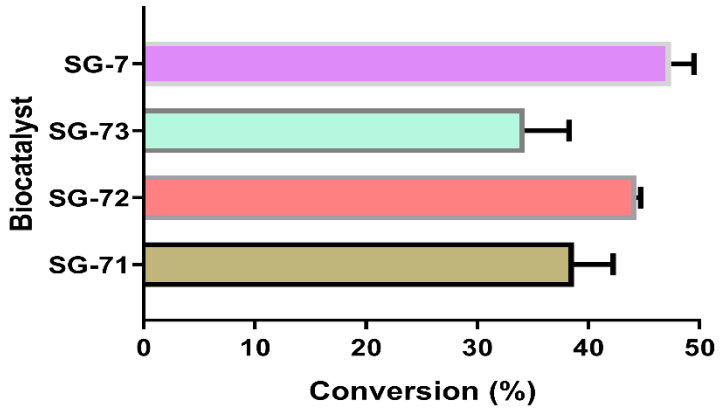


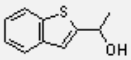
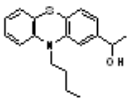
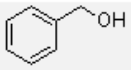
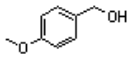
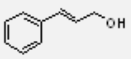
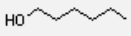
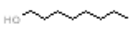
Figure 21. Evaluation of the synergic effect. Reaction conditions: 1.5 mL glass vial, 100 mM racemic 1-phenylethanol, 2 equiv. vinyl acetate, 610.5 µg CaL-B, 1 mL *n*-hexane, 1000 rpm, 30 °C, 4 hours.

Almost maximum conversion was achieved for the reaction catalyzed by SG-7 which was prepared using a DES as additive, whilst the lowest conversion value (~35%) was achieved for the reaction catalyzed by SG-73, which was obtained by adding a mixture of ChCl and fructose solutions (1:1 volumetric *ratio*). The concern that only one component of the DES increased the enzyme's activity was eliminated when it was observed that the conversion values in case of the reactions mediated by SG-71 and SG-72 (in which separate components of the DES were used) were lower than the conversion value of the transesterification of racemic 1-phenylethanol mediated by SG-7. Based on the obtained results it can be said that the use of DESs as additives to lipase encapsulation *via* sol-gel technique is justified as it positively affects the enzyme's activity and stability.

4.2.2.6. Expanding the substrate domain of the optimum biocatalyst

After process optimization was performed using the transesterification of racemic 1-phenylethanol with vinyl acetate as model reaction the optimum biocatalyst's performance was tested for the acylation of nine other alcohols, all with industrial relevance, as described in **Table 5**.

Table 5. Results obtained for the acylation of several alcohols with vinyl acetate mediated by SG-7.

Substrate code	Substrate name	Substrate structure	Substrate enantiomeric excess [%]	Product enantiomeric excess [%]	Conversion [%]
A	1-(benzo[*b*]thiophen-2-yl)ethan-1-ol		99.9	99.9	50
B	1-(10-butyl-10*H*-phenothiazin-2-yl)ethan-1-ol		99.9	99.9	50
C	benzyl alcohol		NAP	NAP	99.9
D	(4-methoxyphenyl)methanol		NAP	NAP	99.9
E	3-phenylpop-2-en-1-ol		NAP	NAP	99.9
F	1-pentanol		NAP	NAP	99.9
G	1-hexanol		NAP	NAP	99.9
H	1-octanol		NAP	NAP	99.9
I	1-dodecanol		NAP	NAP	99.9

*NAP- not applicable. Reaction conditions: 100 mM substrate, 2 equiv. vinyl acetate, 1 mL *n*-hexane, 1000 rpm, 30 °C, 4 hours. SG-7 was added in each case in order to respect the optimum *ratio* of 20:1 (w/w %) between substrate and enzyme.

Excellent results were obtained for the transformation of all tested substrates (**A-I** in **Table 5**) mediated by SG-7. Enantiopure products (product enantiomeric excess >99%) were obtained with maximum conversion values (50%) starting from compounds **A** and **B**, whilst compounds **C-I** were efficiently acylated with almost quantitative conversions (>99%). The versatility and stability of the novel biocatalyst SG-7 recommends it for a potential industrial production and application.

4.2.3. Conclusions

In this chapter of the thesis the novel use of DESs as additives in the encapsulation of CaL-B *via* sol-gel technique was discussed. Using the optimum DES, a mixture of ChCl:Fructose 1:1 (mol/mol % as a 30% aqueous solution), an efficient, stable, versatile and active biocatalyst (SG-7) was prepared and compared with similar biocatalysts prepared by using the more popular ILs as additives. The optimum biocatalyst was used for the efficient EKR of racemic 1-phenylethanol through transesterification with vinyl acetate. Using the optimal reaction conditions the best performing biocatalyst was able to efficiently transform nine other alcohols with industrial relevance, making SG-7 production suitable for a potential industrial scale-up.

Chapter 5. Experimental section (experimental data)

Chapter 6. General conclusion

In this thesis the successful immobilization of CaL-B by entrapment in tailored sol-gel matrices was employed and the obtained biocatalysts were used mainly for the enzymatic synthesis of aroma esters. An enzymatic procedure for anisyl propionate synthesis was successfully optimized which can produce the desired ester with 90% yield starting from a substrate concentration of 1 M in just 6 hours, which is the first reported efficient enzymatic synthesis of anisyl propionate.

The immobilization of CaL-B in tailored sol-gel matrices in the presence of poly-hydroxy compounds (as additives) has proven to be an efficient encapsulation method as the obtained biocatalysts were used for the successful synthesis of seven natural short-chain aroma esters. Based on the obtained results after process optimization the desired products can be obtained with excellent yields in short time (1 hour) and are easy to isolate from the reaction medium, therefore the proposed process is a strong candidate for a potential industrial scale-up and it also can be considered a green and sustainable process (supported by the evaluation of green metrics).

The novel use of DESs as additives in the encapsulation of CaL-B *via* the sol-gel technique was also described in this thesis. A mixture of ChCl: Fructose in 1:1 molar *ratio* (as a 30% aqueous solution) was used to prepare an efficient and stable biocatalyst that was successfully applied for the EKR of racemic 1-phenylethanol. Using the previously determined optimal conditions the mentioned biocatalyst (SG-7) was successfully applied in the acylation of nine other alcohols with industrial relevance. This was the first application of DESs as additives for the CaL-B encapsulation in sol-gel matrices, which opens the road for further research on this subject.

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