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VIRTUTE ET SAPIENTIA

FACULTATY OF CHEMISTRY

DISSERTATION THESIS

Student: MARIA CRISTINA GHEȚU

Coordinator: Assoc Prof. dr. MĂDĂLINA TUDORACHE

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VIRTUTE ET SAPIENTIA

FACULTATY OF CHEMISTRY

DEPARTAMENT OF ORGANIC CHEMISTRY, BIOCHEMISTRY AND CATALYSIS

DISSERTATION THESIS

DESIGNING BIOCATALYSTS BASED ON THE MODEL OF ENZYMES CO-IMMOBILIZATION WITH APPLICATION FOR CASCADE BIOCATALYSIS

MASTER: CHEMISTRY OF ADVANCED MATERIALS

Student: MARIA CRISTINA GHEȚU

Coordinator: Assoc Prof. dr. MĂDĂLINA TUDORACHE

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Introduction

Multi-enzymatic reactions are processes composed of minimum two reactions steps catalyzed by enzymes. There are some inherited advantages of such systems arising from the environmentally friendly nature of biocatalysts, along with their high substrate specificity, high product chemo-, stereo-, and enantioselectivity. Design of such eco-friendly systems proposes great advantages over typical industrial processes.

Cascade reactions represent a minimum of two consecutive processes occurring in the presence of enzymes. Commonly known as one pot processes, they provide an alternative for synthesizes of various compounds that would be obtained in a series of different steps, because there is no need for intermediate isolation and purification between each step. Therefore, making the cascade processes advantageous for a facile way of producing various substances with higher yields.

Lipases are enzymes which catalyzes the reaction on ester bonds. These enzymes have a high preference for water-insoluble substrates, and their most attractive feature is their inherit ability for interfacial catalysis. One of the most studied is lipase B from *Candida antarctica*.

Epoxide hydrolases are another class of enzymes which directs the hydrolytic ring opening of epoxides, resulting in the corresponding vicinal diols.

Enzyme co-immobilization is a process in which different enzymes are attached on a support, matrix, or form a network. This approach in enzymatic catalysis provides better catalytic activity, enhanced stability, and the possibility of reusing the biocatalyst over various reaction cycles, thus overcoming the disadvantages imposed by free enzyme catalysis.

Monoterpenes are natural compounds widely occurring in plants. These compounds have a typical hydrocarbon structure, however, their oxygenated derivates, known as monoterpenoids have a wide range of applications for flavor and fragrance products, being employed as such from centuries. There are various processes which are based on terpenes to terpenoid valorization, however, the majority employs the use of whole cell biocatalyst, which attracts some disadvantages that would be overcame by pure enzyme catalysis.

In this paper, we detail the steps performed to design a biocatalyst based on lipase and epoxide hydrolase co-immobilization with applications for monoterpenes valorization to flavor and fragrance products.

CHAPTER I: General notions

1.1. Enzymes in biocatalysts

Biocatalysis represents a chemical transformation which occurs inside or outside of the cell catalyzed by biological components, such as enzymes or antibodies. High catalytic efficiency, substrate selectivity and reaction specificity are some of the properties that make biocatalysis an attractive field. Those proprieties may be translated into the biocatalyst recognizing the substrate and obtaining the desired product with little to no side reactions. Biocatalysis is considered a part of green chemistry because of its eco-friendly nature [1].

Enzymes are biocatalysts that catalyze reactions in a living organism. They are proteins, meaning that enzymes are composed of one or more polypeptide chains. The sequence of the composing aminoacids is determined during the protein synthesis inside the living organism. As a protein, the enzyme is composed of a primary structure – the sequence of the composing aminoacids –, the secondary structure – the conformation of the aminoacid chain –, tertiary structure – the over-all 3D structure of the polypeptide chain –, and sometimes the quaternary structure – made by multiple proteins subunits [2].

Enzymes are molecules with complex proprieties, however, there are some misconceptions about them. Some of those misconceptions arise from their complicated structure and mechanism of action, and enzymes might be considered too sensitive, too expensive, or to be catalytically active only with their natural substrates or only in their natural environment [3].

Overall, enzymes are very efficient catalysts and have some major advantages. A typical reaction rate of enzyme-mediated processes is faster by a factor of 10^8–10^{10} the noncatalyzed reactions and are far above the values achieved by chemical catalysts. For example, chemical catalysts are generally employed in concentrations of a mole percentage of 0.1–1%, whereas most enzymatic reactions can be performed at reasonable rates with a mole percentage of 10^{-3}–10^{-4}% of catalyst, which clearly makes them more effective by some orders of magnitude. The high catalytic efficiency for enzymatic reactions is explained by the close contact between the enzyme and substrate because the substrate is located inside the protein structure, exactly on the catalytic site during the bioconversion [4].

A general example of biocatalysis mediated by enzymes is the yeast-mediated transformation of sugars into alcohol, commonly known as fermentation. Due to the protein nature of the enzymes, they are very sensible to changes in the reaction medium, because it may conduct to changes in their

structure that could result in a modification of the active site and, therefore, the enzyme can lose its catalytic activity [5].

Another downside of employing enzymes as biocatalysts is that for them to be used for a specific substrate, the enzyme needs to be purified, which is a time-consuming and highly costly process. An alternative would be the use of whole cells which represent a convenient strategy because in this case the enzyme acts in its natural cellular environment, which provides protection against destabilization and degradation [5]. However, the employment of whole cell catalysis is not without disadvantages [6].

Some downsides of using whole cells to mediate transformations would be the restriction of accessibility for the substrate to the catalytic site, which would result in low conversion degrees, and interaction with other enzymes, which would result in possible side reactions. To overcome such restrictions, surface displayed enzymes are usually used in whole cells reactions. Therefore, both whole cell and pure enzyme catalysis have advantages and disadvantages, however, by making a compromise between the two, enzyme catalysis is still the highly useful, especially in scientific research field, providing promising possibilities for industrial applications [6].

Today, over 3700 enzymes have been recognized by the International Union of Biochemistry and Molecular Biology. For identification purposes, every enzyme has a four-digit number in the general form ‘EC a.b.c.d’, where EC stands for ‘Enzyme Commission’, and ‘a’ denotes the main type of reaction, ‘b’ indicates the substrate class or the type of transferred molecule, ‘c’ shows the nature of the co-substrate, and ‘d’ is the individual enzyme number. Enzymes have been classified into six categories according to the catalyzed type of reaction in the following order: oxidoredictases, transferases, hydrolases, lyases, isomerases, and ligases [3].

1.1.1. Lipases

Lipases are triacylglycerol acylhydrolases, with the code E.C. 3.1.1.3, and are catalyze reactions on ester bonds with preference on water–insoluble substrates, such as monoterpenoids. They are produced by microorganisms either alone or together with other hydrolases. Fungi microorganisms are preferred as a source of lipase because they generally produce extracellular enzymes, facilitating the enzyme recovery from fermentation broth [7].

Lipases have the ability of interfacial catalysis, meaning that the biocatalyst becomes more active in presence of a substrate partially soluble in aqueous environment. They possess a wide range of catalytic properties and are frequently used as a crude extract for synthesis of chiral building blocks and enantiomeric compounds [7].

Lipase B from *Candida antarctica* (CALB) is one of the most studied biocatalysts due to high selectivity and catalytic activity. CALB is highly enantioselective and efficient in various organic reactions in a wide range of reaction environments, such as solventless or organic media. Its activity is higher in water environment than in organic media, but in immobilized form, CALB shows improved catalytic activity than the free enzyme, being able to withstand higher reaction temperatures. CALB can be immobilized both by physical and chemical means. An example of immobilized CALB is the commercially available Novozym® 435 [8].

1.1.2. Epoxide hydrolases

Epoxide hydrolases, abbreviated as EHs, having the code EC 3.3.2.9, are enzymes that catalyze the hydrolytic opening of epoxide rings resulting in the corresponding vicinal diols. EHs have been largely studied as biocatalysts for production of enantiopure epoxides and diols, two classes of chiral building blocks useful in the pharmaceutical industry. Specifically, the applications of EHs are usually favored because they possess the ability to transform a relatively large substrate spectrum with excellent stereo-, regio-, and chemoselectivity without the presence of a cofactors. These enzymes are very under operative conditions [9].

There is a family of atypical EHs named limonene-1,2-epoxide hydrolases (LEHs, EC 3.3.2.8) according to the natural substrate of the first isolated member, limonene oxide. LEHs were discovered after an interesting enzymatic activity from the bacteria *Rhodococcus erythropolis* DCL14 strain was discovered. This particularly strain was able to grow in the presence of both (+)- and (-)-limonene as the sole carbon and energy source [9].

Metagenomic investigations showed the existence of new thermophilic strains, such as CH55-LEH, which shows increased optimal temperature and melting point compared to previously known Re-LEH. Apart from the higher thermal stability, CH55-LEH maintained high substrate specificity on a boarder pH range, achieving a maximum around the value of 8. CH55-LEH shows high affinity for substrates related to limonene-1,2-epoxide resulting in enantio-conversion to corresponding vicinal diols. This biocatalyst promises great results for hydrolytic ring opening applications due to its increased stability [10].

1.2. Enzyme immobilization

Some of the drawbacks of enzymatic catalysis are overcame, such as the expensive process of purifying and isolating the enzymes being replaced by recombinant DNA techniques able to produce most enzymes for a reasonable price. Advances in protein engineering made possible to tailor enzymatic characteristics, such as substrate specificity, activity, selectivity, stability, or optimum pH

values, making enzymes more available than ever for potential applications. However, when it comes to possible industrial uses of enzymes as catalysts, it is clear that their lack of long-term operational stability and the difficulty of their separation from the reaction environment and reuse, makes them unfit [11].

Enzymatic immobilization is a technique that consists of confinement of protein molecules onto a support or matrix by chemical or physical means, leading to enhanced stability of enzymes, easier recovery from the reaction environment, improved catalytic performance and the possibility of reuse of the biocatalyst. Therefore, by immobilization approach, the drawbacks of pure enzyme catalysis compared to whole cell one are overcome, making enzymes suitable catalysts [11].

Enzyme immobilization is an important aspect of the biocatalytic process especially for industrial biotechnology. Immobilized enzymes offer many advantages, as previously mentioned, and, sometimes, as in the case of lipases, the enzyme is apparently activated after this immobilization step [12]. Depending on the immobilization technique employed, the properties of the biocatalyst, such as stability, selectivity, rate constant value, optimum pH or temperature, might be altered. The immobilization approaches developed comprises different strategies: adsorption, covalent attachment, cross-linking, entrapment, and encapsulation [13].

1.2.1. Immobilization on support surface

Support binding can be achieved both by physical and chemical means. The physical attachment can be achieved by hydrophobic and van der Waals interactions. Immobilization on a support can be done by ionic or covalent interactions, as well. Physical interactions between enzymes and support are usually very weak and leaching of the biological material may occur, but this method is highly cost effective. However, even thou the ionic and covalent interactions employ stronger forces and prevent leaching, they can lead to the enzyme deactivation due to conformational changes of the recognition site, preventing the interaction between the active site and substrate. The support material can be synthetic resigns, biopolymers, or inorganic polymers [11].

The easiest, cheapest, and oldest method employed is the adsorption onto a water-insoluble macroscopic carrier. It may be equally well applied to isolated enzymes as well as to whole cells [14].

The downside of this method is that forces employed in the support attachment are relatively weak, such as London forces, ionic interactions, hydrogen bonding. As a result of the weak binding forces, loss of catalytic activity is relatively low, but desorption from the carrier might occur. Also, minor changes in the reaction parameters, such as a variation of substrate concentration, solvent changes, temperature, or pH variations, could cause the leaching of the biological material [14].

However, the most appealing feature of this type of immobilization is the simplicity of the procedure, and various supports might be used, such as activated charcoal, alumina, silica, cellulose, controlled-pore glass, and synthetic resins [14].

1.2.2. Immobilization into a matrix

Some other immobilization approach for enzymes is entrapment, which refers to the inclusion of the biomolecule in a gel-like polymeric matrix. The polymers used for this method are usually organic polymers or silica sol-gel [11]. Encapsulation refers to the inclusion of an enzyme within a semi-permeable membrane, such as lipid vesicles. These are by far the best approaches which avoid any negative influence of the protein structure of the biocatalysts and prevent their aggregation and denaturation [13]. However, physical restrains of an enzyme might be too weak and lead to leaching of the biomolecules, therefore, the addition of covalent attachment might be necessary [11].

Cross-linking of the enzyme aggregates or crystals using a bifunctional reagent that result in carrier less macroparticles. This method is worth mentioning, because the presence of a carrier leads to a 'dilution' of catalytic activity due to the introduction of a large non-catalytic ballast [11]. The disadvantage of cross-linked enzyme crystals is that enzyme crystallization is an extremely laborious process and expensive. The other possible approach is cross-linked enzyme aggregates, which refers to the precipitation of physical protein aggregates [13]. The most used linker is glutaraldehyde, and the immobilization is based on the reaction between protein surface and amino group of the linker [15].

Overall, every immobilization method has its own downsides, meaning that there is not a standard method suitable for every process. Depending on the structure of the enzyme and the applications the biocatalyst is designed for, an immobilization method is chosen [13].

1.3. Multi-enzymatic reactions

Multi-enzymatic reactions represent a combination of several enzymatic transformations in a continuous process. They offer considerable advantages, such as less time demand, costs and chemicals for product recovery may be reduced, reversible reactions can be driven to completion and the concentration of harmful or unstable compounds can be kept to a minimum. A particular case of multi-enzymatic reactions is the bienzymatic ones, which refer to a series of reactions which use two different enzymes with specificity for different substrates [16].

Multi-enzymatic reactions are of great importance for technology to succeed in industrial process development, such as synthesis of pharmaceutical, cosmetic, and nutritional compounds. Different strategies to construct multienzyme structures have been widely reported. Enzymes complexes are designed by three types of routes: fusion proteins, enzyme scaffolds, or immobilization.

As a result, enzyme complexes can enhance cascade enzymatic activity through substrate channeling [17].

Biocatalysis has made tremendous advances in the field of synthesis of industrially important products and intermediates, especially by employing immobilized enzymes. By immobilization, the biocatalysts show improved operational temperature, improved convenience in separation from the reaction mixture, reducing costs. Therefore, immobilized multi-enzymatic biocatalysts gain an increased interest for potential industrial applications due to economic and environmental considerations [17].

1.3.1. Cascade processes in biocatalysis

Cascade reactions represent a chemical process that comprises at least two consecutive reactions coupled in such a way that each subsequent reaction occurs only in virtue of the chemical functionality formed in the previous step. It has the great advantage that the isolation of intermediates is not required because each reaction composing the sequence occurs spontaneously. Another advantage of cascade processes over others are that the reaction conditions do not change among the consecutive steps of a cascade and no new reagents are added after the initial step. Cascade processes are also known as one-pot procedures because the lastly mentioned similarly allow at least two reactions to be carried out consecutively without any isolation of intermediates, but do not preclude the addition of new reagents or the change of conditions after the first reaction. Although often composed solely of intramolecular transformations, cascade reactions can also occur intermolecularly [18].

The concept of performing multi-step syntheses in one-pot has gained some attention in the past period because from an environmental point of view, cascades represent a very promising approach. There are, however, some technological and scientific challenges to be overcame to reach industrial scale implementation of cascades. One of the most common challenges for the practicability of a cascade reaction is the combination of biocatalysts from different sources, which often have different optimal reaction conditions and show undesired side reactions [18].

Overall, multi-enzymatic cascade reactions offer considerable advantages: the demand of time, costs and chemicals for product recovery may be reduced, reversible reactions can be driven to completion and the concentration of harmful or unstable compounds can be kept to a minimum. They can be employed for the asymmetric synthesis of chiral alcohols, amines and amino acids, as well as for C-C bond formation [19]. As for example, the stereoselective three-enzyme cascade synthesis of diasteromerically pure γ-oxyfunctionalized α-amino acids [18].

1.3.2. Simultaneous biocatalytic reactions

On the other hand, a simultaneous reaction consists of any of two or more chemical reactions occurring at the same time in the same system, independently of each other. These types of processes are usually employed in industrial reactions, and are usually used in adjustment of the enzyme feed rate which helps to maximize the value of the product less the cost of the enzymes [20].

In a multi-enzymatic system, simultaneous reactions are not preferred. This reasoning is due to some factors intervening during the reaction. For example, competition of different substrates for the same active site, a product of a reaction might act as an inhibitor for another enzyme present in the system, or the presence of competitive inhibition phenomenon are just some aspects worth considering if one is to develop a multi-enzymatic simultaneous process [20].

An example of a simultaneous multi-enzymatic reaction which overcomes the mentioned drawbacks is the simultaneous multi-enzymatic hydrolysis and lactic fermentation, which would result in low lactose yogurt. This is a single stage process, which couples the fermentation process with the addition of β-galactosidase which would convert the lactose while it is produced [21].

1.4. Monoterpenes

Every year, large amounts of carbon dioxide are assimilated by plants, and part of this amount is converted and released back into the environment. A significant part of those substances released are volatile organic compounds, which include monoterpenes and sesquiterpenes [22].

Monoterpenes have the general formula $C_{10}H_{16}$ and are composed of two linked C_5H_8 isoprene units, therefore, represent a class of hydrocarbons [22]. They are known to act mainly as deterrent agents against herbivores, as well as antifungal defenders and to attract pollinators. Higher terpenes are present in mammals as well, and act as stabilizers of cell membranes, metabolic pathways, or regulators of enzymatic reactions [23].

Two of the most encountered monoterpenes are pinene, present in the wood plants as a constituent of their resign, and limonene, present in citrus plants. The term ‘terpene’ refers only to hydrocarbons, whereas ‘terpenoids’ is the term which refers to the oxygenated derivates of terpenes, and are manly produced by flowers, and are used as flavor and fragrance products [22].

Released monoterpenes are transformed in the atmosphere within hours in reactions with molecular oxygen, ozone, hydroxyl radicals, nitrogen oxides, chlorine atoms by photolysis or chemical reactions. The products resulting from these transformations constitute secondary aerosols that are transported to the soil by rain. Therefore, it would represent a great advantage to be able to develop a

method in which the widely occurring monoterpenes could be safely converted in flavor and fragrances [22].

Due to their wide impact on the global market, terpenes valorization is an important topic. Accounting for the need for a natural and sustainable approach for industrial processes, the bioconversion of such compounds might have an added value. Therefore, biocatalytic approach in monoterpenes conversion is an important aspect worth considering when developing a process. However, any strategy which proposes the valorization of this class of compounds has some challenges arising from the fact that this class of compounds is highly chemical instable, has low water solubility, high volatility, high toxicity of both substrates and products, relatively low yields, and high fermentation cost [24].

Most bioconversion processes of monoterpenes reported in the last years employed the use of whole cell biocatalysts due to the use of microbial cell conversions is easier to apply than to use purified enzymes. This might be advantageous in some cases due to low yield reported in enzyme catalyzed processes due to enzyme high substrate specificity. However, the high toxicity of both terpenes and terpenoids possess a high risk in affecting the cell catalytic activity [25]. Therefore, it would be of great interest to develop a biocatalyst based on enzyme immobilization able to convert monoterpenes into flavor and fragrance products, thus minimizing the disadvantages of using whole cell catalysts, and maximizing the possibility of obtaining enantiopure products.

1.5.1. Limonene

Limonene is the most abundant monocyclic monoterpene and second most abundant volatile organic compound indoors. It is the main component of citrus essential oil [22]. It has a precursory role for some other monocyclic monoterpenes, such as carveol, carvone, α-terpineol, pulegone, or 1,8-cineole [26].

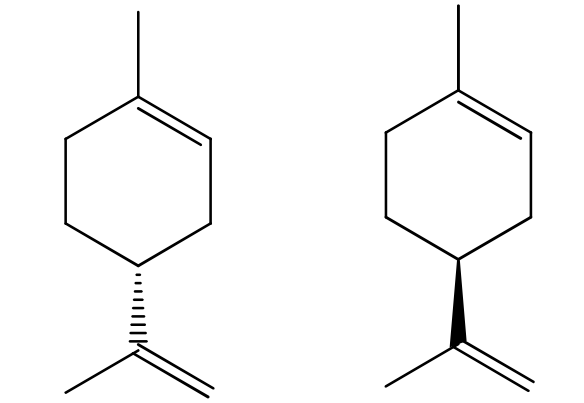


Image 1. Structure of a) R-(+)-limonene and b) S-(-)-limonene.

Limonene is an optically active compound. It exists in two enantiomeric forms: S and R, presented in image 1. The R-(+)-limonene isomer is known as d-limonene and it is the main component in essential oils from citruses peels. The l-limonene is mainly found in essential oils from pine needles and spearmint [26].

This monoterpene is widely used as flavor and fragrance additive in many products, such as perfumes, beverages, detergents, soap, or other house cleaning products. In addition to its additive use, limonene is also important as a starting material for the synthesis of other natural compounds, such as p-cymene. This wide range of application combined with vulnerability to ozone aided oxidation make limonene a highly commercially important molecule [26].

1.5.1. α-Phellandrene

α-Phellandrene is the main constituent of evergreen trees essential oils, but it might be also found in other essential oils from eucalypt, menthe, citruses, pine needles in lower amounts. This compound is often used in fragrances. α-Phellandrene is described as having a very distinctive citrus, green, black pepper-like scent [27].

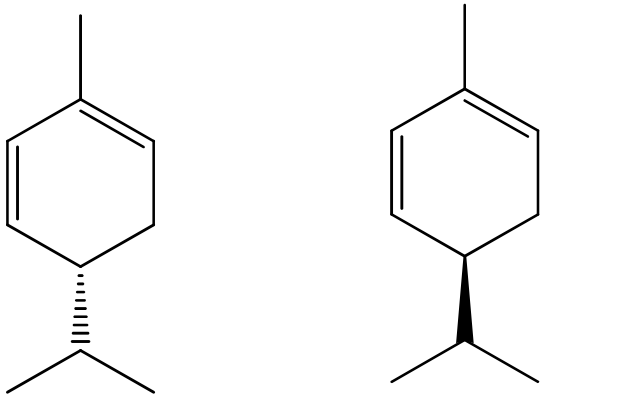


Image 2. Structure of a) S-(+)-α-phellandrene and b) R-(-)- α-phellandrene.

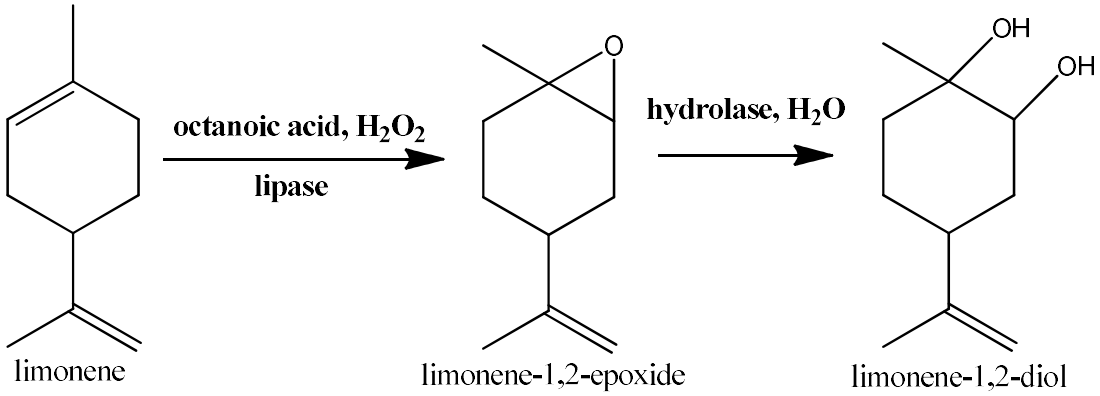
This monoterpene exist is the form of two enantiomers presented in image 2, the enantiomers have different physicochemical and olfactive properties. Biological activity of α-phellandrene was studies and it was reported to be inactive as an antimicrobial agent [27].

Chiral monoterpenes usually occur in nature as a single enantiomer, but the isomers possess different olfactory properties. Therefore, a stereo- and enantioselective approach in their valorization is of high interest for pharmaceutical and chemical industries large scale applications [27].

1.5. Aim of this thesis

Due to the wide occurrence of monoterpenes, the chiral nature of these molecules and the most used approach in their valorization being whole cell catalysis, we propose a bienzymatic biocomposite designed by co-immobilization for cascade conversion of R-(+)-limonene to flavor and fragrance products.

We based our study on literature reports of lipase-mediated epoxidation on limonene to limonene oxide using fatty acids [28] using a cheap oxidizing agent, hydrogen peroxide [29], cupelled with a thermophilic epoxide hydrolase able to withstand higher reaction temperatures [10]. Therefore, we developed a biocomposite able to convert monoterpenes into monoterpenoids with potential applications for industrial processes, being able to overcome potential drawbacks of using whole cell catalysts, such as loss of catalytic activity due to high toxicity of substrates and products in large amounts, or the presence of secondary mediated by other biocatalysts present in the system. The reaction scheme is presented in scheme 1.



Scheme 1. Proposed scheme for limonene bioconversion.

Our process is composed of a two-step cascade bienzymatic conversion of our substrate. In the first step, uses an indirect lipase-mediated conversion of monoterpenes [28], meaning that the lipase oxidized a fatty acid present in the system, which in our case is octanoic acid, to its corresponding peracid by means of hydrogen peroxide as an oxidizing agent. The then formed octanoic peracid converts the R-(+)-limonene substrate into limonene oxide. In the second step of our process, the hydrolase directs the hydrolytic ring opening of the epoxide intermediate toward the formation of an enantiopure vicinal diol, more exactly (1S,2S,4R)-limonene-1,2-diol.

The biocatalyst is based on the adsorption of CH55-LEH hydrolase on the surface on commercially available lipase B from *Candida antarctica* immobilized on the surface of a hydrophobic acrylic resign in the form of Novozym® 435. Therefore, in our study we optimized the biocatalytic process, characterized the obtained material, and propose possible applications for our biocatalyst.

CHAPTER II: Experimental

2.1. Substances and solutions

For our study various substances and solutions were used. It is worth mentioning that the vast majority of the substances used were provided by Sigma Aldrich, meaning octanoic acid, R-(+)-limonene, α-phellandrene, limonene oxide, (1S, 2S, 4R)-(+)-limonene-1,2-diol, hydrogen peroxide 30%, ethyl acetate, pyridine, p-nitrophenyl palmitate, p-nitrophenol, the acid-trapping salts, as well as the three lyophilized lipase A and B from *Candida antarctica*, and lipase from *Aspergillus niger*, and two immobilized lipases from *Candida cylindracea* and *Pseudomonas cepacia*, both immobilized in a sol-gel matrix. The few differently sourced substances and solutions are mentioned further.

The phosphate-buffered saline (PBS) with a pH around 8 contains sodium chloride (NaCl), potassium chloride (KCl), disodium phosphonate (Na_2HPO_3), dipotassium phosphonate (K_2HPO_3) in water, and the pH is adjusted using sodium hydroxide (NaOH).

The tris-HCl buffer used as the environment of choice for some of our studies due to its relatively neutral pH. This buffer was prepared by mixing 0.1 M of tris(hydroxymethyl)aminomethane with a concentration of 12.1 g/L with 44.2 mL of 0.1 M hydrochloric acid to obtain a pH of 7.2.

Some commercially available immobilized lipases were studied. Six different immobilized enzymes provided by Strem Chemicals in collaboration with Novozymes®. From their range we studied the behavior of Lipozyme® TL IM – lipase from *Thermomyces lanuginosus* immobilized on a non-compressible silica gel carrier - , Novozym® 435 – lipase B from *Candida antarctica* immobilized in hydrophobic acrylic resign -, Transenzyme – lipase from *Geobacillus stearothermophilus* immobilized in acrylic resign -, Lipozyme® RM IM – lipase from *Rhizmucor miehei* which is immobilized on an anionic exchange resin carrier. The immobilized lipases were all stored in the fridge.

The hydrolases studied are not commercially available. These enzymes were provided by Dr. Daniela Monti from Chemical Institute of Molecular Recognition, C. N. R., Milan, Italy, and her team. The hydrolases were provided in the form of enzymatic solutions. For out studies we used Re-LEH, which has the optimum temperature around room temperature, and Tomsk-LEH and CH55-LEH, both thermophilic enzymes [10]. The solutions provided to us have the following concentrations: 1.81 mg/mL CH55-LEH, 1.27 mg/mL Tomsk-LEH, and 2.63 mg/mL Re-LEH. The hydrolase samples were stored at -40 ^{o}C and defrost only when preparing samples.

2.1.1. Sample preparation and pre-treatment

A sample contain the following components in order:

1. Octanoic acid – in a concentration of 1.6 M;
2. Substrate – with concentration of 1.59 M;
3. Phosphate-buffered saline (PBS) with the pH of 8.00 and concentration of 0.04 M – it is used to maintain a constant volume among all samples;
4. Lipase – 43.89 μg/mL is the concentration of the free lipases, and 9.66 mg/mL is the concentration of immobilized lipases;
5. Hydrolase – with a concentration of 2.81% v/v in the final solution;
6. Hydrogen peroxide (H_2O_2) 30% - its concentration of 0.44 mM;
7. Acid-trapping reagent – 0.3 milimols of acid-trapping salt.

For the lyophilized lipases, an enzyme solution with a concentration of 1 mg/mL was prepared beforehand by solubilizing the enzyme powder in PBS with pH 8.

The experimental procedure consists of sample reaction for 24 hours at temperatures between 25 ^{o}C and 50 ^{o}C, 1000 rotations per minute in a thermoshaker. The extraction step is 1:1 volume ratio of sample and ethyl acetate, followed by 30 minutes of agitation at room temperature at 1500 rotation per minute. Depending on our study goal, here might be an additional derivatization step consisting of 100 μL of sample previously extracted and agitated mixed with 150 μL pyridine, and 66 μL acetic anhydride, 24 hours at 25 ^{o}C at 1000 rotations per minute in a thermoshaker reaction. The derivatization step is usually employed for the bienzymatic system to increase the resolution of diol detection.

The final solution is analyzed using gas chromatography. We developed a series of methods of analysis using different chromatographic columns to better tailor the methods to our system.

2.1.2. Enzyme co-immobilization-based biocatalyst preparation

The immobilization procedure is based on the adsorption of the hydrolase on the surface of a support already containing a lipase. Our biocomposite candidates were CH55-LEH hydrolase and Novozym® 435, and Re-LEH and Lipozyme® RM IM.

For both materials, the same procedure was followed. 32 μL of hydrolase solution with a concentration of 2.63 mg/mL Re-LEH, or 1.81 mg/mL CH55-LEH were added on 11 mg of immobilized lipase. The samples were prepared in a 2 mL Eppendorf. The vials were left open in the fridge for 4 to 5 days to dry. After completely drying the biocomposite was washed with 1 mL PBS pH 8. This step was repeated three times.

2.2. Methods of analysis

2.2.1. Identification/ quantification of substrate/products

Gas chromatography (GC) was the method of choice for the sample analysis, giving their volatile nature. We used different chromatographic columns for a proper detection, more exactly GC with a polar chiral column and a flame ionization detector (GC*-FID) which provide a better detection for the limonene-1,2-diol products. This method is used for the bienzymatic acetylated sample. The other chromatographic methods use another GC with flame ionization detector (GC-FID) having a different column than the previous, and GC with mass spectrometer detection (GC-MS). Both GCs use the same non-polar separation column.

The GC*-FID analysis uses hydrogen as a carrier gas. The injector has a temperature of 230 ^{o}C. Injection mode is split. Flow control mode is pressure. The carrier gas has the following parameters: pressure of 0.74 bar, total flow 23.9 mL/min, column flow 1.90 mL/min, linear velocity 50.2 cm/sec, purge flow 3.0 mL/min, split ratio 10. The temperature regime of the column starts from 50 ^{o}C with a holding time of 20 minutes. The temperature increases with 10 ^{o}/min till 150 ^{o}C. The holding time at this temperature is 10 minutes. The analysis time for a sample is 40 minutes. The column stationary phase is a β-cyclodextrin modified with S-hydroxypropyl.

The GC-FID analysis aslo uses hydrogen as a carrier gas. The injector has a temperature of 230 ^{o}C. Injection mode is split. Flow control mode is linear velocity. The carrier gas has the following parameters: pressure of 1.82 bar, total flow 20.5 mL/min, column flow 1.59 mL/min, linear velocity 45.0 cm/sec, purge flow 3.0 mL/min, split ratio 10. The temperature regime of the column starts from 50 ^{o}C without holding time. The temperature increases with 7 ^{o}/min till 250 ^{o}C. The holding time at this temperature is 5 minutes. The analysis time for a sample is 34.57 minutes. The column stationary phase is a (5%-phenyl)-methylpolysiloxane.

The GC-MS analysis uses helium carrier gas. The injector has a temperature of 230 ^{o}C. Injection mode is split. The carrier gas has the following parameters: flow mode constant flow with vacuum compensation, gas saver flow 10 mL/min, gas saver time 2 minutes, split flow 30 mL/min, split ratio 30. MS transfer line has a temperature of 250 ^{o}C. The temperature regime of the column starts from 50 ^{o}C with a holding time of 3 minutes. The temperature increases with 5 ^{o}/min till 230 ^{o}C. The holding time at this temperature is 1 minutes. The analysis time for a sample is 40 minutes. The column stationary phase is a (5%-phenyl)-methylpolysiloxane.

The GC*-FID method is used to quantify the ratio between the S and R isomers of the limonene-1,2-diol. For sample analysis the sample is acetylated, this step increasing the resolution for

the enantiomer separation, but might derivatize some unreacted substrate as well. Therefore, this method cannot be reliably used to calculate the substrate conversion, but it is useful to determine the yield of limonene-1,2-diol, and enantiomeric excess (ee%).

The other two methods of analysis are exclusively for extracted samples. Therefore, the substrate can be properly quantified. GC-MS is used to identify the products of the enzymatic system. Once the structures are attributed, the method of choice for analysis is GC-FID. Both methods use the same stationary phase, meaning that the resulting chromatograms are relatively similar.

To properly calculate the conversion of limonene and the products yield, calibration curves were constructed. For R-(+)-limonene quantification, eight solutions were prepared in ethyl acetate. The concentration varied between 3.32 M and 0 M. The samples were homogenized by agitation at room temperature in a vortex for 30 minutes at 1500 rotations per minute. The same procedure was followed for limonene oxide, with a concentration range between 3.26 M to 0 M.

The procedure for the limonene-1,2-diols is the same for the most part, the concentration range being between 3.32 M and 0 M. Homogenization done as well at room temperature in a vortex for 30 minutes at 1500 rotations per minute. The difference was that 100 µL of sample were derivatized with 150 µL pyridine, and 66 µL acetic anhydride, and reacted for 24 hours at 25 °C at 1000 rotations per minute in a thermoshaker. The solutions were analyzed using the GC*-FID.

It is worth mentioning that the curve for (1S, 2S, 4R)-(+)-limonene-1,2-diol has a correction factor equal to 0.30, because the calibration curve was obtained using a previously employed GC*-FID method with a higher split ratio. Another calibration curve could not be prepared due to commercial unavailability of (1S, 2S, 4R)-(+)-limonene-1,2-diol.

All mentioned calibration curves are presented in the supplementary information (figures A to C), and have a correlation coefficient close to 1, therefore, they all present good linearity.

For the sample analysis, the formulas 1 to 4 were used to calculate the conversion degree of R-(+)-limonene, the yield of limonene oxide intermediate, or limonene-1,2-diol products, and the enantiomeric excess of (1S, 2S, 4R)-(+)-limonene-1,2-diol.

1) $$C\% = \frac{\text{initial concentration of limonene} - \text{concentration of limonene from figures 1 or 3}}{\text{initial concentration of limonene}} * 100$$

2) $$\text{epoxide yield}\% = \frac{\text{concentration of epoxide from figures 2 or 4}}{\text{initial concentration of limonene}} * 100$$

3) $$\text{diol yield}\% = \frac{\text{concentration of diol from figure 5} * \text{correction factor}}{\text{initial concentration of limonene}} * 100$$

4) $$\text{enantiomeric excess}\% = \frac{\text{peak area S} - \text{pe are R}}{\text{peak area S} + \text{pe are R}} * 100$$

2.2.2. Characterization of the biocatalyst

Fourier-transform infrared (FTIR) spectroscopy was used to characterize the prepared biocomposites. FTIR spectra of the materials were recorded using Bruker Tenso-II FTIR spectrometer with Diffuse Reflectance "Smart Accessory". The absorption spectra were collected between 400 and 4000 cm^{-1}, at room temperature. The registered spectra have an average of 64 scans with a resolution of 16 cm^{-1}. The samples were not pre-treated before analysis.

The spectra were collected from solid samples by mixing a small amount with potassium bromide (KBr). Besides the biocomposites, the hydrolase solutions, Novozym® 435 and Lipozyme® RM IM beads, and lyophilized lipase B from *Candida antarctica* were analyzed as well.

2.2.3. Determination of enzyme loading

The loading of CH55-LEH hydrolase on the surface of the acrylic beads of Novozym® 435 was determined employing a calibration curve for BSA. The curve was constructed by solubilizing the protein in tris-HCl buffer, varying the protein concentration between 0.1 to 1 mg/mL. The liquid samples were analyzed using UV-VIS spectrophotometer Specord 250, Analytik Jena, and the absorbance was registered at 280 nm. The calibration curve is presented in the supplementary information (figure G).

For the quantification of the amount of CH55-LEH hydrolase adsorbed on the Novozym® 435 beads, the absorbance of an initial enzymatic solution of CH55-LEH with a concentration of 1.81 mg/mL, as well as the absorbance of the washing solution after immobilization. This last solution is composed of all three washing solutions mixed and homogenized before analysis. Both absorption values were recorded at 280 nm.

2.2.4. Determination of enzymatic activity

The washing solution of the biocomposite and an enzymatic solution of 2.63 mg/mL Re-LEH were analyzed using a UV-VIS spectrophotometer Specord 250, Analytik Jena between 214 and 400 nm. For a better comparison, 11 mg of Novozym® 435 and other 11 mg of Lipozyme® RM IM were washed three times with 1 mL of PBS pH 8 following the same procedure as for the biocomposite. This washing solution was analyzed as well between 200 and 400 nm.

The determination of the enzymatic activity is based on the conversion of p-nitrophenyl palmitate catalyzed by the lipase to p-nitrophenol, which has an absorption peak in the near-UV region.

A calibration curve was prepared for the quantification of p-nitrophenol. The curve was prepared by varying the amount of p-nitrophenol between 0.5 and 0 mM in ethanol. The absorption was registered at 347 nm using a UV-VIS spectrophotometer Specord 250, Analytik Jena.

The procedure employed to determine the enzymatic activity of immobilized lipases consists on the construction of two calibration curves of the amount of formed p-nitrophenol by different amounts of both Novozym® 435 and Lipozyme® RM IM. The amount of immobilized lipase was varied between 1 and 11 mg. The enzymes were mixed with 900 µL of tris-HCl buffer 0.05 M with a pH of 7.2, and 100 µL of p-nitrophenol palmitate 0.025 M in ethanol. Then, were incubated for 30 minutes at 37 °C. After, the reaction was ceased by the addition of 250 µL of a solution of 0.1 M Na_2CO_3. The samples were then centrifugated at 12000 rpm for 2 minutes. The analysis was done on the liquid sample at 410 nm using a UV-VIS spectrophotometer Specord 250, Analytik Jena.

All calibration curves are presented in the supplementary information (figures D to F).

For the determination of the enzymatic activity of our samples, the same procedure was followed as for bare Novozym® 435 and Lipozyme® RM IM. The immobilized lipases were analyzed before the immobilization of the hydrolase, as well as after. The activity of the resulting biocomposite was determined before the washing procedure, as well as after each washing step, and after up to 3 reaction cycles.

The washing solution was analyzed as well, each washing solution separately, to determine if the lipase might be lost during the procedure. The protocol is the similar, only the amounts are slightly changed, 250 µL of washing solution is mixed with 650 µL of tris-HCl buffer 0.05 M with a pH of 7.2, and 100 µL of p-nitrophenol palmitate 0.025 M in ethanol. Followed by incubation, reaction cessation, centrifugation, and UV-VIS analysis.

The enzymatic activity of the CH55-LEH hydrolase was determine before and after the co-immobilization. The determination was realized by preparing a sample containing 1.6 M limonene-1,2-epoxide, 1.6 M octanoic acid, 0.04 M PBS pH 8, and 2.81% v/v hydrolase solution. The enzymatic activity value before co-immobilization was determined using the calibration curve for limonene-1,2-epoxide constructed using GC-FID method, presented in the supplementary information (figure B). The value after the co-immobilization was determined from the percentage of hydrolase loading on the Novozym® 435 surface.

CHAPTER III: Results and discussions

3.1. Mono-enzymatic system

3.1.1. Comparison between free enzyme and immobilized enzyme

The behavior of lyophilized enzymes was compared with the one of immobilized enzymes. This study focuses on how the system components influence parameters, such as limonene conversion and epoxide yield, depending on lipase source and state (free or immobilized), and the effect of immobilization on enzymatic activity.

For the study of lipases available in lyophilized form, solutions with the concentration of 1 mg/mL lipase in PBS pH 8 were prepared prior. We tested lipase from *Aspergillus niger*, and lipase A and B, both from *Candida antarctica*.

The samples were prepared, pre-treated, and analyzed using the GC-FID method. The conversions and epoxide yields were obtained using the calibration curves are presented in figures 1 and 2.

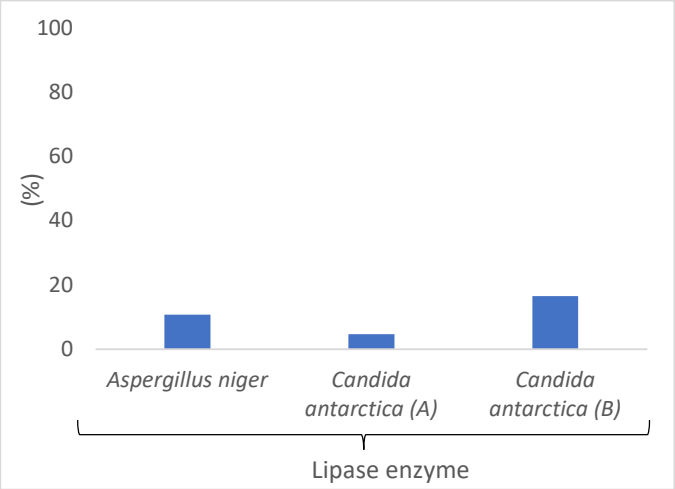


Figure 1. Conversion of R-(+)-limonene obtained with lyophilized lipases.

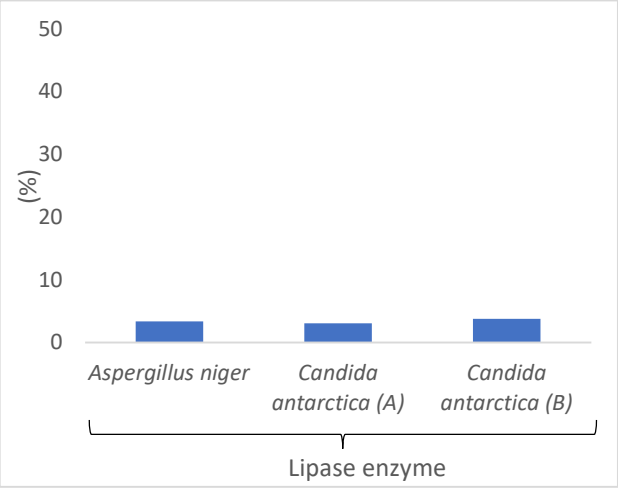


Figure 2. Yield of limonene oxide obtained with lyophilized lipases.

(1.6 M R-(+)-limonene, PBS pH 8 0.04 M, 1.60 M octanoic acid, 0.44 mM hydrogen peroxide, and 43.89 µg/mL lipase. Reaction: 24 h, 25 °C, 1000 rpm. Liquid-liquid extraction - 1:1 = sample : ethyl acetate (v/v) → 30 min stirring at room temperature)

In figure 1 are presented the values for limonene conversion obtained using different lyophilized enzymes. The conversion increases in the following order: lipase A from *Candida antarctica*, lipase from *Aspergillus niger*, and the highest value is obtained for lipase B from *Candida antarctica*. The lowest value for conversion is 5% in the case of lipase A, and the maximum one is 16% for lipase B. The conversion percent obtained for lipase from Aspergillus niger is 11%. Therefore, there is only a 5% difference between the lipase from *Aspergillus niger* and lipase B from *Candida antarctica*.

In figure 2, there are represented the yield values for limonene oxide, the product of the enzymatic transformation of limonene. The yield values are not as different as in the case of the conversion. The values are contained between 3 to 4%. The lowest value is again obtained when lipase A is used, and the highest in the presence of lipase B. The value for the yield obtained when lipase from *Aspergillus niger* is employed is only 0.4% smaller than the maximum value obtained for lipase B.

The highest values for conversion and yield were obtained for lipase B from *Candida antarctica*. However, considering that we previously worked with liquid lipase from *Aspergillus niger*, in our succeeding studies we chose to work with lyophilized lipase from *Aspergillus niger*, that would provide a better understanding of the variation of efficiency of our system when other factors are varied.

For the study of lipases available in immobilized form, we tested six different immobilized lipases commercially available. We tested Lipozyme® TL IM which contains lipase from *Thermomyces lanuginosus* immobilized on a non-compressible silica gel carrier, Novozym® 435 which is lipase B from *Candida antarctica* immobilized in hydrophobic acrylic resign, Transenzyme which is lipase from *Geobacillus stearothermophilus* immobilized in acrylic resign, and Lipozyme® RM IM which is lipase from *Rhizmucor miehei* immobilized on an anionic exchange resin carrier.

The sample preparation and pre-treatment before analysis, as well as analysis method employed are the same as previously mentioned. The conversions and epoxide yields were obtained from the corresponding calibration curves are presented in figures 3 and 4.

In figure 3, it can be observed how the conversion varies when different immobilized enzymes are used. The lowest limonene conversions were obtained in when the sol-gel immobilized lipases were employed. The conversion value obtained for Lipozyme® TL IM is also in the lower end. This is to be expected because the lipase from *Thermomyces lanuginosus* is thermophilic, and its optimum temperature is higher than our reaction temperature. From information available on Strem Chemicals, the temperature range for this immobilized lipase is between 50 and 75 ^{o}C. We chose to use this lipase in our succeeding studies because its optimum range is at very increased temperatures. This proposes two problems. First, the hydrolase can become denatured at such increased temperatures, and second, the remaining three immobilized lipases can convert higher amounts of limonene at lower temperatures, therefore, there would not be a need to input higher energy in our system.

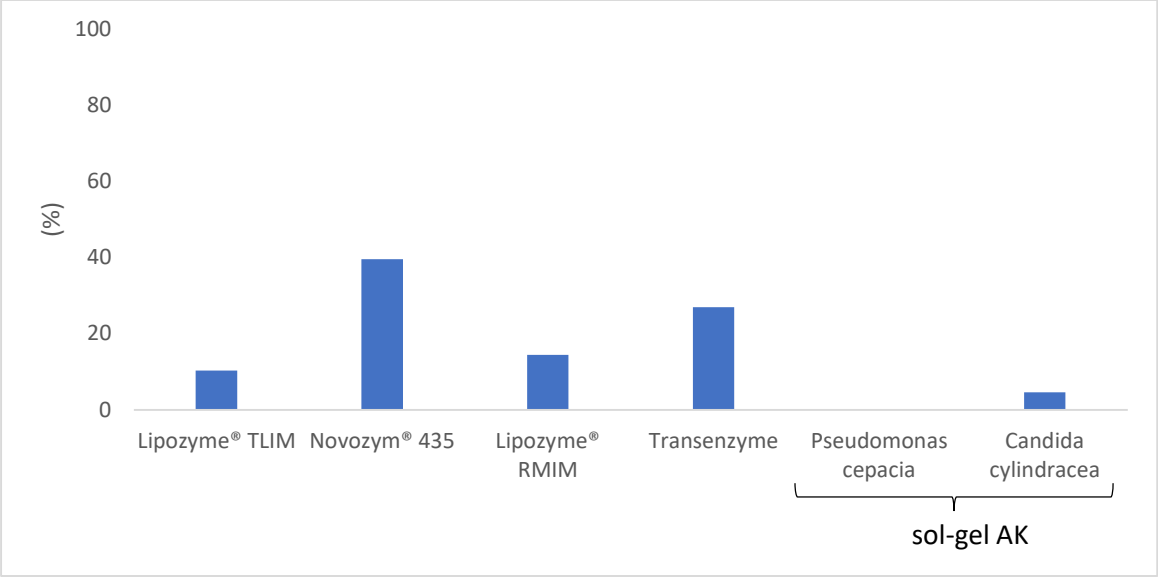


Figure 3. Conversion of R-(+)-limonene obtained with immobilized lipases. (1.6 M R-(+)-limonene, PBS pH 8 0.04 M, 1.60 M octanoic acid, 0.44 mM hydrogen peroxide, and 9.66 mg/mL immobilized lipase. Reaction: 24 h, 25 °C, 1000 rpm. Liquid-liquid extraction - 1:1 = sample : ethyl acetate (v/v) → 30 min stirring at room temperature)

From the remaining three immobilized lipases we chose Novozym® 435 and Lipozyme® RM IM because they provided high conversions of limonene at room temperature. Even thou Transenzyme performed compared to Lipozyme® RM IM, we choose to go further with our tests with two lipases immobilized on different supports for a proper comparison.

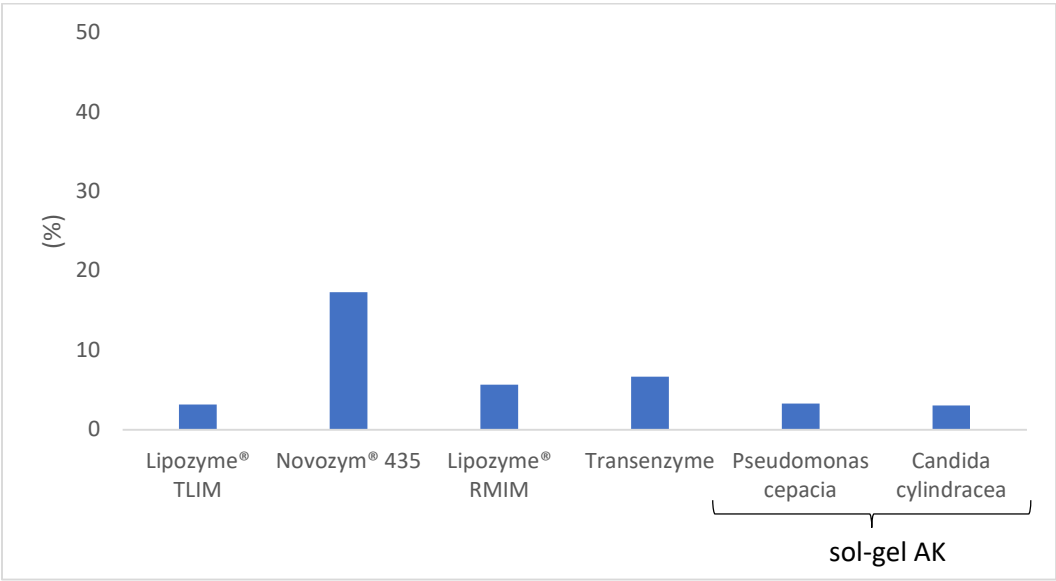


Figure 4. Yield of limonene oxide obtained with immobilized lipases. (1.6 M R-(+)-limonene, PBS pH 8 0.04 M, 1.60 M octanoic acid, 0.44 mM hydrogen peroxide, and 9.66 mg/mL immobilized lipase. Reaction: 24 h, 25 °C, 1000 rpm. Liquid-liquid extraction - 1:1 = sample : ethyl acetate (v/v) → 30 min stirring at room temperature)

The yield values from figure 4 follow the same trend as the values for limonene conversion from figure 3. This supports our choice to continue our studies using Novozym® 435 and Lipozyme® RM IM.

As shown in figures 3 and 4, Novozym® 435 provided the most efficient in converting limonene to limonene oxide. The conversion was 40% and the yield is around 17%. We previously shown in figures 8 and 9 that lipase B from *Candida antarctica* performs better to indirectly convert limonene, it was to be expected that its immobilized form in Novozym® 435 would provide higher efficiency.

Therefore, we proposed for our next study to test the behavior of lipase from *Aspergillus niger*, Novozym® 435 and Lipozyme® RM IM, along systems without hydrogen peroxide and without biocatalysts, when the reaction temperature is varied between 25 and 50 °C.

3.1.2. Variation of reaction temperature

For the study of the influence of reaction temperature on R-(+)-limonene conversion in the presence of the lipase as the only biocatalysts we varied the reaction temperature between 25 and 50 °C. This range of reaction temperatures would show best the influence it has on the process.

From the tested immobilized lipases, we chose Novozym® 435 and Lipozyme® RM IM, because these two performed the best in the given conditions, and characteristic information about these products is available on Strem Chemicals website.

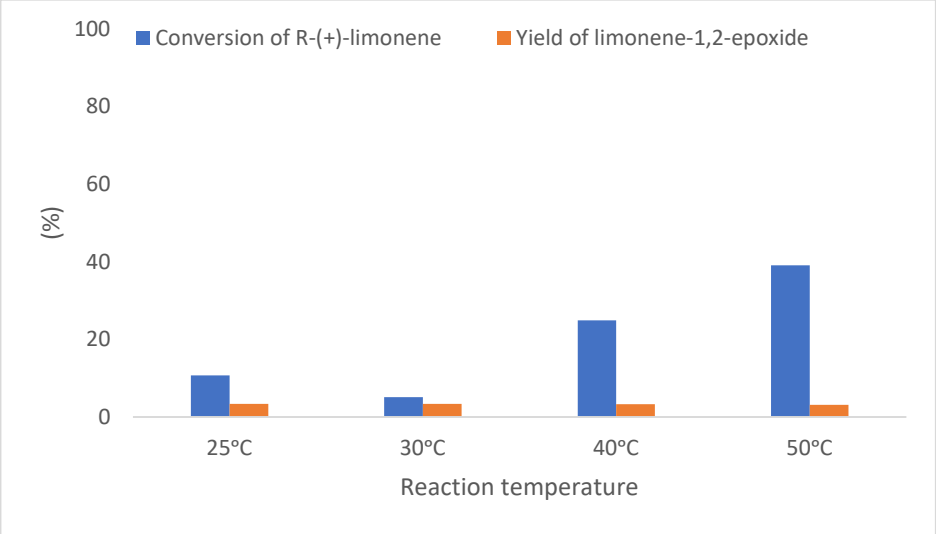


Figure 5. Influence of reaction temperature on samples containing lyophilized lipase from *Aspergillus niger*. (1.6 M R-(+)-limonene, PBS pH 8 0.04 M, 1.60 M octanoic acid, 0.44 mM hydrogen peroxide, and 43.89 µg/mL free lipase. Reaction: 24 h, 25-50 °C, 1000 rpm. Liquid-liquid extraction - 1:1 = sample : ethyl acetate (v/v) → 30 min stirring at room temperature)

The samples were prepared, pre-treated, and analyzed using the GC-FID method. The conversions and epoxide yields were obtained using the calibration curves are presented in figures 5 to 9, each representing the influence of the reaction temperature on each type of sample.

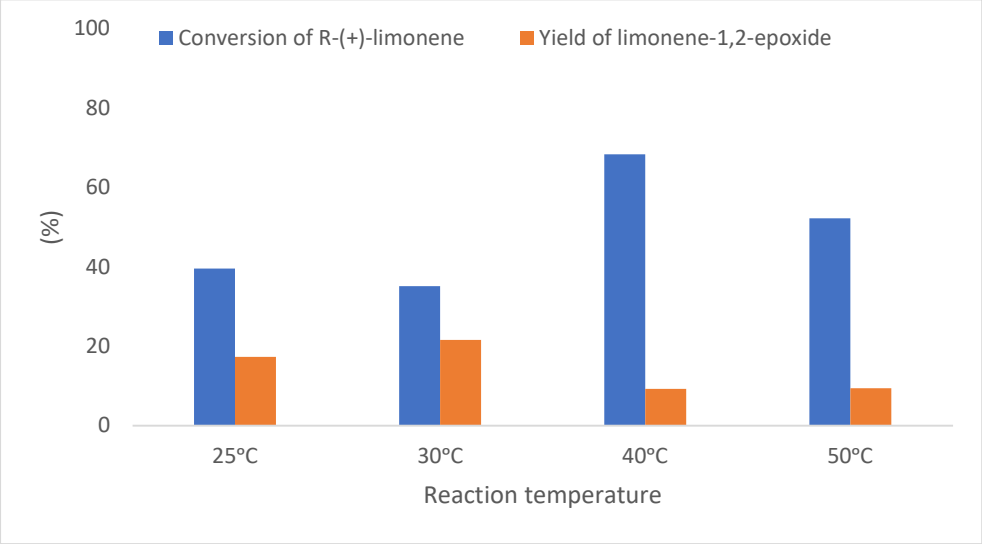


Figure 6. Influence of reaction temperature on samples containing Novozym® 435. (1.6 M R-(+)-limonene, PBS pH 8 0.04 M, 1.60 M octanoic acid, 0.44 mM hydrogen peroxide, and 9.66 mg/mL immobilized lipase. Reaction: 24 h, 25-50 °C, 1000 rpm. Liquid-liquid extraction - 1:1 = sample : ethyl acetate (v/v) → 30 min stirring at room temperature)

At room temperature, the sample containing Novozym® 435 had the most promising results, having a conversion of 40%, and a yield around 17%. The second performance is also for an immobilized lipase in the sample containing Lipozyme® RM IM. This result proves the better efficiency of immobilized enzymes over the lyophilized one. The other two samples had lower performances. The sample without a biocatalyst managed to produce a small amount of limonene oxide, but the sample without hydrogen peroxide did not. This is explained by the role of hydrogen peroxide in our system to oxidize limonene to its corresponding epoxide. Therefore, when no oxidizing agent is present, no epoxide was detected in our extracted sample.

For the next samples set, we modified the reaction temperature with +5 °C, meaning a reaction temperature of 30 °C. From the figures, it can be observed that the values for conversion of limonene and epoxide yield did not visibly increase in the mono-enzymatic samples. As for the previous samples, this set follows the same trend, meaning that the systems with immobilized lipases performed better than the one containing lyophilized lipase from *Aspergillus niger*. From figures 14 and 15, there can be observed that the behavior of the samples without biocatalyst and the one without hydrogen peroxide behave differently than the ones realized at a reaction temperature of 25 °C. In both cases, the conversion of limonene is increased visibly than in the previous set. The most interesting aspect is that the conversion when no oxidizing agent is present is drastically increased, however, the substrate is not

converted into limonene oxide. This means that by increasing the reaction temperature with as low as 5 °C, more R-(+)-limonene tends to transform, but taking into account that without hydrogen peroxide the lipase cannot epoxidize limonene, the reaction is not directed toward our desired product.

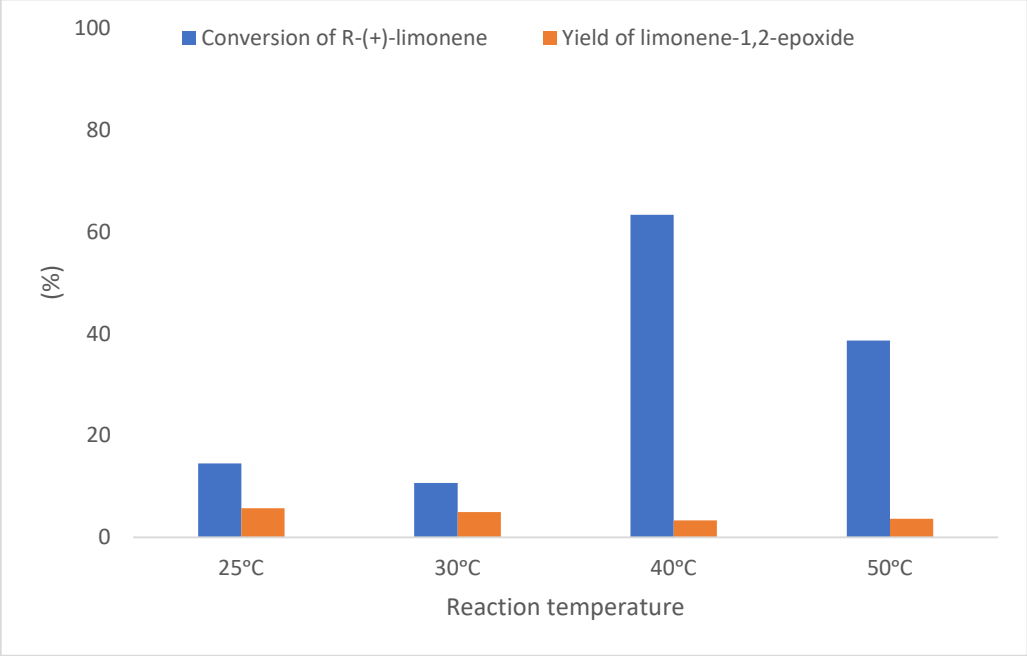


Figure 7. Influence of reaction temperature on samples containing Lipozyme® RM IM. (1.6 M R-(+)-limonene, PBS pH 8 0.04 M, 1.60 M octanoic acid, 0.44 mM hydrogen peroxide, and 9.66 mg/mL immobilized lipase. Reaction: 24 h, 25-50 °C, 1000 rpm. Liquid-liquid extraction - 1:1 = sample : ethyl acetate (v/v) → 30 min stirring at room temperature)

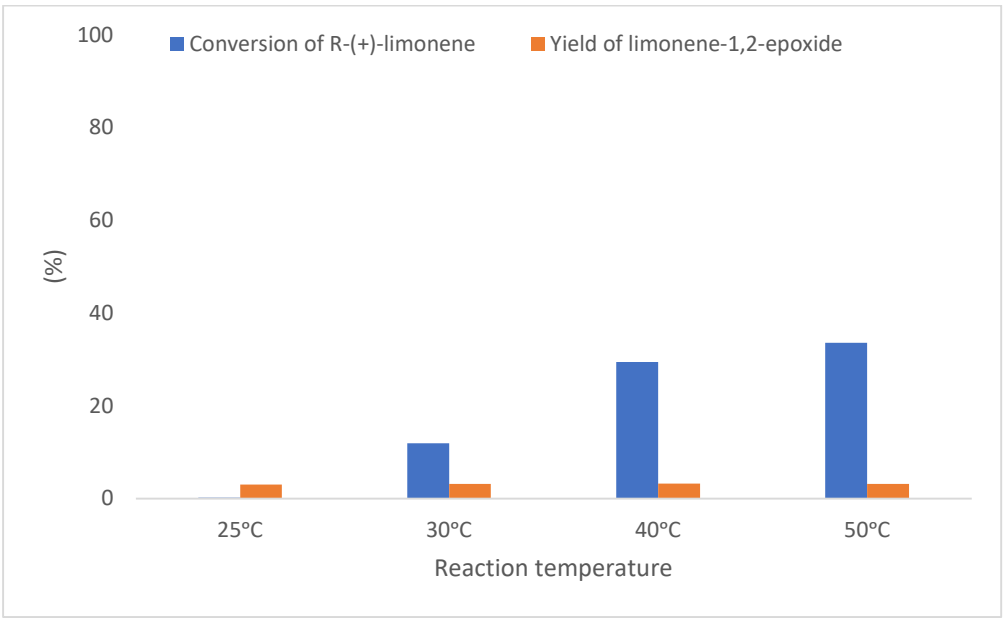


Figure 8. Influence of reaction temperature on samples without biocatalyst. (1.6 M R-(+)-limonene, PBS pH 8 0.04 M, 1.60 M octanoic acid, 0.44 mM hydrogen peroxide. Reaction: 24 h, 25-50 °C, 1000 rpm. Liquid-liquid extraction - 1:1 = sample : ethyl acetate (v/v) → 30 min stirring at room temperature)

For the next samples set, we modified the reaction temperature with +5 °C, meaning a reaction temperature of 30 °C. From the figures, it can be observed that the values for conversion of limonene and epoxide yield did not visibly increase in the mono-enzymatic samples. As for the previous samples, this set follows the same trend, meaning that the systems with immobilized lipases performed better than the one containing lyophilized lipase from *Aspergillus niger*. From figures 14 and 15, there can be observed that the behavior of the samples without biocatalyst and the one without hydrogen peroxide behave differently than the ones realized at a reaction temperature of 25 °C. In both cases, the conversion of limonene is increased visibly than in the previous set. The most interesting aspect is that the conversion when no oxidizing agent is present is drastically increased, however, the substrate is not converted into limonene oxide. This means that by increasing the reaction temperature with as low as 5 °C, more R-(+)-limonene tends to transform, but taking into account that without hydrogen peroxide the lipase cannot epoxidize limonene, the reaction is not directed toward our desired product.

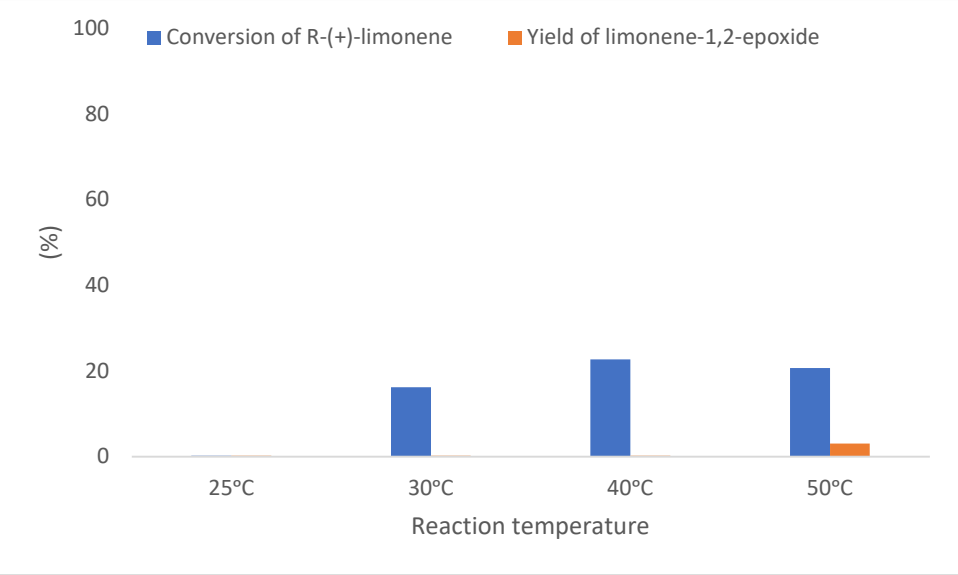


Figure 9. Influence of reaction temperature on samples containing lyophilized lipase from *Aspergillus niger* and no hydrogen peroxide. (1.6 M R-(+)-limonene, PBS pH 8 0.04 M, 1.60 M octanoic acid, and 43.89 µg/mL free lipase. Reaction: 24 h, 25-50 °C, 1000 rpm. Liquid-liquid extraction - 1:1 = sample : ethyl acetate (v/v) → 30 min stirring at room temperature)

The next samples were reacted at a temperature of 40 °C, meaning a modification from the initial temperature with +10 °C. from figures 5 to 9, it can be observed that the efficiency parameters are drastically increased compared to the ones corresponding to a reaction temperature of 25 °C. As for the previous sets, the trending is the same. The samples containing immobilized enzymes had the most promising results.The best conversion degree was obtained for the sample containing Novozym® 435 as biocatalyst and its value is 68%, which is higher than we expected giving the ratio between lipase and substrate, around 250 mg limonene to 11 mg immobilized lipase. This result may be due to a

combination of factors, such as the increased enzymatic activity of the lipase in immobilized form, which was proved by our previous study, and the higher energy input in the system. All the factors combined result in more than half of the substrate quantity being converted. It is observed that the highest amount of epoxide was also obtained in the presence of Novozym® 435. As expected, no limonene oxide was found present in the sample without hydrogen peroxide.

The last sample set was reacted at a temperature of 50 °C. As before, the highest conversion degree and the highest epoxide yield are registered for Novozym® 435. This establish Novozym® 435 as our preferred biocatalyst for the epoxidation of limonene. The sample containing lyophilized lipase from *Aspergillus niger* has a drastic increase in the conversion value. The same can be observed for the sample without biocatalyst and the one without hydrogen peroxide. In all these cases, the increase can be attributed to the high energy input in the samples. Our substrate for those studies, limonene, is not a very stable molecule and this combined with a high reaction temperature may explain the increase in conversion.

In this study, the efficiency of the mono-enzymatic system was registered at different reaction temperatures. Even thou when no enzyme is present there is some epoxide obtained due to the presence of hydrogen peroxide, this result in low limonene oxide obtained and low substrate conversion. When the enzyme is present, but there is no hydrogen peroxide, the biocatalytic process cannot take place.

In table 1 are presented the values obtained from the study on reaction temperature. From conversion values of samples containing lipase from *Aspergillus niger*, it can be observed that as the reaction temperature is increased also does the amount of substrate converted. This is explained by the higher energy input that converts more limonene, because from yield variation is clear that the amount of epoxide does not increase as well.

An interesting aspect is that the maximum conversion degree obtained for a reaction temperature of 50 °C is only 39% which is comparable to the values obtained for Novozym® 435 at lower temperatures of reaction, as it can be observed from table 1. This means that same amount of product can be obtained with a lower energy input.

Table 1 . Values of substrate conversion and epoxide yield from the data presented in figures 5 to 9. (1.6 M R-(+)-limonene, PBS pH 8 0.04 M, 1.60 M octanoic acid, 0.44 mM hydrogen peroxide, and 9.66 mg/mL immobilized lipase or 43.89 µg/mL free lipase. Reaction: 24 h, 25-50 °C, 1000 rpm. Liquid-liquid extraction - 1:1 = sample : ethyl acetate (v/v) → 30 min stirring at room temperature)

	Conversion of R-(+)-limonene (%)				**Yield of limonene oxide (%)**			
	25 °C	*30 °C*	*40 °C*	*50 °C*	*25 °C*	*30 °C*	*40 °C*	*50 °C*
Lyophilised lipase from Aspergillus	11	5	25	39	3	3	3	3

niger								
Novozym® 435	40	35	68	52	17	22	9	9
Lipozyme® RM IM	14	11	63	39	6	5	3	4

Form epoxide yields values of samples containing Novozym® 435, it can be observed that the highest amount of limonene oxide is obtained at a reaction temperature of 30 °C. If the temperature is increased further the amount of epoxide decreases. This is explained by the increase energy input which provides a higher probability of limonene oxide to convert in side-reactions.

The results obtained for the two immobilized lipases is also sustained by the information available on the website of Strem Chemicals. Novozym® 435 has its optimum temperature between 30 and 60 °C, and the pH range between 5 and 9. Lipozyme® RM IM has its optimum parameters between 30 – 50 °C, and pH between 7 and 10. This information supports the obtained results and explain why when the temperature of reaction is 50 °C, the conversion degree when Lipozyme® RM IM is used decreases more drastically than with Novozym® 435.

Overall, the drastic increase in R-(+)-limonene conversion at 40 °C reaction temperature compared to room temperature means that a higher energy input settles the advantages of increasing the reaction temperature. However, this increase comes with a downside, it also increases the possibility of loosing limonene oxide in random side reactions. This could also affect the efficiency of the bienzymatic system. Therefore, for our next quest in the optimization of the mono-enzymatic system, we decided to manage a way of increasing the epoxide yield.

3.1.3. Testing acid-trapping reagents

The variation of reaction temperature showed that an increased energy input increases the conversion degree to as high as 68% for Novozym® 435 at 40 °C, which was the goal of that specific study. However, the limonene oxide yield registered for 40 °C is only 9%, which is not even comparable to the valued obtained at 30 °C of 22%. This drastic decrease in the intermediate yield was expected due to the relatively unstable nature of limonene oxide and the increased reaction temperature. It was inevitable that a higher reaction temperature would increase the probability of side reactions. Therefore, our new quest in optimizing our system was to increase the epoxide yield at 40 °C.

A possible solution to hopefully increase the yield toward the formation of the epoxide and ceasing the cause of the side reactions would be the use of acid-trapping reagents. These are salts of

weak acids which have a double role, to protect the lipase and to neuter the residual acid, in our case the octanoic acid, which might cause the ring-opening of limonene oxide [30].

A study on lipase-mediated epoxidation of α-pinene shows that the corresponding epoxide could be degraded by acidic conditions. The researching team tested the effect of some alkali compounds that would constrain the acidic protons and, therefore, would increase the epoxidation yield. They proposed different acid-trapping reagents, such as trisodium citrate ($Na_3C_6H_5O_7$), sodium bicarbonate ($NaHCO_3$), sodium carbonate (Na_2CO_3), disodium phosphate (Na_2HPO_4). Their reasoning was that trisodium citrate and disodium phosphate are usually components of pH buffers and, therefore, would not have a negative effect on the lipase activity. The other two salts would theoretically be converted in carbon dioxide and water by the acidic media [30].

The study also concluded that the best acid-trapping reagent is trisodium citrate. This one had the best compatibility with the lipase. The other reagents tested, especially the carbonates, proved to be highly alkaline and, therefore, would deactivate the lipase during the reaction resulting in a drastic decrease of the conversion degree [30].

We decided to test the effect of their proposed trapping reagents on our mono-enzymatic system. The samples were prepared, pre-treated, and analyzed using the GC-FID method. The results are presented in figure 10. We tested trisodium citrate ($Na_3C_6H_5O_7$), sodium bicarbonate ($NaHCO_3$), sodium carbonate (Na_2CO_3), and disodium phosphate (Na_2HPO_4).

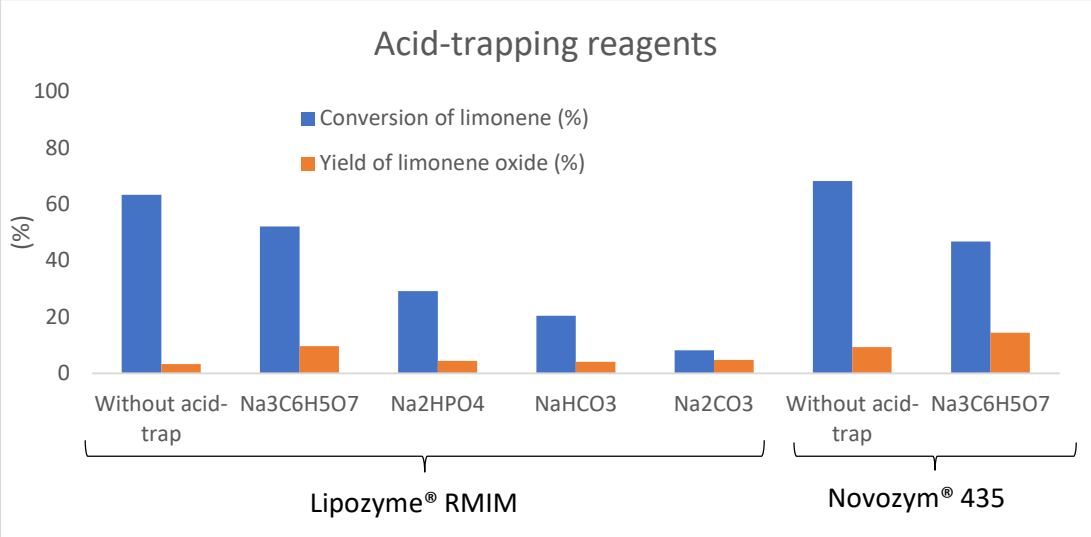


Figure 10. Efficiency parameters for different acid-trapping reagents at 40 °C. (1.6 M R-(+)-limonene, PBS pH 8 0.04 M, 1.60 M octanoic acid, 0.44 mM hydrogen peroxide, 9.66 mg/mL immobilized lipase, and 0.26 M acid-trapping reagent. Reaction: 24 h, 40 °C, 1000 rpm. Liquid-liquid extraction - 1:1 = sample : ethyl acetate (v/v) → 30 min stirring at room temperature)

Right off, it can be clearly observed that samples containing no acid-trap reagent registered the highest R-(+)-limonene conversion. However, the limonene oxide yield is low, as shown previously

when the influence of the reaction temperature was studied. This means that an increased energy input converts a more of the substrate but it also increases the epoxide degradation during the reaction which is undesirable.

In samples containing acid-trapping reagents, the highest conversions were registered for trisodium citrate followed by disodium phosphate which was to be expected due to literature reports on the good compatibility between it and lipases, especially Novozym® 435. The samples containing sodium carbonate registered a drastic decrease in R-(+)-limonene conversion which was caused by its high alkalinity which inactivates the lipase [30].

The epoxide yields registered clearly show that the trisodium citrate managed to preserve the epoxide only when coupled with Novozym® 435. The yield was slightly increased in this case. Trisodium citrate coupled with Lipozyme® RM IM did not affect the epoxide yield in a meaningful way. However, the carbonates and the phosphate not only drastically decreased the conversion, but also the limonene oxide yield, meaning that their action on the lipase activity is undesirable.

Overall, trisodium citrate had the best outcome of all trapping reagents employed in this study managing to preserve the epoxide, and affecting the R-(+)-limonene conversion the least, meaning it has a good compatibility with the lipase. Considering the effect of the on the enzyme activity and limonene oxide yield, we decided to include trisodium citrate in our samples.

3.2. Bienzymatic system

3.2.1. Enzyme screening

From the the previously obtained data, the best reaction temperature is 40 ^{o}C, but the temperature increase would also produce a decrease of limonene oxide yield which was managed to be solved by the addition of trisodium citrate acid-trapping reagent. Therefore, we started the optimization of the bienzymatic process by screening to find the best couple lipase-hydrolase for our system. However, the presence of a second enzyme can affect the behavior of the samples unexpectedly, therefore, samples containing lyophilized lipase from *Aspergillus niger* were prepared as a performance reference. We decided to perform the enzyme screening without the acid-trapping reagent present in the system to avoid any possible side effects of trisodium citrate on the enzymatic activity of the hydrolase.

From the three hydrolases tested, CH55-LEH and Tomsk-LEH are thermophilic. From the data provided by Ferrandi et all [1], the optimum temperature for Re-LEH is around 30 ^{o}C, for Tomsk-LEH is around 40 ^{o}C, and CH55-LEH goes for as far as 60 ^{o}C. We tested the two immobilized lipases with all three hydrolases, however, for lipase from *Aspergillus niger*, we chose only Re-LEH and CH55-

LEH. The reason for our choice was that the optimum temperature for Re-LEH is around room temperature, and we also wanted to compare the performance of CH55-LEH at room temperature, due to its high thermal stability.

The samples were prepared, pre-treated, and analyzed using both GC-FID, and GC*-FID methods. The efficiency parameters are presented in figures 11 and 12 and the efficiency values are in table 2.

In the figure 11 are presented the values for R-(+)-limonene conversion among all combinations between lipases and hydrolases. The samples containing lipases in immobilized form had a reaction temperature of 40 °C because in our previous study at this reaction temperature we recorded the best performance.

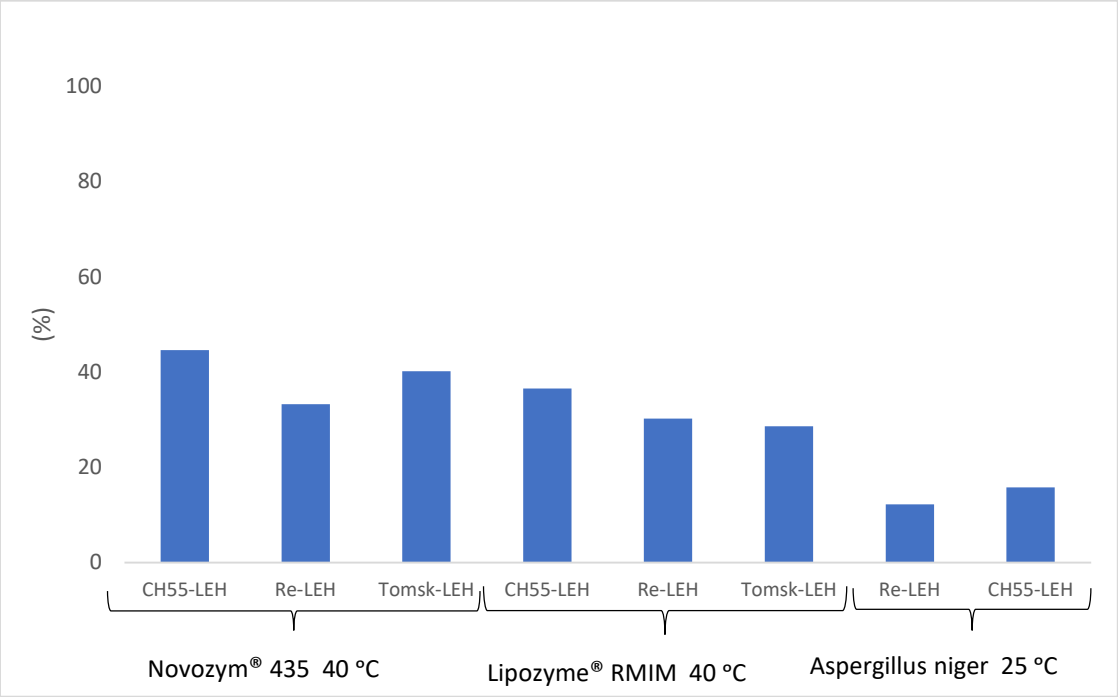


Figure 11. Conversion of R-(+)-limonene among different bienzymatic systems. (1.6 M R-(+)-limonene, PBS pH 8 0.04 M, 1.60 M octanoic acid, 0.44 mM hydrogen peroxide, 43.89 µg/mL free lipase or 9.66 mg/mL immobilized lipase, and 2.81 % v/v hydrolase. Reaction: 24 h, 25 or 40 °C, 1000 rpm. Liquid-liquid extraction - 1:1 = sample : ethyl acetate (v/v) → 30 min stirring at room temperature)

Among the samples containing lipase Novozym® 435, the highest values for substrate conversion were recorded for samples containing thermophilic hydrolases. This result was expected, because CH55-LEH and Tomsk-LEH have the optimum temperature range above room temperature. The difference between the two conversion is only 5%.

The samples containing Lipozyme® RM IM behaved quite different compared to the ones containing Novozym® 435. The conversion decreases from CH55-LEH to Tomsk-LEH, which means that the sample containing Re-LEH performed better than the one containing Tomsk-LEH. However,

the sample containing Re-LEH has a conversion degree only 1% higher than the one containing Tomsk-LEH.

Among samples prepared with lyophilized lipase from *Aspergillus niger*, the one containing CH55-LEH resulted in a higher conversion degree than the one containing Re-LEH. This means that hydrolase CH55-LEH is more active even at room temperature than Re-LEH.

By comparing the samples with the reference couple of lipase and hydrolase, more exactly, lipase from *Aspergillus niger* and hydrolase Re-LEH, every other combination converted more substrate than this initial mixture. The samples containing immobilized lipase is that they performed better in our previous studies. This combined with the increased reaction temperature used for this sample sets may explain the increased conversion degree.

The drastic decrease in the conversion degree of R-(+)-limonene might be attributed to the free hydrolase present in the system. This enzyme might adsorb randomly on the support of the immobilized lipases resulting in blocking the reagents access to the active sites of the lipase.

Overall, the samples containing immobilized enzymes registered a higher conversion degree than the ones containing free enzyme. Between the samples containing Novozym® 435 and the ones containing Lipozyme® RM IM, the first ones performed better. The highest efficiency was recorder in every set for samples containing hydrolase CH55-LEH.

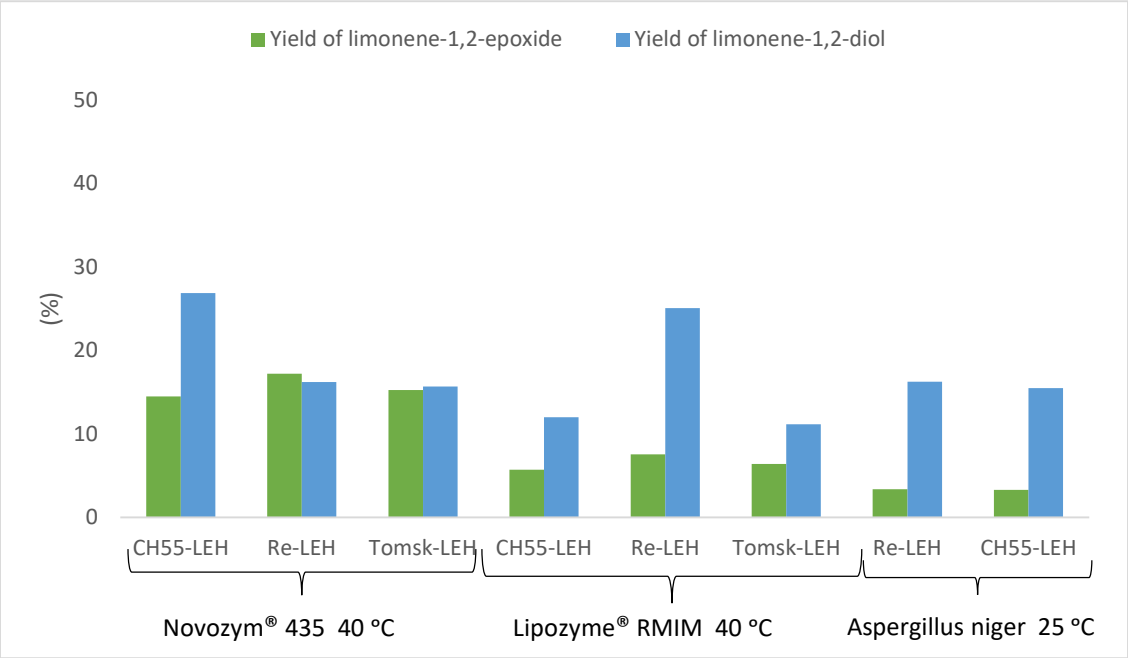


Figure 12. Yield values for limonene-1,2-epoxide and limonene-1,2-diol among different bienzymatic systems. (1.6 M R-(+)-limonene, PBS pH 8 0.04 M, 1.60 M octanoic acid, 0.44 mM hydrogen peroxide, 43.89 µg/mL free lipase or 9.66 mg/mL immobilized lipase, and 2.81 % v/v hydrolase. Reaction: 24 h, 25 or 40 °C, 1000 rpm. Liquid-liquid extraction - 1:1 = sample : ethyl acetate (v/v) → 30 min stirring at room temperature. Diol derivatization - 100 µL extract, 150 µL pyridine, 66 µL acetic anhydride → 24 h, 25 °C, 1000 rpm)

In the figure above are presented the results obtained for epoxide and diol yields. Our ideal bienzymatic system has high yield for limonene-1,2-diol, and small yield value for limonene oxide. However, this ideal case is highly unlikely, because it means that the hydrolase would convert most of the amount of epoxide present in the system in diols. The de-cyclization reaction of epoxide intermediate depends on more factors than the presence of hydrolase. Therefore, our goal for this study is to obtain a bienzymatic system which favors the formation of limonene-1,2-diol.

From figure 12 can be observed that the majority of the bienzymatic systems tested favor the formation of diol products over the formation of epoxide intermediates. In the samples containing lipase from *Aspergillus niger* limonene-1,2-diol is preferentially obtained in both cases. The yield for limonene oxide is as low as 3%, while the yield for diol is around 16%.

However, only the ratio between the two yields is favorable in the *Aspergillus niger* – hydrolase couple, because higher diol yields were obtained in the bienzymatic systems Lipozyme® - Re-LEH, and Novozym® - CH55-LEH. Both yields are around 30%, more exactly, 25% for Lipozyme® RM IM - Re-LEH, and 27% for Novozym® 435 - CH55-LEH. Between these two bienzymatic couples, the one that favors more the formation of limonene-1,2-diol over limonene oxide is Lipozyme® - Re-LEH, which has an epoxide yield of only 8%. The other sample, Novozym® - CH55-LEH has a yield value for epoxide of 14%, almost double.

The other samples containing the couple Novozym® 435 – hydrolase do not favor the formation of either limonene-1,2-diol, nor limonene oxide. Both products have similar values for yields. On the other hand, the other two samples containing Lipozyme® RM IM favor the formation of diol products, but the yield values are lower than in the case when lipase from *Aspergillus niger* was used.

One other factor to give consideration when choosing the best couple lipase – hydrolase is the value of enantiomeric excess. The obtained values are presented below in table 2.

Table 2. Values of enantiomeric excess of (1S, 2S, 4R)-(+)-limonene-1,2-diol of the samples presented in figures 11 and 12. (1.6 M R-(+)-limonene, PBS pH 8 0.04 M, 1.60 M octanoic acid, 0.44 mM hydrogen peroxide, 43.89 μg/mL free lipase or 9.66 mg/mL immobilized lipase, and 2.81 % v/v hydrolase. Reaction: 24 h, 25 or 40 °C, 1000 rpm. Liquid-liquid extraction - 1:1 = sample : ethyl acetate (v/v) → 30 min stirring at room temperature. Diol derivatization - 100 μL extract, 150 μL pyridine, 66 μL acetic anhydride → 24 h, 25 °C, 1000 rpm)

Reaction temperature (°C)	Lipase	Hydrolase	Enantiomeric excess of (1S, 2S, 4R)-(+)-limonene-1,2-diol (ee%)
40	Novozym® 435	CH55-LEH	96
		Tomsk-LEH	89
		Re-LEH	89
	Lipozyme® RM IM	CH55-LEH	88

		Tomsk-LEH	89
		Re-LEH	97
25	Lyophilized lipase from *Aspergillus niger*	CH55-LEH	89
		Re-LEH	91

The values of the enantiomeric excess obtained from our samples are in majority close to 90%, meaning that our systems favor the formation of the S isomer of limonene-1,2-diol. However, just two couples of lipases – hydrolases obtained values closer to 100%, meaning Lipozyme® RM IM - Re-LEH and Novozym® 435 - CH55-LEH, with the values of 97% and 96%, respectively. Besides these those two samples, the rest favors the S isomer as well, however, the combination of lipases with their respective hydrolases are not as close to produce an enantiopure limonene-1,2-diol.

Overall, the systems containing lyophilized lipase from *Aspergillus niger* coupled with either Re-LEH or CH55-LEH, had an enantiomeric excess around 90%, and favored the formation of limonene-1,2-diol over limonene oxide, however, the conversion of limonene substrate was low. From the samples containing immobilized lipases, only Lipozyme® RM IM - Re-LEH and Novozym® 435 - CH55-LEH stood out, because the enantiomeric excess was closer to 100%, and they both favored the formation of diol product while converting higher amounts of limonene.

3.2.2. Development of enzyme co-immobilization-based composite

After the optimization of the enzymatic cascade process for R-(+)-limonene valorization, we decided to test the biocomposite candidates which had the most promising results in our enzyme screening test in the presence of the acid-trapping reagent of choice. For a better comparison, we decided to prepare samples containing both enzymes immobilized, in the form of a biocomposite, and immobilized lipase coupled with free hydrolase. This would provide a better understanding of how the system efficiency is affected in different conditions.

The samples were prepared, pre-treated, and analyzed using both GC-FID, and GC*-FID methods. The analysis was done using GC-FID method for extracted samples, and GC*-FID for the derivatized samples. The efficiency parameters are presented in figure 13 and table 3.

The bienzymatic cascade system in which the immobilized lipases were coupled with free hydrolase registered low conversions degrees, even lower than the systems in which the immobilized lipases were used alone. This drastic decrease cannot be attributed to the presence of trisodium citrate acid-trap, because, as it can be observed in figure 13, the co-immobilized systems did not register a decrease of the conversion degree, but the opposite.

The Lipozyme® RM IM containing system appear to be more affected in both cases, converting drastically low amounts of R-(+)-limonene each time. This might be due to the support choose for the immobilization, because the anionic resign used for this biocatalyst has a tendency of aggregating in liquid medium and this would block some active sites. This effect would explain only the results for the free hydrolase system and its relatively high diol yield registered. For the Lpozyme® RM IM – Re-LEH biocomposite additional testing needs to be done, because there is a possibility that the lipase desorbs during the washing procedure, and therefore the conversion would be highly affected but not the limonene-1,2-diol yield, as it can be observed.

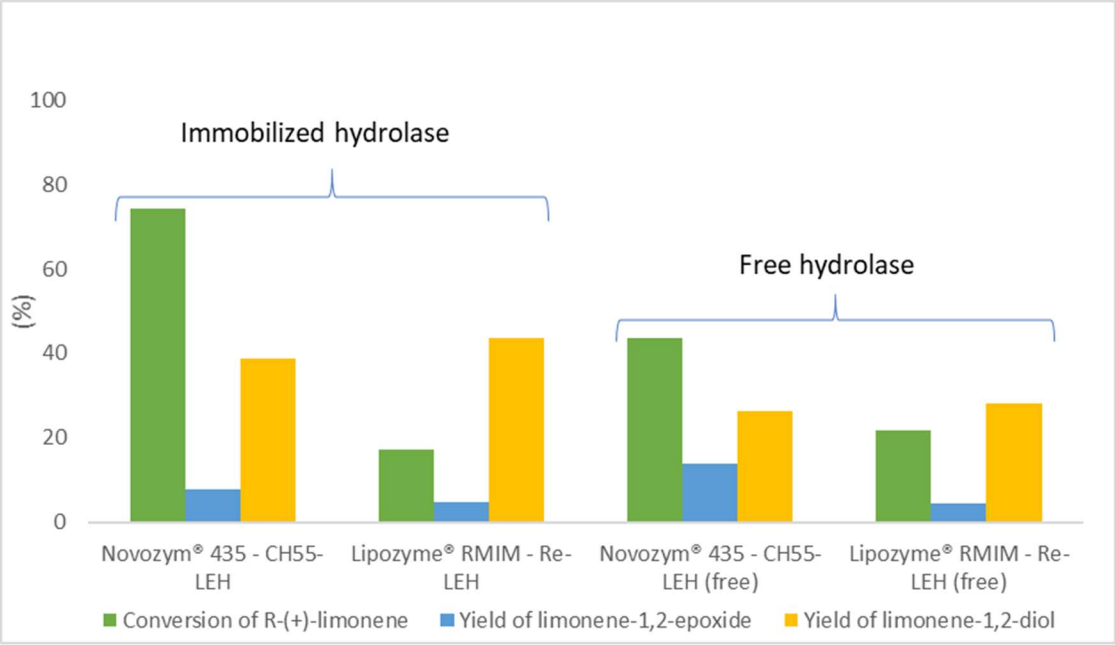


Figure 13. Efficiency parameters for different bienzymatic systems. (1.6 M R-(+)-limonene, PBS pH 8 0.04 M, 1.60 M octanoic acid, 0.44 mM hydrogen peroxide, 9.66 mg/mL immobilized lipase or bienzymatic biocatalyst, 2.81 % v/v hydrolase, and 0.26 M trisodium citrate. Reaction: 24 h, 40 °C, 1000 rpm. Liquid-liquid extraction - 1:1 = sample : ethyl acetate (v/v) → 30 min stirring at room temperature. Diol derivatization - 100 µL extract, 150 µL pyridine, 66 µL acetic anhydride → 24 h, 25 °C, 1000 rpm)

The Novozym® 435 coupled with free CH55-LEH registered low conversion degree and the highest limonene oxide yield among all samples. At first sight, one might be tempted to assume this might be due to the action of the trisodium citrate trapping salt. However, the other samples are not affected in the same way, meaning that there might be another explanation.

By comparing the Novozym® 435 containing samples, it is obvious that the co-immobilization-based composite performed the best. The only difference between the two samples is the state of the hydrolase, free or immobilized. Therefore, we concluded that the reason for the poor performance of the Novozym® 435 coupled with the free CH55-LEH hydrolase system is the tendency of the CH55-LEH to adsorb on the surface of the acrylic support during the reaction. This process taking place

during the reaction time resulted in the blocking of the active sites of both enzymes which explains the low conversion degree and high epoxide yield.

The hydrolase adsorption on support during reaction hypothesis might be applied for the Lipozyme® RM IM coupled with free Re-LEH system. However, if the adsorption would have such an important effect this would lead to an accumulation of epoxide during the reaction, because the hydrolase active site would be blocked by random interactions with the support, and this is not the case. The most affected parameter is the R-(+)-limonene conversion which sustains the aggregation hypothesis previously mentioned.

In table 3 are presented the enantiomeric excess values for all the samples. It can be observed that there is not a high difference between the values for the co-immobilized systems and immobilized lipase coupled with the free hydrolase samples. This means that the hydrolase ability to convert the epoxide to a preferential enantiomer of the limonene-1,2-diol is not affected.

Table 3. Enantiomeric excess of (1S, 2S, 4R)-(+)-limonene-1,2-diol values corresponding to the data from figure 13. (1.6 M R-(+)-limonene, PBS pH 8 0.04 M, 1.60 M octanoic acid, 0.44 mM hydrogen peroxide, 9.66 mg/mL immobilized lipase or bienzymatic biocatalyst, 2.81 % v/v hydrolase, and 0.26 M trisodium citrate. Reaction: 24 h, 40 °C, 1000 rpm. Liquid-liquid extraction - 1:1 = sample : ethyl acetate (v/v) → 30 min stirring at room temperature. Diol derivatization - 100 µL extract, 150 µL pyridine, 66 µL acetic anhydride → 24 h, 25 °C, 1000 rpm)

Biocatalyst	Enantiomeric excess (1S, 2S, 4R)-(+)-limonene-1,2-diol (ee%)
Novozym® 435 – CH55-LEH composite	91
Lipozyme® RM IM – Re-LEH composite	91
Novozym® 435 and free CH55-LEH	89
Lipozyme® RM IM and free Re-LEH	89

The most promising results were obtained from the Novozym® 435 – CH55-LEH biocomposite, which managed to convert around 70% of the R-(+)-limonene present in the initial system. Low amounts of epoxide were found during the analysis, meaning that most of it was converted into the corresponding diol. The limonene-1,2- diol yield was as high as almost 40%, from which 91% is the (1S, 2S, 4R)-limonene-1,2-diol isomer. Therefore, this biocomposite had the most promising results, and was choose for further testing.

3.2.3. Testing of enzyme co-immobilization-based composite

As previously shown, from the two biocomposites developed, meaning Novozym® 435 – CH55-LEH composite and Lipozyme® RM IM – Re-LEH composite, only the first registered promising results. Therefore, we chose to further test only the biocatalyst composed of Novozym® 435 and CH55-LEH co-immobilization for reusability in consecutive reaction cycles. Another possible

application for the developed biocatalyst is the bioconversion of α-phellandrene. For this study we tested both materials, for a better comparison.

The samples were prepared, pre-treated, and analyzed using both GC-FID, and GC*-FID methods. The analysis was done using GC-FID method for extracted samples, and GC*-FID for the derivatized samples. The efficiency parameters are presented in figure 14 and table 4.

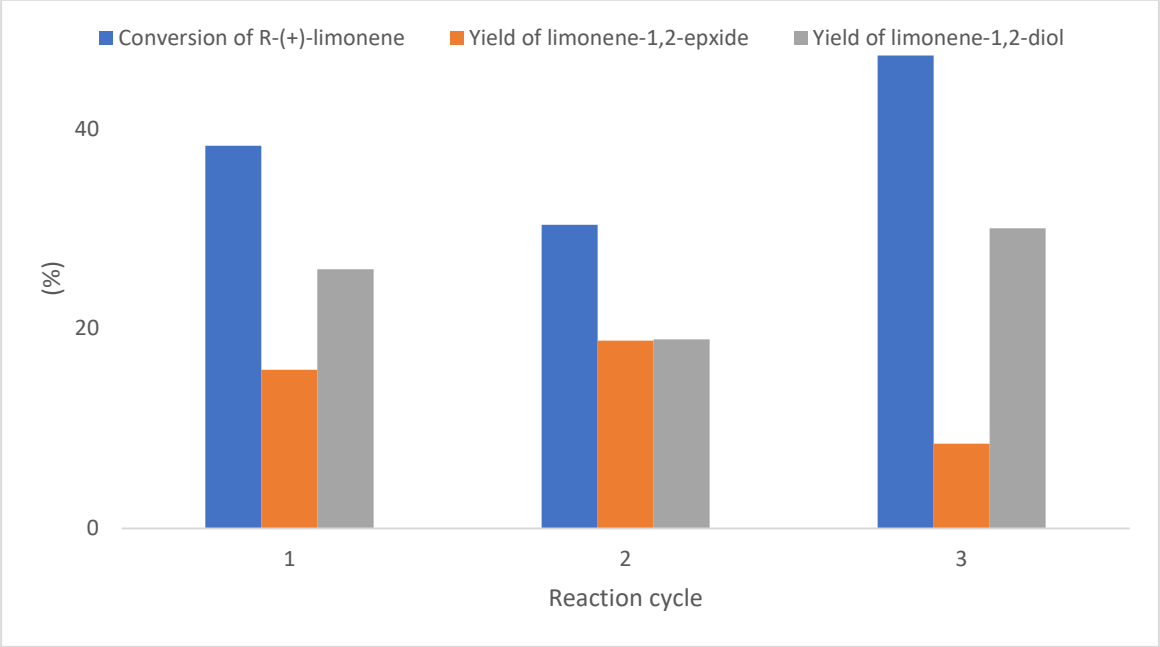


Figure 14. Efficiency parameters of Novozym® 435 – CH55-LEH biocomposite recycling. (1.6 M R-(+)-limonene, PBS pH 8 0.04 M, 1.60 M octanoic acid, 0.44 mM hydrogen peroxide, 9.66 mg/mL immobilized lipase or bienzymatic biocatalyst, and 0.26 M trisodium citrate. Reaction: 24 h, 40 °C, 1000 rpm. Liquid-liquid extraction - 1:1 = sample : ethyl acetate (v/v) → 30 min stirring at room temperature. Diol derivatization - 100 µL extract, 150 µL pyridine, 66 µL acetic anhydride → 24 h, 25 °C, 1000 rpm)

In the figure 14 are presented the efficiency parameters of R-(+)-limonene conversion for the three reaction cycles. The conversion degree varies among the cycles. It registered a decrease of around 8% between the first and second cycle, but it increases with around 17% in the third one. This shows that our material conserved most of its activity among all reaction cycles. The average conversion degree of R-(+)-limonene among all consecutive transformations is 39% ±9, meaning that the biocomposite manages to convert most of the substrate in each process.

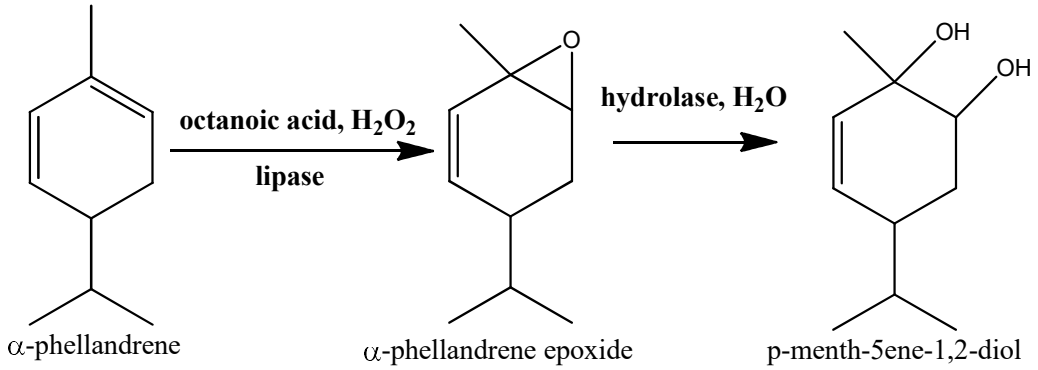
The formation of limonene-1,2-diol is favored over the one of limonene oxide. The diol yield increases in the third cycle compared to the first two. By comparison, the epoxide yield increases from the first cycle to the second, but drastically decreases in the third. However, after each process, the formation of (1S,2S,4R)-limonene-1,2-diol isomer is favored, as it can be observed from the enantiomeric excess of (1S, 2S, 4R)-(+)-limonene-1,2-diol values presented in table 4.

Table 4. Values of the efficiency parameters of Novozym® 435 – CH55-LEH biocomposite recycling presented in figure 14. (1.6 M R-(+)-limonene, PBS pH 8 0.04 M, 1.60 M octanoic acid, 0.44 mM hydrogen peroxide, 9.66 mg/mL bienzymatic biocatalyst, and 2.81 % v/v hydrolase. Reaction: 24 h, 40 °C, 1000 rpm. Liquid-liquid extraction - 1:1 = sample : ethyl acetate (v/v) → 30 min stirring at room temperature. Diol derivatization - 100 µL extract, 150 µL pyridine, 66 µL acetic anhydride → 24 h, 25 °C, 1000 rpm)

Reaction cycle	1st cycle	2nd cycle	3rd cycle	Average ± confidence interval (%)
Conversion of R-(+)-limonene (%)	38	30	47	39±8
Yield of limonene-1,2-epoxide (%)	16	19	8	14±5
Yield of limonene-1,2-diol (%)	26	19	30	25±6
Enantiomeric excess of (1S, 2S, 4R)-(+)-limonene-1,2-diol (ee%)	93	96	95	95±2

The developed biocatalyst was tested for R-(+)-limonene conversion in up to three consecutive reaction cycles. Overall, the material managed to conserve most of its activity after each cycle, the formation of limonene-1,2-diol is favored over the limonene oxide, and the formation of (1S,2S,4R)-limonene-1,2-diol isomer is favored after each cycle.

For another possible application of our developed materials, we tested the bioconversion of α-phellandrene. The process is similarly to the one for R-(+)-limonene and it is presented in scheme 2. The two-step process is based on the epoxidation of one of the double bonds on α-phellandrene cycle with the formation of α-phellandrene epoxide indirectly catalyze by the lipase in the presence of hydrogen peroxide and octanoic acid, followed by the selective hydrolytic ring opening in the presence of the hydrolase.



Scheme 2. Scheme of the α-phellandrene biotransformation.

The sample contents is the same, as previously mentioned, with the only difference being that the R-(+)-limonene is replaced with the same amount of α-phellandrene. For this application we tested both biocomposites Novozym® 435 – CH55-LEH and Lipozyme® RM IM – Re-LEH. The samples were extracted and derivatized. The analysis was done using both GC-FID and GC*-FID methods for a proper quantification of all reaction products.

In table 5 are presented the registered efficiency parameter values for the substrate conversion, and in figure 15 is a graphic representation of the substrate conversion and product yields. It can be observed that both biocatalysts registered promising results in the valorization of a different monoterpene.

In both cases, similar values of the conversion degree of α-phellandrene, α-phellandrene epoxide and p-menth-5-ene-1,2-diol yields were registered, as well as similar enantiomeric excesses for the (1S,2S,4R)-p-menth-5-ene-1,2-diol isomer.

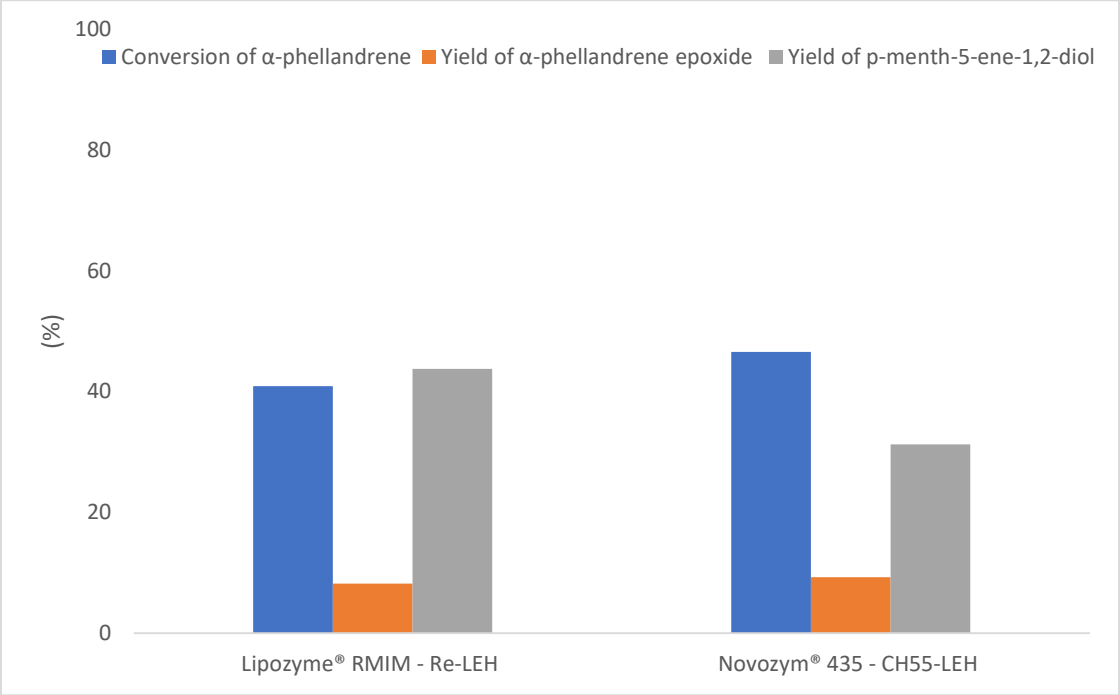


Figure 15. Efficiency parameters of α-phellandrene biotransformation. (1.6 M α-phellandrene, PBS pH 8 0.04 M, 1.60 M octanoic acid, 0.44 mM hydrogen peroxide, 9.66 mg/mL bienzymatic biocatalyst, 0.26 M trisodium citrate. Reaction: 24 h, 40 °C, 1000 rpm. Liquid-liquid extraction - 1:1 = sample : ethyl acetate (v/v) → 30 min stirring at room temperature. Diol derivatization - 100 µL extract, 150 µL pyridine, 66 µL acetic anhydride → 24 h, 25 °C, 1000 rpm)

The formation of p-menth-5-ene-1,2-diol is favored over α-phellandrene epoxide, meaning that most of the converted substrate is then transformed in the desired diol product. Is appears that the biocomposite containing Lipozyme® RM IM lipase managed to yield a higher diol amount compared to the Novozym® 435 containing one, with a difference of around 13%.

The conversion degree is again higher in the case of Lipozyme® RM IM – Re-LEH composite, having a value close to 50%, compared to close to 40% in the case of Novozym® 435 – CH55-LEH composite. However, the overall values presented in table 4 are close to each other, which offers promising perspectives for future applications for α-phellandrene valorization using a bienzymatic cascade process.

Table 5. Values of the efficiency parameters of α-phellandrene biotransformation from figure 15. (1.6 M α-phellandrene, PBS pH 8 0.04 M, 1.60 M octanoic acid, 0.44 mM hydrogen peroxide, 9.66 mg/mL bienzymatic biocatalyst, 0.26 M trisodium citrate. Reaction: 24 h, 40 °C, 1000 rpm. Liquid-liquid extraction - 1:1 = sample : ethyl acetate (v/v) → 30 min stirring at room temperature. Diol derivatization - 100 µL extract, 150 µL pyridine, 66 µL acetic anhydride → 24 h, 25 °C, 1000 rpm)

Biocomposite	Novozym® 435 – CH55-LEH	Lipozyme® RM IM – Re-LEH
Conversion of R-(+)-limonene (%)	41	47
Yield of limonene-1,2-epoxide (%)	9	8
Yield of limonene-1,2-diol (%)	31	44
Enantiomeric excess of (1S,2S,4R)-p-menth-5-ene-1,2-diol (ee%)	96	95

3.3. Correlation between biocatalyst behavior and characteristics

3.3.1. FTIR analysis

The developed materials were characterized using FTIR spectroscopy. The samples were analyzed between 400 and 4000 cm^{-1} and the resulted spectra are presented in figures 16 and 17.

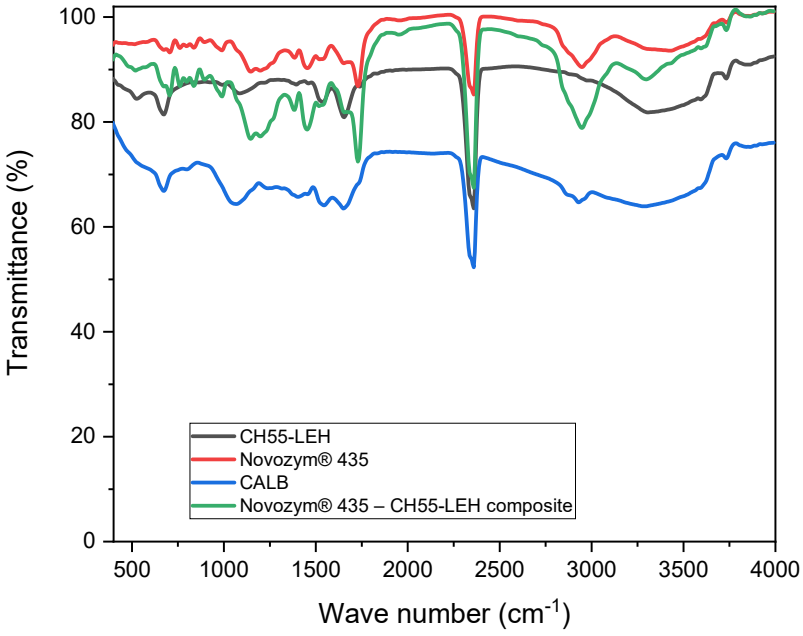


Figure 16. FTIR spectra of free and immobilized lipase and hydrolase components of Novozym® 435 – CH55-LEH composite.

A comparative FTIR analysis of the enzymes before and after immobilization provides significant information about the effect of the immobilization on the protein secondary structure. Due

to the polypeptide and protein repeating units which compose the backbone of enzymes, there are nine characteristic IR bands arising from this type of structure, meaning amide A and B and I to VII. From all these bands, only amide I and amide II have the most prominent vibrations. However, the most sensitive to the protein secondary structure changes is amide I between 1600 and 1700 cm^{-1} [31], and this would be our band of interest.

In figure 16 is presented the FTIR spectra of Novozym® 435 – CH55-LEH composite along with free CALB, bare Novozym® 435 and free CH55-LEH. Right off, the band registered between 2300 and 2400 cm^{-1} corresponds to the asymmetric stretching mode of CO_2 [32], meaning it is an interference and not a part of our materials. The same CO_2 characteristic vibration can be observed in the figure 17. However, due to the protein nature of our samples, we would be focusing only on the amide I region of our samples.

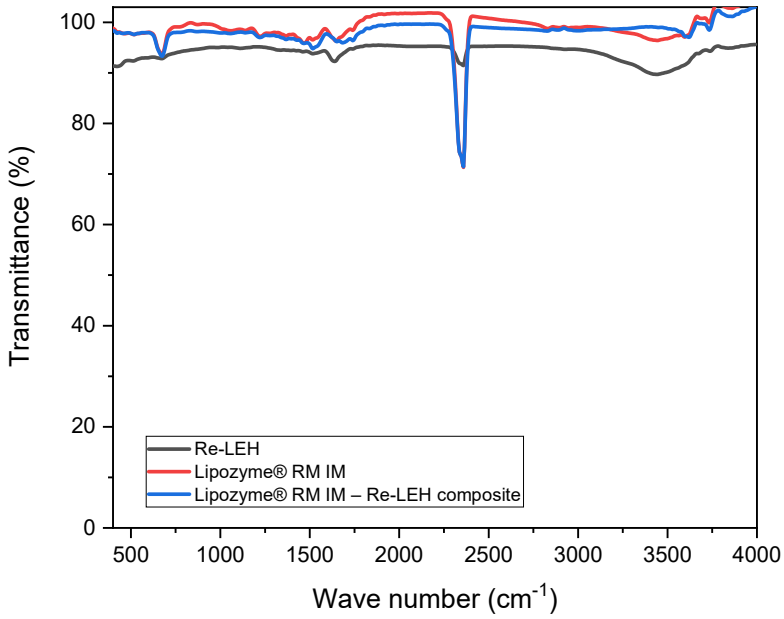


Figure 17. FTIR spectra of free and immobilized lipase and hydrolase components of Lipozyme® RM IM – Re-LEH composite.

By comparison between figure 16 and figure 17, it can be observed that the IR amide I bands of Lipozyme® RM IM – Re-LEH composite and its components is less intense than the ones of Novozym® 435 – CH55-LEH composite and its components. The spectra from figure 16 have bands intense enough that it can be observed the presence of one in the 1000-1200 cm^{-1} region, which corresponds to carbohydrate content and glycoform distribution of protein [33]. This band is visible for

free lipase B from *Candida antarctica*, bare Novozym® 435 and our biocatalyst, being more intense for the lipase than for the CH55-LEH hydrolase.

By comparison between figure 16 and figure 17, it can be observed that the IR amide I bands of Lipozyme® RM IM – Re-LEH composite and its components is less intense than the ones of Novozym® 435 – CH55-LEH composite and its components. The spectra from figure 16 have bands intense enough that it can be observed the presence of one in the 1000-1200 cm^{-1} region, which corresponds to carbohydrate content and glycoform distribution of protein [33]. This band is visible for free lipase B from *Candida antarctica*, bare Novozym® 435 and our biocatalyst, being more intense for the lipase than for the CH55-LEH hydrolase.

Due to the more intense spectra and the fact that we managed to analyze both free lipase B from *Candida antarctica* and CH55-LEH hydrolase, we chose to focus on the characterization of the amide I region of the Novozym® 435 – CH55-LEH composite.

3.3.2. Deconvolution of FTIR data

Amide I band of the FTIR spectra of proteins is the most sensitive region to modifications in the secondary structure. This band is situated between 1600 and 1700 cm^{-1}. Its apparition is mostly caused by the stretching vibration of C=O of the peptide chain, in proportion of 80%. The rest of around 20% is due to the stretching of C-N bond. The exact position of this band is correlated to the backbone conformation of the enzyme and the pattern of hydrogen bonds [31].

The structural component of amide I band are scattered within its region. We choose the following spectral regions of the components from literature data, β-sheets between 1610-1640 cm^{-1}, α-helix between 1640-1660 cm^{-1}, turns between 1660-1690 cm^{-1}. The deconvolution of FTIR data results in a semi-quantitative analysis, giving the percentage of each component [33].

The deconvoluted bands of the graph from figure 16 are presented in the supplementary information in figures K to N. In table 6 are presented the wave numbers of the deconvoluted elements of the amide I region of each enzyme, whereas in table 7 are the percentages of each component.

Table 6. Wave numbers corresponding to the structural elements of amide I region of the developed biocatalyst.

	Wave number (cm^{-1})		
	β-sheet	**α-helix**	**turns**
Free CH55-LEH	1627	1650	1674
Free CALB	1616	1648	1681
Novozym® 435	1603	1649	1678

Novozym® 435 – CH55-LEH biocomposite	1603	1649	1678

From the component positions, it can be observed that the lipase B from *Candida antarctica* records a shift in the wave numbers of components bands after immobilization in the form of Novozym® 435. This means that the adsorption on the acrylic support caused small conformational changes in the secondary structure of the lipase.

The wave numbers of the components are the same in Novozym® 435 and our biocatalyst. Due to the small ratio between lipase and hydrolase used for our system, the CH55-LEH hydrolase might be present in such a small amount that the band positions are not visibly affected. However, the percentage of each component is changed in the final biocatalyst, as seen in table 7.

The free CH55-LEH hydrolase has a preponderant percentage of turns elements and a more of an α-helix structure than β-sheets, but the difference is around 8% between the last two. On the other hand, the free CALB has a higher preference toward the α-helix structure. In the bare Novozym® 435, CALB registers an increase of β-sheet conformation than in the free form, as well as a decrease in the turn elements.

By comparing bare Novozym® 435 with Novozym® 435 – CH55-LEH composite, our material registers a decrease in β-sheet and α-helix elements and an increase of the turn elements. This means that the adsorption of the CH55-LEH hydrolase on the surface of the acrylic support already containing the immobilized lipase determined a change in the conformational structure in the final material.

Table 7. Structural elements of amide I region of the developed biocatalyst.

	β-sheet (%)	**α-helix (%)**	**turns (%)**
Spectral range (cm^{-1})	*1610-1640*	*1640-1660*	*1660-1690*
Free CH55-LEH	24	32	44
Free CALB	8	57	35
Novozym® 435	23	60	17
Novozym® 435 – CH55-LEH biocomposite	17	49	34

The Novozym® 435 – CH55-LEH composite maintained the preference for α-helix conformation of CALB due to its abundance in the final material, but also registered an increase in the proportion of turn elements from the adsorption of the hydrolase.

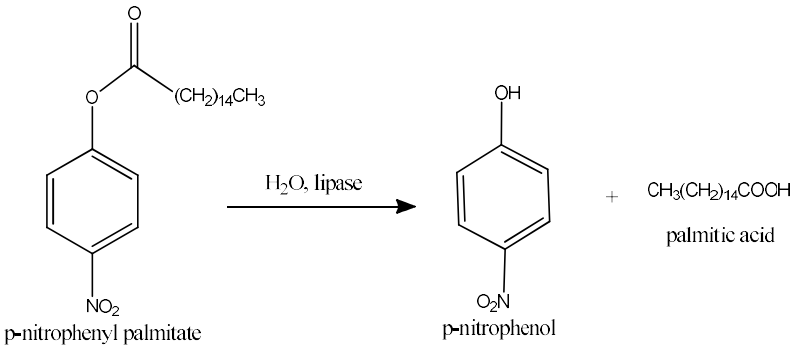
3.3.3. Enzyme loading

The enzyme loading was determined only for the Novozym® 435 – CH55-LEH composite, giving the confirmation of the hydrolase immobilization by the FTIR analysis. The percentage of loading was determined by UV-VIS measurement at 280 nm, because at this wavelength absorb two aromatic amino acids, tryptophan (Trp) and tyrosine (Tyr) and, to a small extent, cystine of the disulfide bonds of the protein tertiary structure [34].

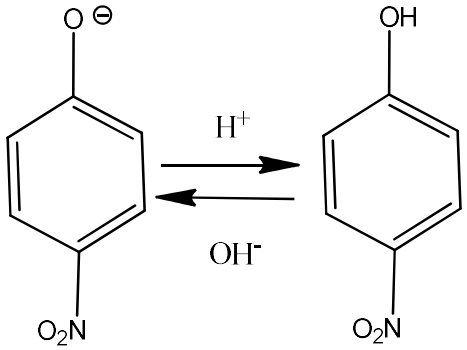
The loading percentage was determined by the protein ratio of an enzymatic solution before immobilization and the washing solutions collected after immobilization. The initial solution registered a concentration of 0.29 mg/mL, and 0.23 mg/mL is the protein concentration after immobilization. From this determination, it resulted that 21% of CH55-LEH hydrolase adsorbed on the surface of the Novozym® 435 beads.

3.3.4. Enzymatic activity

The determination of the enzymatic activity is based on the reaction between the lipase and p-nitrophenyl palmitate. The substrate is converted by the lipase in presence of water to form palmitic acid and p-nitrophenol. The reaction is presented in scheme 3.



Scheme 3. Reaction scheme for determination of lipase enzymatic activity.



Scheme 4. Ionized and non-ionized forms of p-nitrophenol [35].

For the determination of the enzymatic activity, the amount of p-nitrophenol is measured. The quantity of the product detected after the ceasing of the reaction is proportional with the activity of the enzyme. The enzymatic activity of Novozym® 435 – CH55-LEH composite was determined before washing, after each washing step, and after each one, two and three consecutive reaction cycles. The activity of Lipozyme® RM IM – Re-LEH composite was determine before washing, after each of the tree washing steps, as well as after one reaction cycle.

It is worth mentioning, that the calibration curve for p-nitrophenol was constructed at 347 nm where the non-ionized form is preponderant due to the acidic environment, whereas in our samples the

ionized form is in higher amounts due to the relatively neuter pH, and it has an adsorption peak at 410 nm [35]. The equilibrium between the ionized and non-ionized form is presented in scheme 5.

Before the determination of the enzymatic activity of our materials, UV-VIS absorption spectra were registered between 200 and 400 nm of an enzymatic solution of Re-LEH hydrolase. The resulted graph is presented in the supplementary information (figure H). The absorption spectra of the hydrolase has a peak at 280 nm, corresponding to aromatic aminoacids in its composition [34]. In figures 18 to 19 are presented the near-UV absorption spectra of the washing solutions of immobilized lipase, as well as the ones of the resulting biocoposite.

Both Novozym® 435 – CH55-LEH and Lipozyme® RM IM – Re-LEH composites washing solutions have a maximum absorption at 258 nm, which corresponds as well to aromatic aminoacids within the protein chain [36]. However, the absorption peak does not correspond to the one of the hydrolase enzymatic solution. Therefore, there might be a possibility of lipase desorption during the washing procedure.

In figures 18 and 19 are presented the near-UV absorption graphs of bare Novozym® 435 and Lipozyme® RM IM washing solutions. The procedure was the same as the one performed on the developed biocatalysts, the only difference being that this time it was performed on bare immobilized lipases. Because the immobilization approach used for the enzymes was based on adsorption on support, it was to be expected that some enzyme would desorb due to weak interaction between the protein and support material [11].

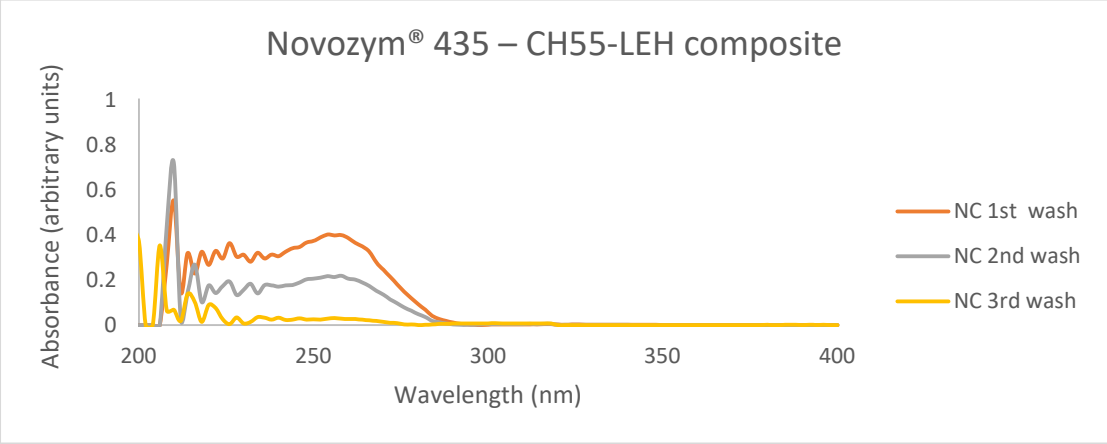


Figure 18. Near-UV absorption spectra of washing solutions of Novozym® 435 – CH55-LEH composite.

From the graph in figure 19 it is clear the peak detected at 258 nm corresponds to some lipase being desorbed during the washing step. It is worth mentioning that the highest amount of lipase CALB desorbs during the first washing step and around the third step the amount becomes negligible in both

cases. The same happens for the Lipozyme® RM IM – Re-LEH composite, the absorption decreases with each washing, but in the third washing solution there is still some non-negligible amount of lipase. the near-UV absorption spectra are in the supplementary information (figures I and J).

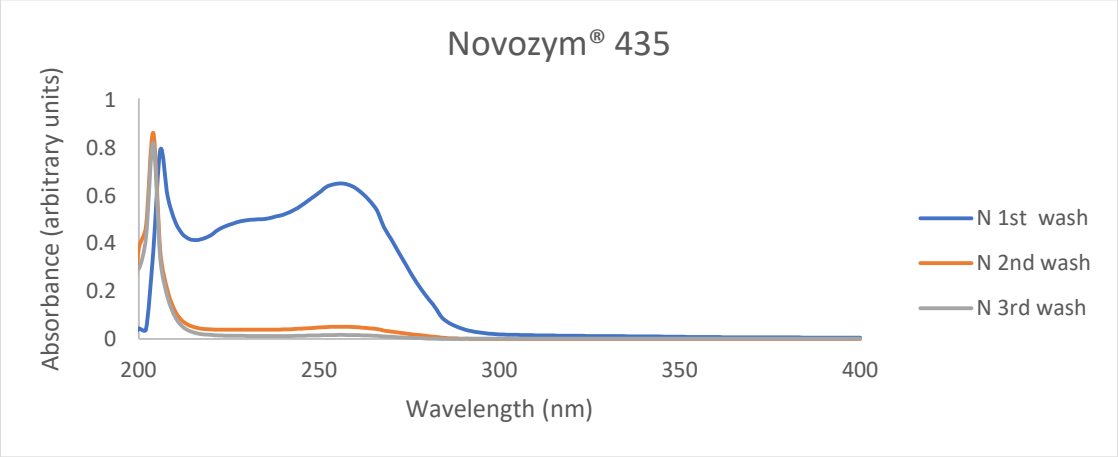


Figure 19. Near-UV absorption spectra of washing solutions of Novozym® 435.

Concluding that the peak detected corresponds to the lipase and not the hydrolase, the enzymatic activity was determined for the Novozym® 435 – CH55-LEH composite before washing, after each washing step, and after each of the three consecutive reaction cycles. The results for both composites are presented in table 8.

Table 8. Enzymatic activity values for the developed biocatalysts.

Novozym® 435 – CH55-LEH composite (washed with PBS pH8)	**Enzymatic activity (µM p-NP/min/mg ptotein)**	**Lipozyme® RM IM – Re-LEH composites (washed with PBS pH 8)**	**Enzymatic activity (µM p-NP/min/mg ptotein)**	**Lipozyme® RM IM – Re-LEH composites (washed with PBS pH 6)**	**Enzymatic activity (µM p-NP/min/mg ptotein)**
Before washing	0.8	*Before washing*	4.2	*Before washing*	9.8
After 1st wash	0.7	*After 1st wash*	6.4	*After 1st wash*	7.5
After 2nd wash	0.7	*After 2nd wash*	4.0	*After 2nd wash*	3.9
After 3rd wash	0.8	*After 3rd wash*	2.7	*After 3rd wash*	2.5
After 1st cycle	0.7	*1st washing solution*	12.0	*1st washing solution*	4.7
After 2nd cycle	0.8	*2nd washing solution*	6.6	*2nd washing solution*	1.6
After 3rd cycle	0.7	*3rd washing solution*	1.5	*3rd washing solution*	0.8
		After 1st cycle	1.5		

For the Novozym® 435 – CH55-LEH composite the activity is maintained constant even after three consecutive reaction cycle. The biocatalyst manages to conserve more than 90% of its initial

activity after each reaction cycle. The leaching of the lipase during the washing procedure is negligible, the activity of the biocatalyst is not significantly affected, and the enzymatic activity value detected for the washing solutions is below 0.003 arbitrary units. Therefore, the desorption of the lipase is not a problem for the Novozym® 435 containing composite.

Table 8 results clearly show that the lipase leaching is a major problem of the Lipozyme® RM IM – Re-LEH composite. The material was washed with PBS having a slightly basic or slightly acidic pH to determine if this would influence the amount of enzyme desorbed during the washing procedure. In both cases significant enzymatic activity is lost during the washing. Using a more acidic/ basic pH for the washing solution is not an option because if could lead to the hydrolase deactivation prior to reaction and would not prevent the lipase leaching in the reaction environment. After the first reaction cycle, the biocatalyst manages to conserve only 53% of the enzymatic activity at the end of the washing procedure.

The enzymatic activity of an enzymatic solution of CH55-LEH (1.81 mg/mL) before the immobilization is 11.14 IU. IU are expressed as μmols of substrate per minute per mL of enzymatic solution. The value after immobilization is 53.06 IU. This increased value represents the enzymatic activity apparently induced by the presence of the lipase in the co-immobilized bienzymatic biocatalyst.

Conclusions

In conclusion, we developed a biocatalyst based on enzyme co-immobilization composed of the couple CH55-LEH hydrolase and Novozym® 435, which is commercially available lipase B from *Candida antarctica*. The immobilization approach is based on the adsorption of the hydrolase on the support surface of Novozym® 435 already containing the lipase in immobilized form.

Adsorption approach in immobilization, even if it is the cheapest and easiest method, comes with disadvantages, such as weak enzyme-support interaction which would result in the enzyme randomly desorbing from the surface. However, our enzymatic activity and near-UV absorption studies show a good stability of both CALB and CH55-LEH on the surface of the acrylic polymer beads.

The biocatalyst had a hydrolase loading of 21% and was characterized using FTIR analysis. The deconvoluted amide I region of the Novozym® 435 – CH55-LEH composite registered a change in the conformation of the secondary structure of the proteins which was different compared to bare Novozym® 435 beads. The absorption bands that the co-immobilization of the CH55-LEH hydrolase did not cause major conformational changes which might have resulted in the loss of enzymatic activity.

Our developed biocatalyst showed outstanding results in R-(+)-limonene conversion to (+)-limonene-1,2-diol, the system even favors the formation of the (1S, 2S, 4R)-(+)-limonene-1,2-diol isomer. Our Novozym® 435 – CH55-LEH composite managed to convert over 60% more substrate than Novozym® 435 alone or coupled with free CH55-LEH hydrolase.

The biocatalyst reusability was tested for up to three reaction cycles. After each cycle the material managed to conserve more than 90% of its initial activity. This proposes promising results for potential industrial uses of the enzyme co-immobilization-based composite.

As another possible application for the developed Novozym® 435 – CH55-LEH composite is the bioconversion of α-phellandrene to its corresponding vicinal diol. This opens the possibility of applications of our developed biocatalyst for valorization of various monoterpenes candidates.

The results obtained from our studies propose great future perspectives for developing enzyme co-immobilization-based biocatalysts for cascade valorization of monoterpenes to flavor and fragrance products with possible applications for industrial processes.

Acknowledgments

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Bibliography

[1] Sheldon, R.A, Woodley, J.M., Role of Biocatalysis in Sustainable Chemistry. Chem. Rev., 118 (2018) 801:838.

[2] Feher, J., Quantitative Human Physiology, Academic Press, 2017, Chapter 2. Protein Structure, pgs. 13:140.

[3] Faber, K., Biotransformations in Organic Chemistry. A Textbook. Sixth revised and corrected edition, Springer, 2011, Chapter 1. Introduction and Background Information: Enzyme Properties and Nomenclature, pgs. 11: 27.

[4] Chapman, J., Ismail, A.E., Dinu, C.Z., Industrial applications of enzymes: Recent advances, techniques, and outlooks. Catalysts, 8 (2018) 20:29.

[5] Renge, V.C., Khedkar, S.V., Nandurkar, N. R., Enzyme Synthesis By Fermentation Method: a Review. SRCC, 2 (2012) 585:590.

[6] Lin, B., Tao, Y., Whole-cell biocatalysts by design. Microb. Cell Fact., 16 (2017) 1:12.

[7] Pera, L.M., Romero, C.M., Baigori, M.D., Castro, G.R., Catalytic properties of lipase extracts from Aspergillus niger. Food Technol. Biotechnol., 44 (2006) 247:252.

[8] Kundys, A., Białecka-Florjańczyk, E., Fabiszewska, A., Małajowicz, J., Candida antarctica Lipase B as Catalyst for Cyclic Esters Synthesis, Their Polymerization and Degradation of Aliphatic Polyesters. J. Polym. Environ., 26 (2018) 396:407.

[9] Bassanini, I., Ferrandi, E.E., Monti, D., Riva, S., Studies on the Catalytic Promiscuity of Limonene Epoxide Hydrolases in the Non-hydrolytic Ring Opening of 1,2-Epoxides. ChemBioChem, 21 (2020) 1868:1874.

[10] Ferrandi, E.E., Sayer, C., Isupov, M.N., Annovazzi, C., Marchesi, C., Iacobone, G., Peng, X., Bonch-Osmolovskaya, E., Wohlgemuth, R., Littlechild, J.A., Monti, D., Discovery and characterization of thermophilic limonene-1,2-epoxide hydrolases from hot spring metagenomic libraries. FEBS J., 282 (2015) 2879:2894.

[11] Sheldon, R.A., Enzyme immobilization: The quest for optimum performance. Adv. Synth. Catal., 349 (2007) 1289:1307.

[12] Fernandez-Lafuente, R., Armisén, P., Sabuquillo, P., Fernández-Lorente, G., Guisán, J. M., Immobilization of lipases by selective adsorption on hydrophobic supports. Chem. Phys. Lipids, 93 (1998) 185:197.

[13] Homaei, A.A., Sariri, R., Vianello, F., Stevanato, R., Enzyme immobilization: An update. J. Chem. Biol., 6 (2013) 185:205.

[14] Jesionowski, T., Zdarta, J., Krajewska, B., Enzyme immobilization by adsorption: A review. Adsorption, 20 (2014) 801:821.

[15] Sheldon, R.A., Cross-Linked Enzyme Aggregates as Industrial Biocatalysts. Pharm. Process Chem., 213 (2010) 159:181.

[16] Ricca, E., Brucher, B., Schrittwieser, J. H., Multi-enzymatic cascade reactions: Overview and perspectives. Adv. Synth. Catal., 353 (2011) 2239:2262.

[17] Hwang, E.T., Lee, S., Multienzymatic Cascade Reactions via Enzyme Complex by Immobilization. ACS Catal., 9 (2019) 4402:4425.

[18] Enoki, J., Meisborn, J., Müller, A.C., Kourist, R., A multi-enzymatic cascade reaction for the stereoselective production of γ-oxyfunctionalyzed amino acids. Front. Microbiol., 7 (2016) 1:8.

[19] Schrittwieser, J.H., Velikogne, S., Hall, M., Kroutil, W., Artificial Biocatalytic Linear Cascades for Preparation of Organic Molecules. Chem. Rev., 118 (2018) 270:348.

[20] Sisak, C., Nagy, E., Burfeind, J., Schügerl, K., Technical aspects of separation and simultaneous enzymatic reaction in multiphase enzyme membrane reactors. Bioprocess Eng., 23 (2000) 503:512.

[21] Martins, A.R., Monteiro, R.L., Fernandes de Medeiros Burkert, J., Burkert, A.V.C., Simultaneous enzymatic hydrolysis and lactic fermentation to obtain a yogurt with low lactose content. Cienc. agrotec., 36 (2012) 551:559.

[22] Marmulla, R., Harder, J., Microbial monoterpene transformations-a review. Front. Microbiol., 5 (2014) 1:14.

[23] De Carvalho, C.C.C., Da Fonseca, M.M.R., Biotransformation of terpenes. Biotechnol. Adv., 24 (2006) 134:142.

[24] Sales, A., Pastore, G.M., Bicas, J.L., Biotransformation of limonene to limonene-1,2-diol by two Colletotrichum species. Process Biochem., 86 (2019) 25:31.

[25] Çorbacı, C., Biotransformation of terpene and terpenoid derivatives by Aspergillus niger NRRL. 326, Biologia, 75 (2020) 1473:1481.

[26] Sales, A., Afonso, L.F., Americo, J.A., Rebelo, M.F., Pastore, G.M., Bicas, J.L., Monoterpene biotransformation by Colletotrichum species. 40 (2018) 561:567.

[27] Işcan, G., Kirimer, N., Demirci, F., Demirci, B., Noma, Y., Başer, K.H.C., Biotransformation of (-)-(R)-α-phellandrene: Antimicrobial activity of its major metabolite. Chem. Biodivers., 9 (2012) 1525:1532.

[28] Wiemann, L.O., Faltl, C., Sieber, V., Lipase-mediated epoxidation of the cyclic monoterpene limonene to limonene oxide and limonene dioxide. Z. Naturforsch., 67 (2012) 1056:1060.

[29] Aouf, C., Durand, E., Lecomte, J., Figueroa-Espinoza, M.C., Dubreucq, E., Fulcrand, H., Villeneuve, P., The use of lipases as biocatalysts for the epoxidation of fatty acids and phenolic compounds. Green Chem., 16 (2014) 1740:1754.

[30] Su, W., Li, Q., Liu, Y., Qin, Y., Liu, H., Tang, A., Improved efficiency of lipase-mediated epoxidation of α-pinene using H2O2 in single- phase systems. Molecular Catalysis, 508 (2021) 579:585.

[31] Kong, J., Yu, S., Fourier transform infrared spectroscopic analysis of protein secondary structures. Acta Biochim. Biophys. Sin., 39 (2007) 549:559.

[32] Kauffman, K.L., Culp, J.T., Goodman, A., Matranga, C., FT-IR study of CO2 adsorption in a dynamic copper(II) benzoate-pyrazine host with CO2-CO2 interactions in the adsorbed state. J. Phys. Chem. C, 115 (2011) 1857:1866.

[33] Tudorache, M., Gheorghe, A., Viana, A.S., Parvulescu, V.I., Biocatalytic epoxidation of α-pinene to oxy-derivatives over cross-linked lipase aggregates. J. Mol. Catal. B Enzym., 134 (2016) 9:15.

[34] Pathak, M., Mishra, R., Agarwala, P.K., Ojha, H., Singh, B., Singh, A., Kukreti, S., Binding of ethyl pyruvate to bovine serum albumin: Calorimetric, spectroscopic and molecular docking studies, Thermochim. Acta, 633 (2016) 140:148.

[35] Peng, Y., Fu, S., Liu, H., Lucia, L.A, Determining esterase activity. BioResources, 11 (2016) 10099:10111.

[36] Schmid, F.X., Biological Macromolecules: UV-visible Spectrophotometry. Encycl. Life Sci., (2001) 1:4.

SUPPLEMENTARY INFORMATION

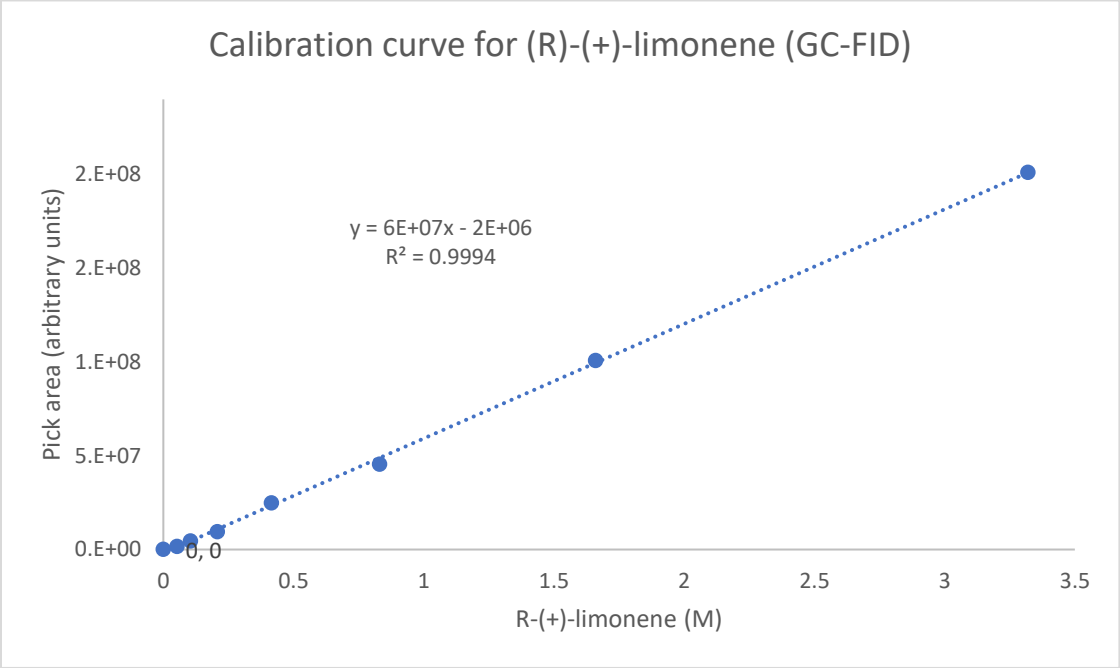


Figure A. Calibration curve for R-(+)-limonene in ethyl acetate analyzed using GC-FID.

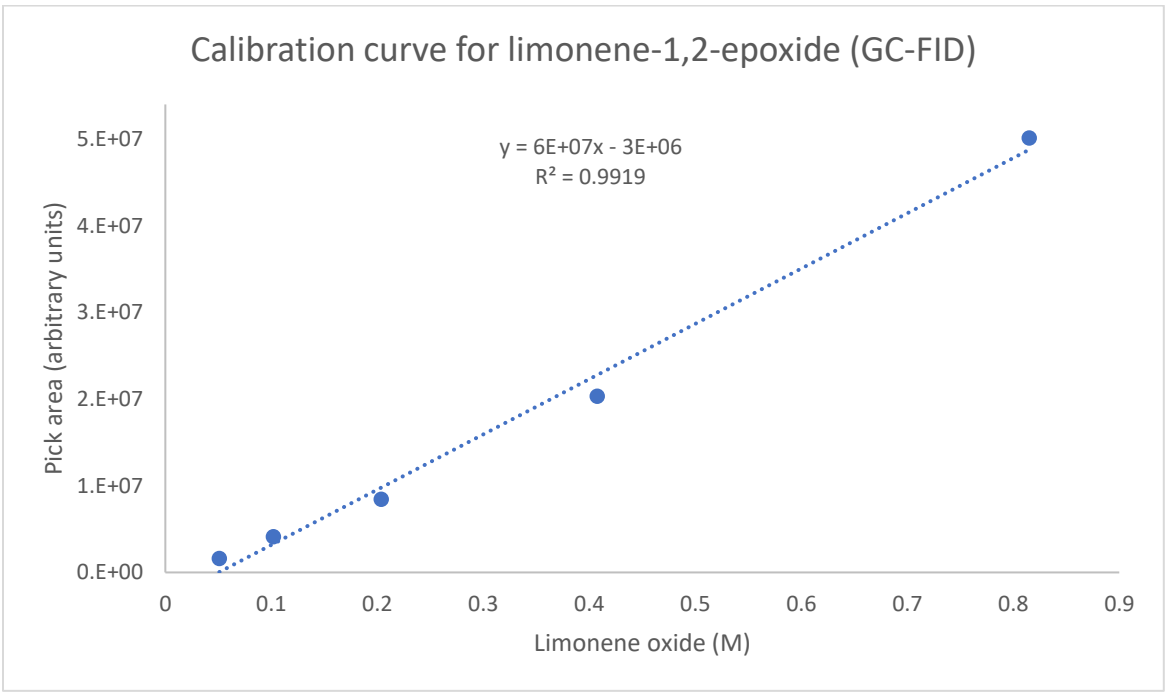


Figure B. Calibration curve for limonene oxide in ethyl acetate analyzed using GC-FID.

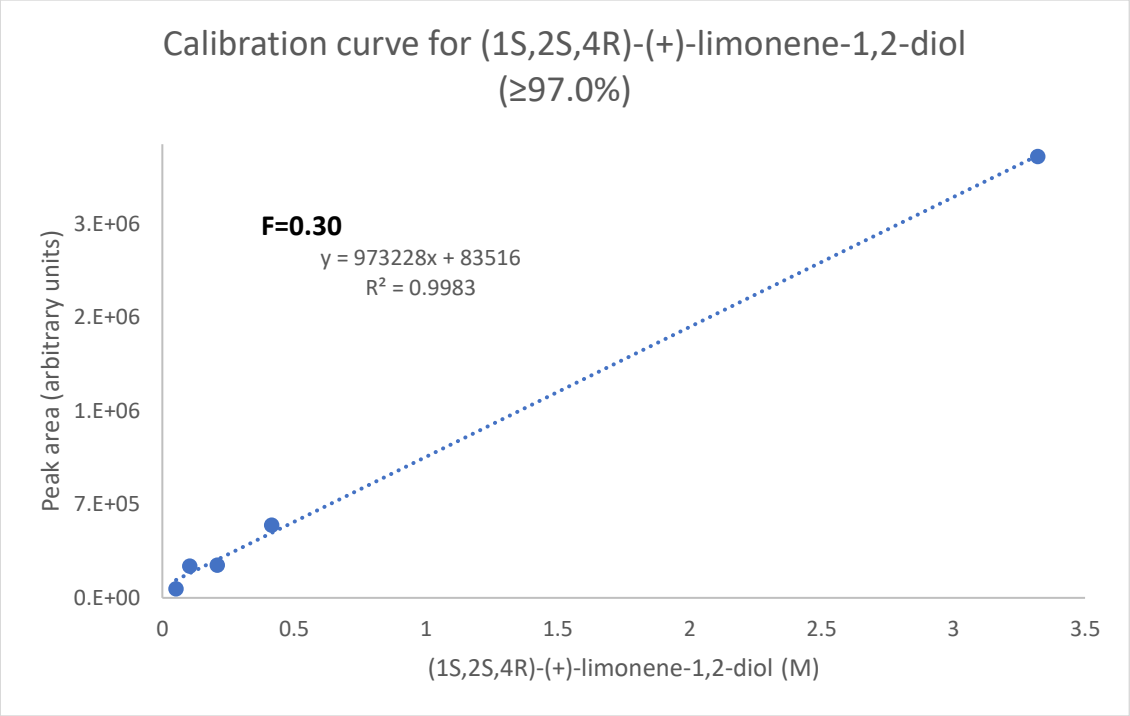


Figure C. Calibration curve for (1S,2S,4R)-(+)-limonene-1,2-diol in ethyl acetate analyzed using GC*-FID.

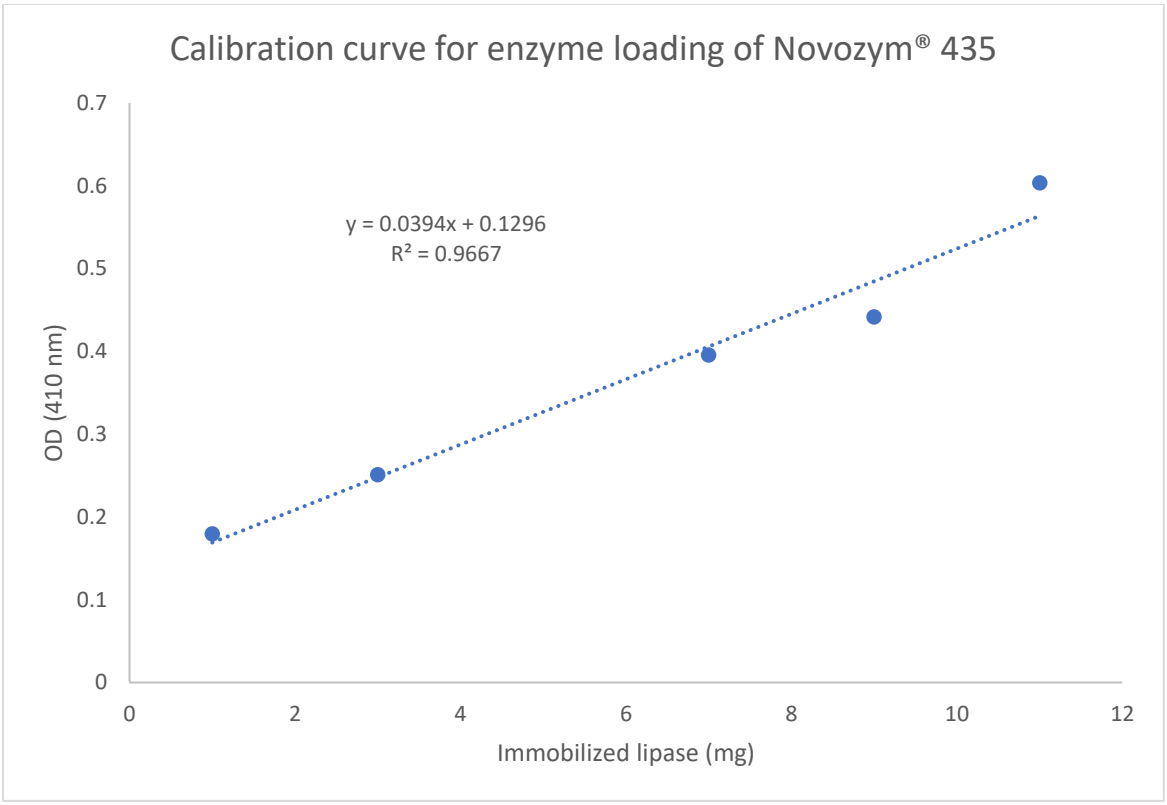


Figure D. Calibration curve of the enzymatic activity of Novozym® 435.

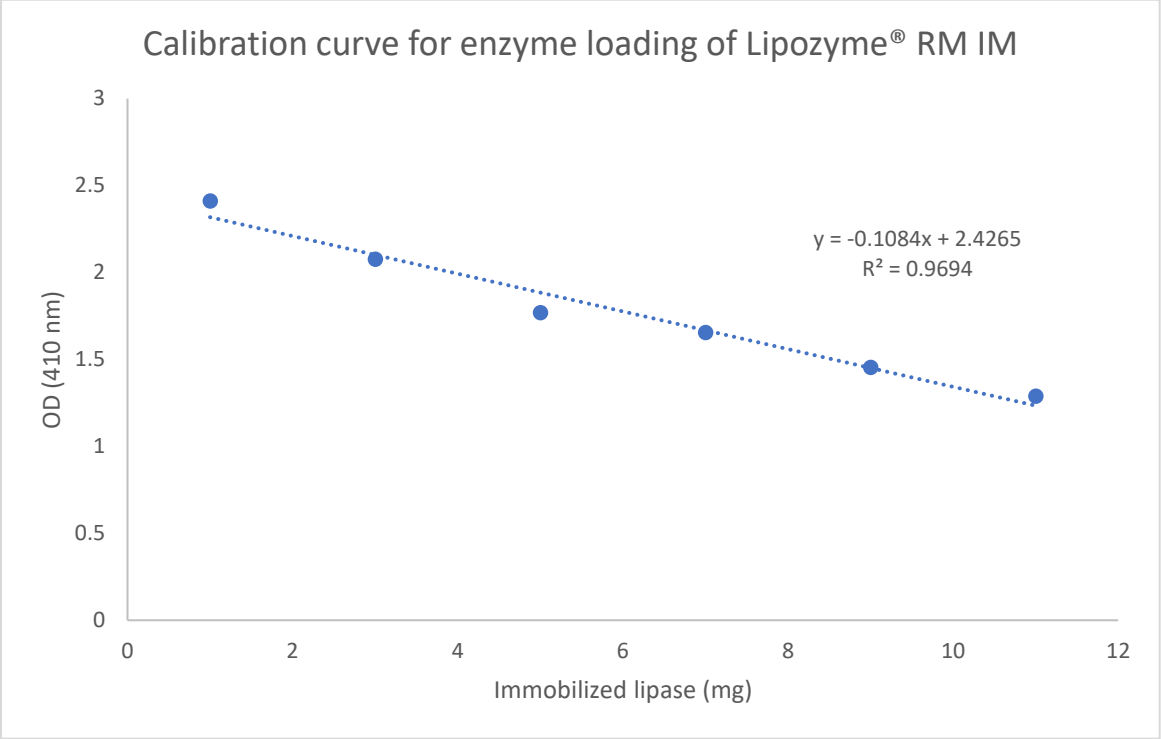


Figure E. Calibration curve of the enzymatic activity of Lipozyme® RM IM.

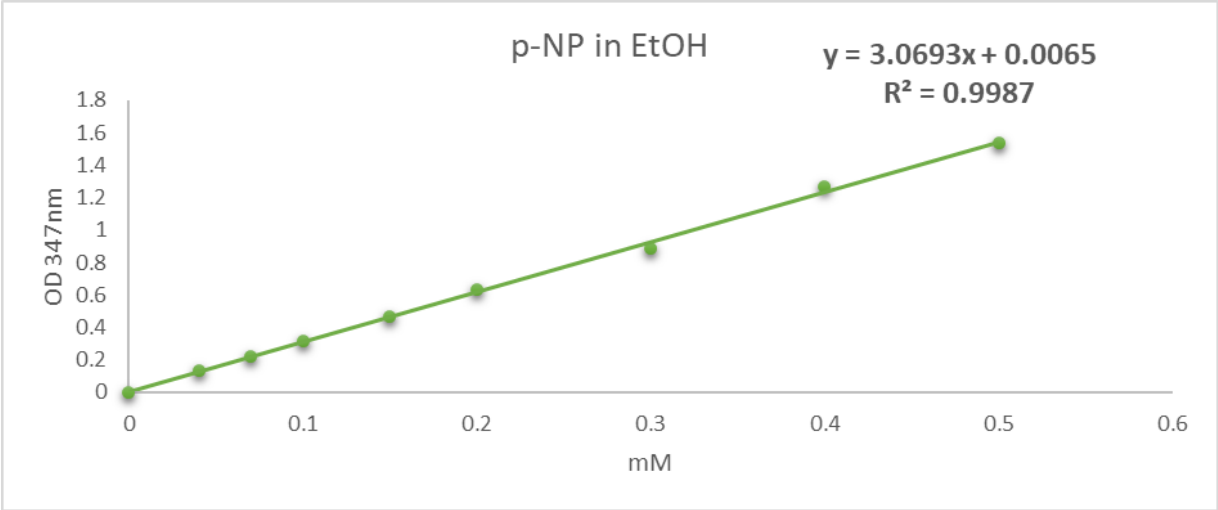


Figure F. Calibration curve of p-nitrophenol in ethanol.

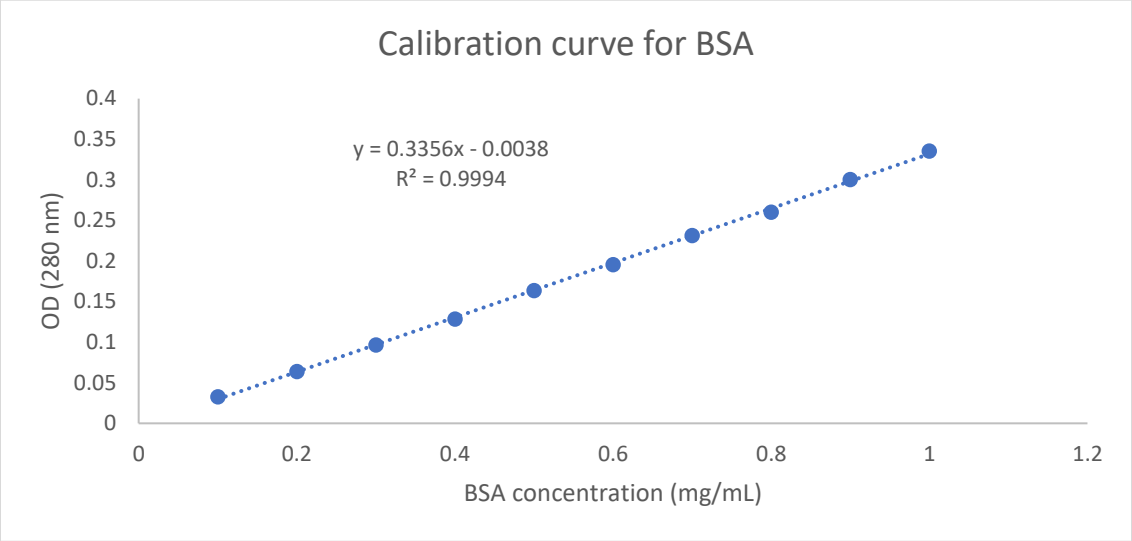


Figure G. Calibration curve of BSA in tris-HCl buffer.

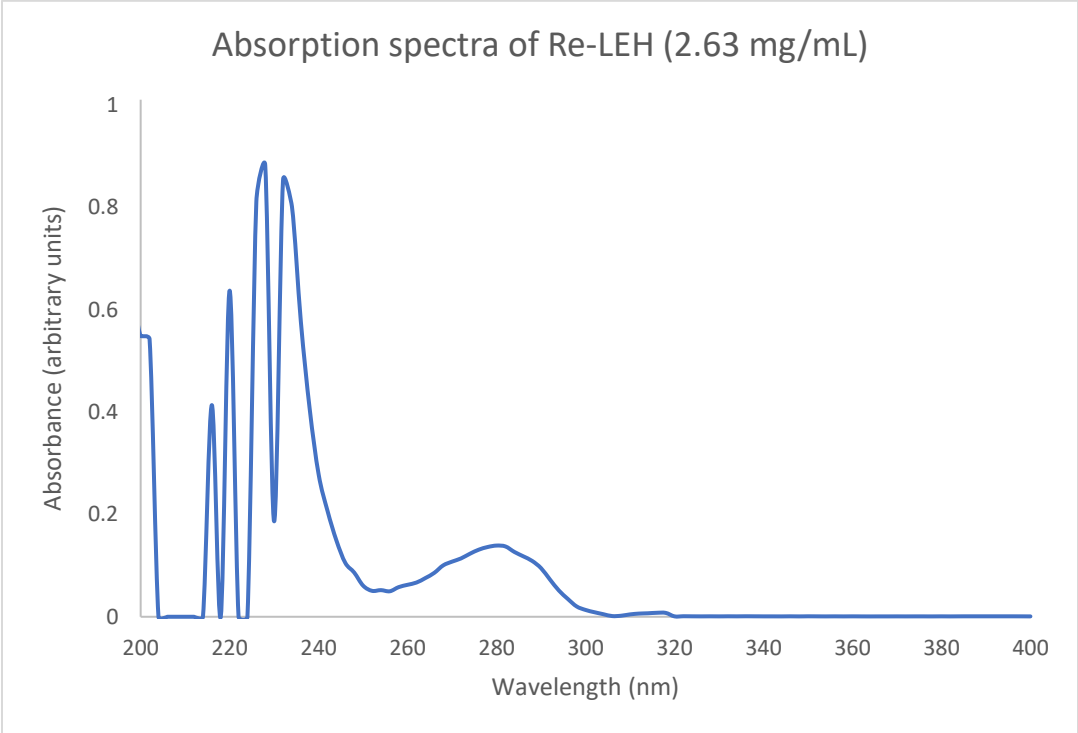


Figure H. Near-UV absorption spectra of an enzymatic solution of Re-LEH with a concentration of 2.63 mg/mL.

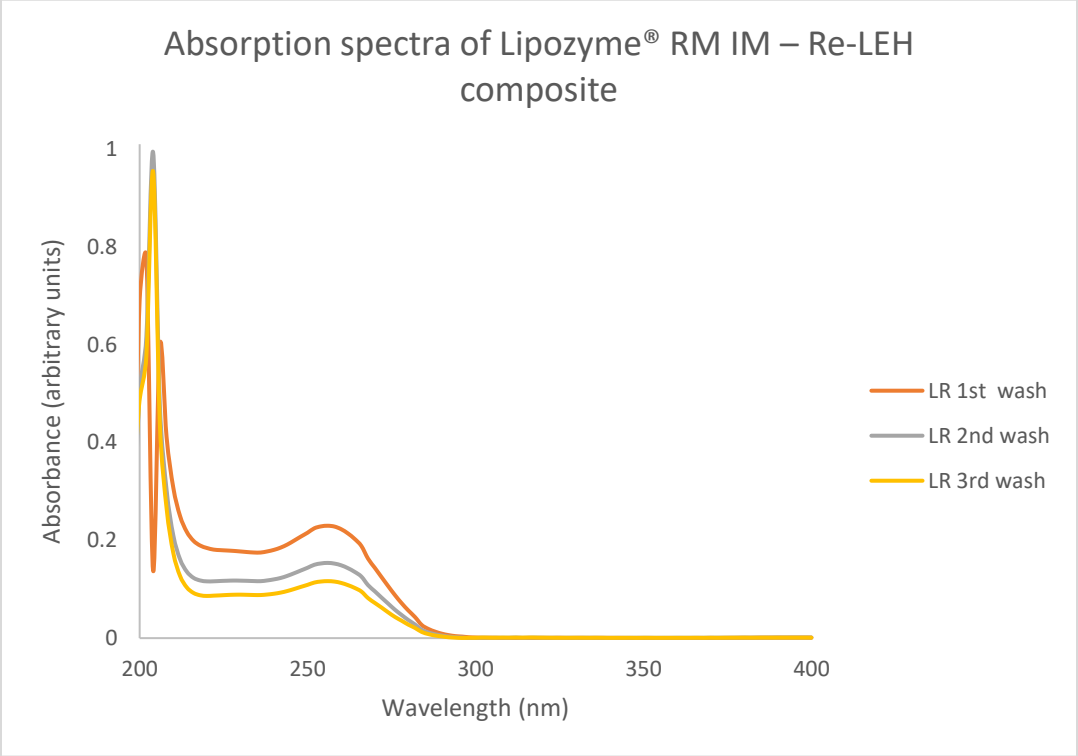


Figure I. Near-UV absorption spectra of washing solutions of Lipozyme® RM IM – Re-LEH composite.

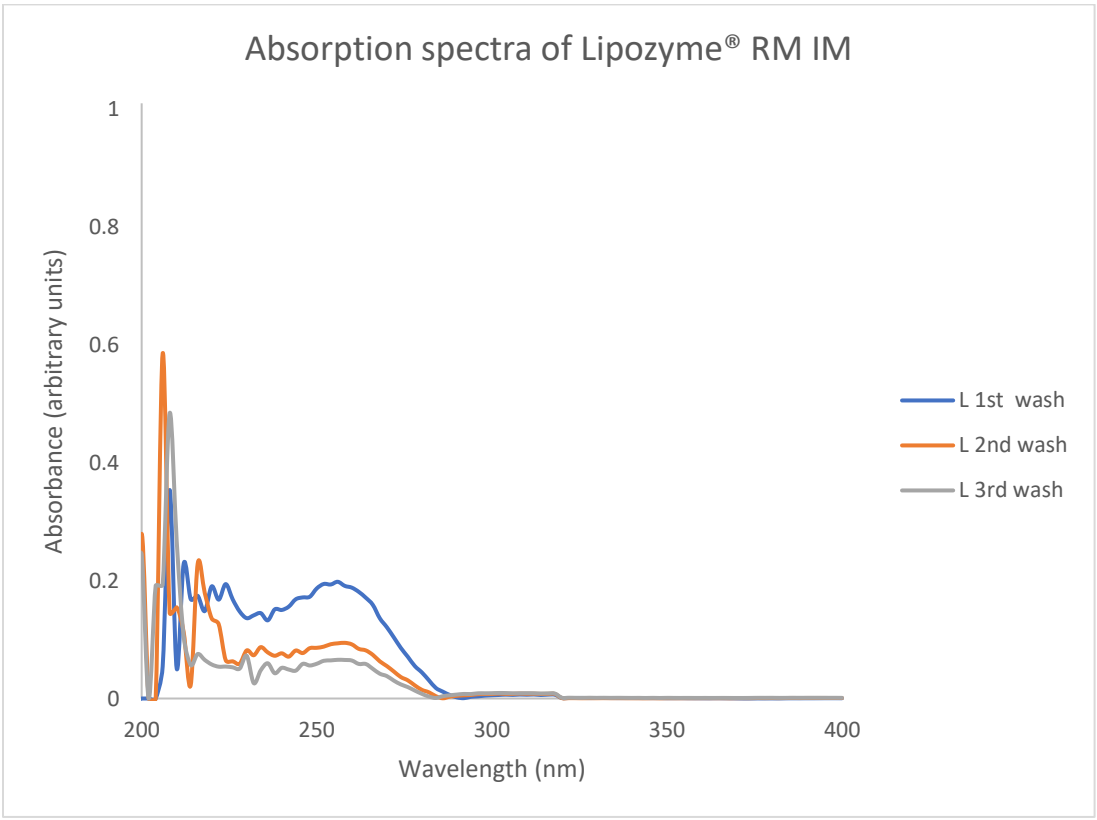


Figure J. Near-UV absorption spectra of washing solutions of Lipozyme® RM IM.

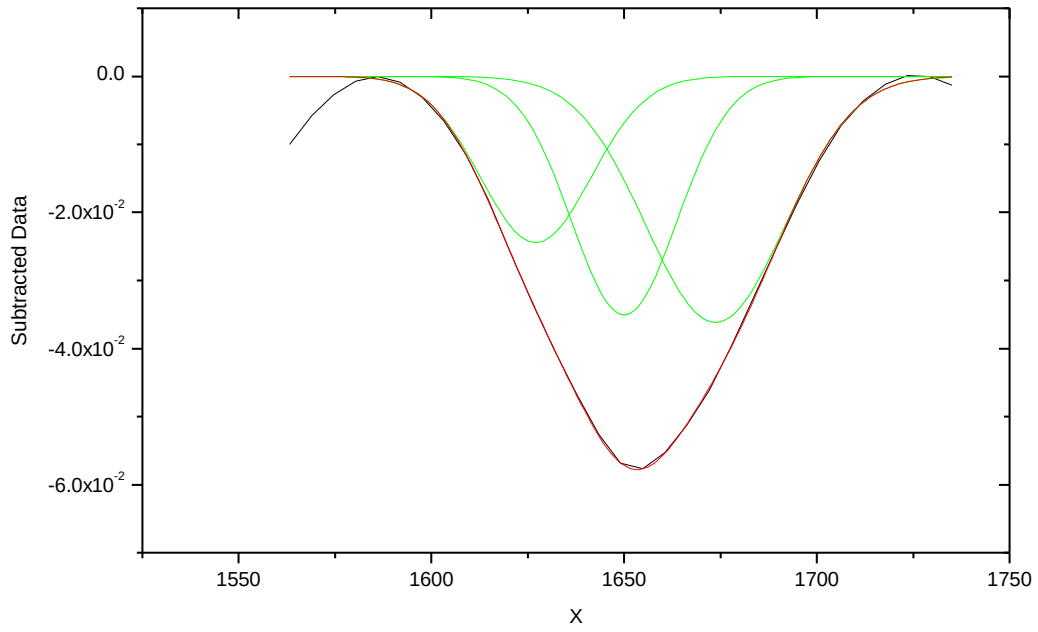
Peak Analysis

Data Set:% ([Book1]FitPeaks1,@WL,Input.IDTR1.IDTC2) Date:6/16/2021

Baseline:Line

Chi^2=-- Adj. R-Square=-- # of Data Points=% ([Book1]FitPeal

SS=-- Degree of Freedom=% ([Book1]FitPeaks1,@WL,RegStats.C1.DOF)



Fitting Results

Peak Index	Peak Type	Area Intg	FWHM	Max Height	Center Grvty	Area IntgP
1.	Gaussian	-0.88127	33.92317	-0.02441	1627.06395	-23.69462
2.	Gaussian	-1.20211	32.2342	-0.03503	1650.02108	-32.32089
3.	Gaussian	-1.63591	42.53986	-0.03614	1673.71108	-43.98449

Figure K. Deconvoluted amide I band of free CH55-LEH from figure 16.

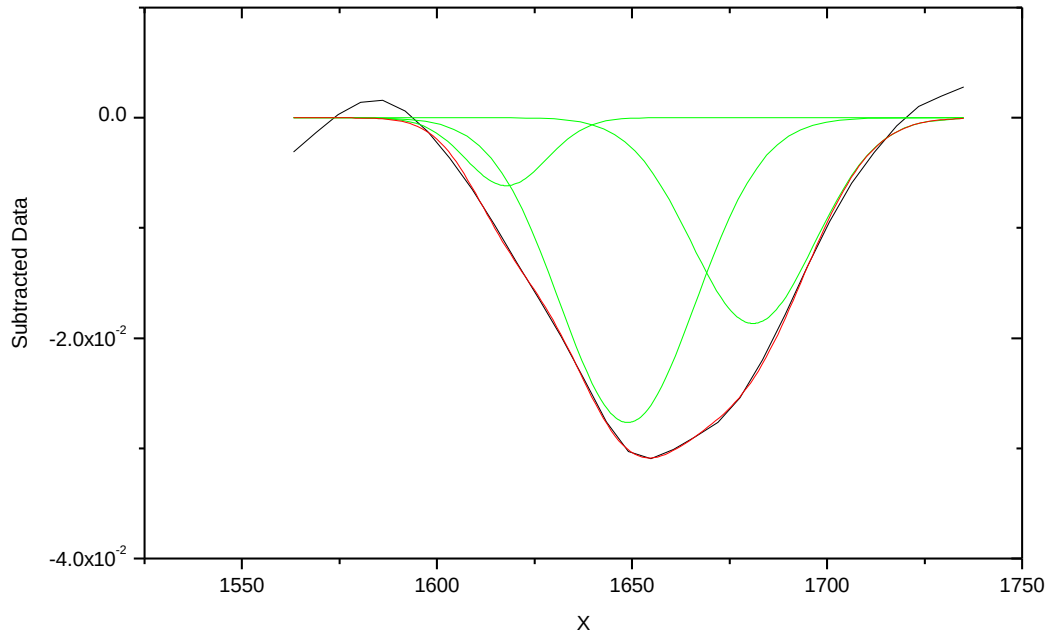
Peak Analysis

Data Set:% ([Book1]FitPeaks2,@WL,Input.IDTR1.IDTC2) Date:6/16/2021

Baseline:Line

Chi^2=-- Adj. R-Square=-- # of Data Points=% ([Book1]FitPeal

SS=-- Degree of Freedom=% ([Book1]FitPeaks2,@WL,RegStats.C1.DOF)



Fitting Results

Peak Index	Peak Type	Area Intg	FWHM	Max Height	Center Grvty	Area IntgP
1.	Gaussian	-0.15988	24.378	-0.00616	1617.7398	-7.57894
2.	Gaussian	-1.20758	41.02756	-0.02765	1648.88968	-57.2444
3.	Gaussian	-0.74206	37.3594	-0.01867	1681.04379	-35.17667

Figure L. Deconvoluted amide I band of free lipase B of *Candida antarctica* from figure 16.

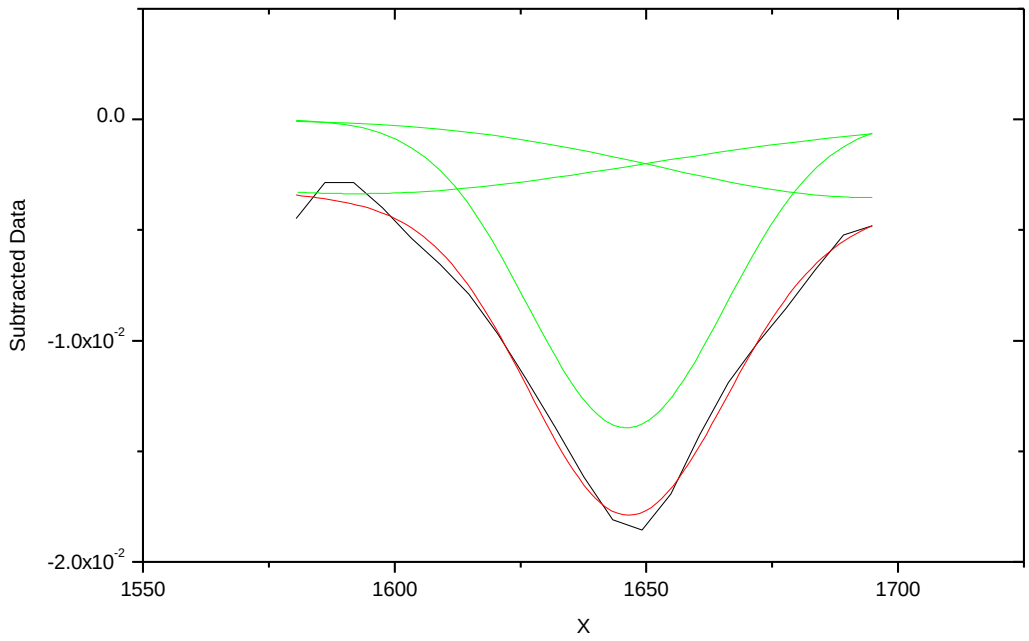
Peak Analysis

Data Set:% ([Book1]FitPeaks3,@WL,Input.IDTR1.IDTC2) Date:6/16/2021

Baseline:Line

Chi^2=-- Adj. R-Square=-- # of Data Points=% ([Book1]FitPeak

SS=-- Degree of Freedom=% ([Book1]FitPeaks3,@WL,RegStats.C1.DOF)



Fitting Results

Peak Index	Peak Type	Area Intg	FWHM	Max Height	Center Grvty	Area IntgP
1.	Gaussian	-0.26096	133.54202	-0.00336	1592.1256	-23.18832
2.	Gaussian	-0.6782	46.02967	-0.01393	1646.09518	-60.2643
3.	Gaussian	-0.18622	99.11765	-0.00353	1694.59205	-16.54738

Figure M. Deconvoluted amide I band of bare Novozym® 435 from figure 16.

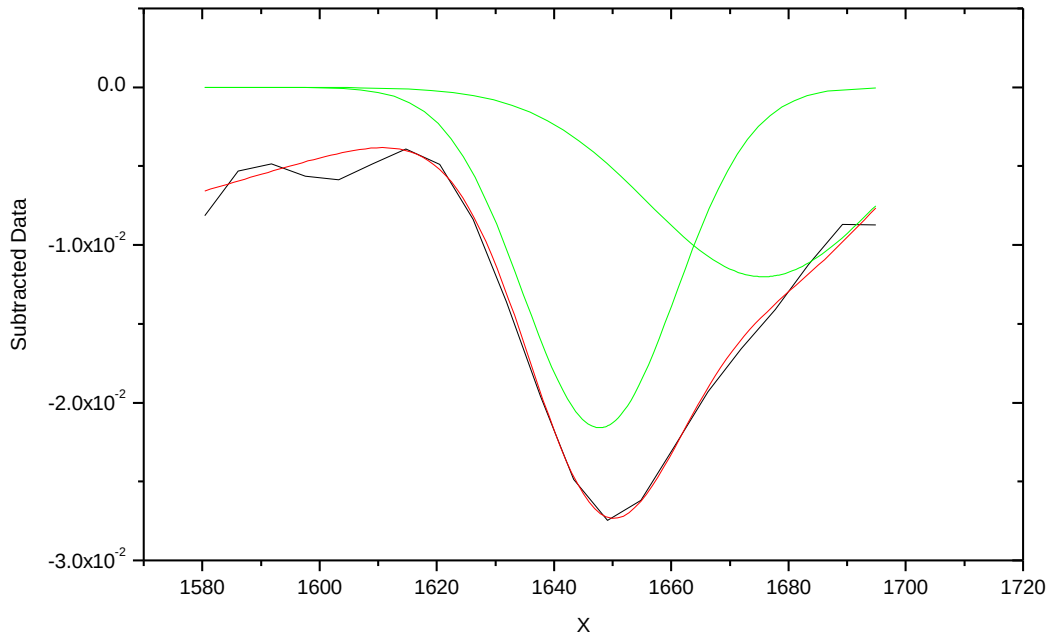
Peak Analysis

Data Set:% ([Book1]FitPeaks4,@WL,Input.IDTR1.IDTC2) Date:6/16/2021

Baseline:Line

Chi^2=-- Adj. R-Square=-- # of Data Points=% ([Book1]FitPeal

SS=-- Degree of Freedom=% ([Book1]FitPeaks4,@WL,RegStats.C1.DOF)



Fitting Results

Peak Index	Peak Type	Area Intg	FWHM	Max Height	Center Grvty	Area IntgP
1.	Gaussian	-0.2446	126.7799	-0.00974	1532.57083	-16.92171
2.	Gaussian	-0.7044	30.6636	-0.02158	1647.77035	-48.73042
3.	Gaussian	-0.4965	46.57293	-0.01201	1675.72771	-34.34787

Figure N. Deconvoluted amide I band of Novozym® 435 – CH55-LEH composite from figure 16.