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Şcoala Doctorală de Studii Inginereşti

New synthetic routes for biocompounds using native and immobilized enzymes Doctoral thesis - Abstract

for obtaining the scientific title of doctor at
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in the field of Chemical Engineering

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Biocatalysis is defined as the field (branch) in which natural microorganisms or macromolecules such as enzymes are used to catalyze chemical reactions [1].

It offers substantial benefits such as: low cost of goods, reduced number of synthetic steps in a process, reduced environmental impact, improved reaction safety and selectivity. [2].

Studies on the immobilization of enzymes, concerning the attachment of the biocatalyst to a desired material through physical, chemical, electrical or mechanical interactions, have shown that as a result of immobilization biocatalysts can improve their activity and stability in a wider range of operating conditions. [3].

In the context of current trends in biocatalysis, the objective of this doctoral thesis was to obtain new biocatalysts by immobilization of lipases in sol-gel, of laccase and peroxidase by covalent binding and adsorption, as well as their utilization to achieve kinetic resolution of racemic substrates, synthesis of D-glucaric acid, and degradation of dyes, respectively.

The optimization of kinetic resolution of racemic substrates was performed using the experimental factorial program and a packed-bed continuous flow system.

This thesis is structured in 4 chapters:

The **first chapter** of the thesis presents a survey of the available literature, emphasizing the motivation for choosing this topic, as well as the fundamental aspects regarding the need to approach and develop these studies.

This chapter also presents the current state of research in the field of obtaining new biocatalysts with lipases from *Pseudomonas fluorescens* (Amano AK), *Burkholderia cepacia* (Amano PS), *Candida antarctica* B (Cal B), *Candida antarctica* A (Cal A), *Trametes versicolor* lacase and horseradish peroxidase obtained by various immobilization techniques, the necessity to obtain new biocatalysts with increased stability at elevated temperature and in organic environments, ways of optimizing racemic resolution reactions using a continuous-flow "packed-bed" system, and synthesis possibilities of aldaric acids.

The **second chapter** presents the original contributions of the doctoral thesis.

Lipase stabilization by immobilization in sol-gel matrices

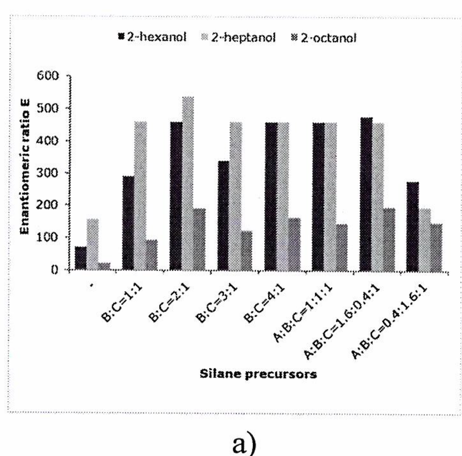
The immobilization of lipases from different sources in sol-gel matrices containing vinyl functional groups was performed. Thus, a vinyl-polysiloxane type hybrid matrix was obtained, in which the presence of vinyl groups influences both the properties of the sol-gel matrix and its hydrophobicity, but also the interaction with the enzyme.

In this study lipases from *Pseudomonas fluorescens* (Amano AK), *Burkholderia cepacia* (Amano PS), *Candida antarctica* B (Cal B) and *Candida antarctica* A (Cal A) were immobilized by sol-gel technique using binary and ternary systems of precursor silanes containing vinyl groups.

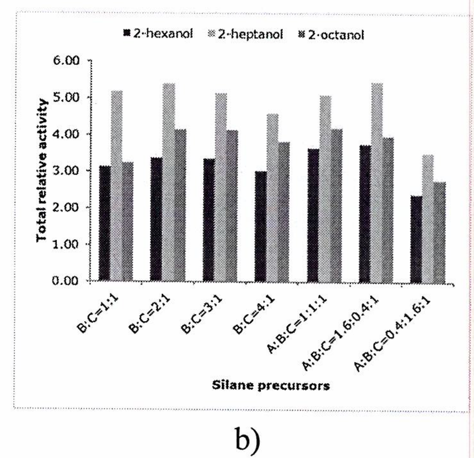
The obtained biocatalysts were tested in acylation reactions of 3 aliphatic secondary alcohols (2-hexanol, 2-heptanol and 2-octanol) in n-hexane medium in a batch system, to determine the influence of the vinyl groups present in the sol-gel matrix on the enzymatic activity and enantioselectivity (Figure 1).

A wide variety of biocatalysts have been obtained, showing high catalytic efficiency on the model substrates.

Cal B lipase entrapped in a matrix obtained from the precursor silanes PhTMOS: VTMOS: TMOS, at 1.6: 0.4: 1 molar ratio, exhibited an activity value of 1.32 $mol \cdot h^{-1} mg^{-1}$ and enantiomeric excess > 95% for the kinetic resolution of 1-hexanol [4,5].



a)



b)

Figure 1. Influence of the molar ratio of precursor silanes on the total relative activity (a) and the enantiomeric ratio E (b), in the enantioselective acylation reaction in n-hexane, at 40°C, of 2-hexanol, 2-heptanol and 2-octanol, using biocatalysts obtained with lipase from Candida antarctica B immobilized by entrapment in sol-gel combined with adsorption (A=PhTMOS, B=VTMOS, C= TMOS).

The values for the total relative activity are close. It must be noticed that the values of the total relative activity were high, between 3 and 5, which denotes a consistent activation of the enzyme following immobilization.

The highest enantiomeric ratio was obtained for the 2-heptanol substrate and the lowest values for 2-octanol, but the values were in all cases very high, over 100. For 2-hexanol and 2-heptanol the values have been over 200, demonstrating almost total enantioselectivity.

Characterization of immobilized biocatalysts

The obtained biocatalysts were characterized by structural, morphological and thermal behavior, using scanning electron microscopy, fluorescence microscopy, FT-IR spectroscopy, atomic force microscopy and thermal analysis in order to find information that can be correlated with the catalytic efficiency in the transesterification reaction.

The SEM analysis showed that in the case of the SGE-1 method, regardless of the molar ratio of the precursor silanes, the biocatalyst have a porous structure (sometimes more compact) of microchannel type, which allows the access of the substrate to the active center of the enzyme.

For biocatalysts obtained using a single silane precursor, TMOS, the morphology of the sol-gel matrix is amorphous, with compact and irregular blocks.

The morphology of the SGE-A biocatalyst obtained by entrapment in sol-gel followed by adsorption on a solid support (Celite 545) was different, the xerogel particles being distributed both on the surface of the solid support and inside the pores.

Confocal fluorescence microscopy analysis showed that the enzyme is distributed relatively evenly both on the surface and inside the xerogel matrix.

FT-IR analysis confirmed that all precursors were included in the sol-gel matrix, and the presence of their functional groups is essential for the activity and operational stability of the entrained enzyme.

All the studied materials were characterized by a content of nanostructures with well-defined nano units, uniform in size. The mesopore size varies between 2-4 nm for all tested preparations.

The roughness observed in the AFM images depends on the nature of the silane precursors, and the surface was uniform and fine for all preparations, without large aggregates.

The DTG curves of *Candida antarctica* B lipase preparations showed that the maximum rate of thermal decomposition was shifted to higher temperatures (for the native enzyme the maximum peak was recorded around 300°C and for the immobilized preparations at temperatures higher than 350°C depending on the preparation), which indicates better protection of the enzyme at high temperatures after immobilization.

The stability in organic solvents and the thermostability of immobilized lipases were also tested. The efficiency of an enzyme in an „*in vitro*" reaction can sometimes be influenced by the low operational stability in organic solvents and temperature resistance.

The obtained materials were subjected to prolonged exposure in organic solvents with different polarities, in order to evaluate their potential as industrial biocatalysts.

Following these studies, it can be stated that the long-term stability of lipases after immobilization was excellent in all organic solvents tested. Therefore, they can be used as catalysts in reaction media of different polarities, depending on the requirements of the reaction system. Also, these results demonstrated the possibility of using preparations immobilized by both techniques in continuous processes that require long-term stability.

To determine the thermostability of the biocatalysts, they were preincubated for 24 hours at temperatures between 40-70°C in n-hexane, followed by the activity assay in the acylation reactions of the secondary alcohols 2-hexanol, 2-heptanol and 2- octanol, with vinyl acetate at 40°C, in n-hexane medium as well.

The enzyme preparations obtained with lipase from *Candida antarctica* B (Cal B) showed the best thermal stability results in the investigated temperature range.

Study of the operational stability of biocatalysts in batch system

Due to the relatively high cost and processing requirements, the utilization of enzymes at industrial level is still limited. Therefore, the use of immobilized enzymes in repeated reaction cycles became very important [6]. An enzyme can be reused until the activity decreases to less than 25% of the initial value [7].

Stability of Amano AK, Amano PS and Cal B lipases immobilized by sol-gel entrapment and sol-gel entrapment combined with adsorption, using a ternary system of precursor silanes PhTMOS: VTMOS: TMOS at a molar ratio of 1,6: 0,4 : 1, was studied in repeated batch acylation reaction of 2-hexanol with vinyl acetate in n-hexane at 40°C [8, 9].

Excellent results were obtained for the operational stability of Cal B lipase, as more than 80% of the activity was recovered after 14 reaction cycles. The results were good in case of Amano AK lipase (after 11 reaction cycles more than 30% of the activity was recovered) , while Amano PS lipase had lower operational stability after immobilization than the native enzyme (Figure 2).

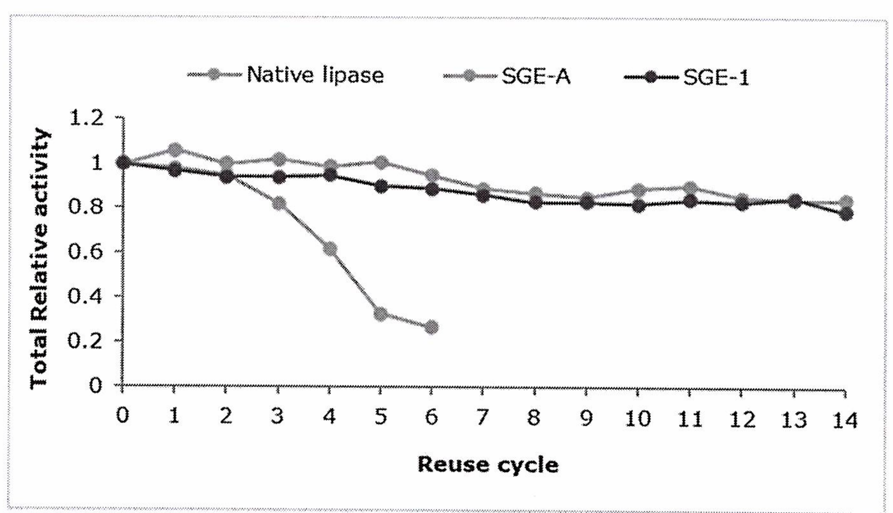


Figure 2. Influence of reuse of native and immobilized enzyme from *Candida antarctica* B on the relative activity in acylation of 2-hexanol at 40°C in n-hexane (native lipase, SGE-A sol-gel entrapment combined with adsorption and SGE-1-sol-gel entrapment).

Acylation of racemic substrates in a continuous system

In addition to immobilization, process engineering can provide additional means to improve the efficiency of biotransformations, reducing the impact of catalyst costs on the product.

One of the most effective ways to intervene in process engineering in order to increase the productivity and service life of the biocatalyst is to use a continuous operation regime [10]. The reactors used are of several types, but in most cases the column type configuration with fixed catalyst layer (PBR) is preferred, in which the immobilized biocatalyst is loaded in the reactor and the substrate solution is pumped at a set flow rate.

In addition to having a faster and higher catalytic activity compared to batch reactors, as well as long-term operational stability, this configuration reduces the cost of the process [11, 12]. Previous studies have included the optimization of the immobilization protocol, tested in batch process reactions, later improved for its use in continuous bed continuous system (PBR) type reactors. To optimize the parameters that have an effect on the kinetic resolution in a continuous system, two secondary alcohols (one aliphatic and one aromatic), 2-octanol and 1-phenylethanol (rac-1 and rac-2), were selected as model substrates.

The selection of lipase immobilized by sol-gel was based on the previously presented studies, both in terms of optimization of the sol-gel matrix and detailed characterization of thermal stability and reuse of biocatalysts immobilized in batch system.

The optimization of the continuous enzymatic kinetic resolution of 2-octanol and 1-phenylethanol was performed using an experimental factorial program.

To better elucidate and understand the interaction and effects of the studied variables (substrate concentration, flow rate and temperature) on the response variables, all experimental data were combined, generating response area graphs (Figure 3).

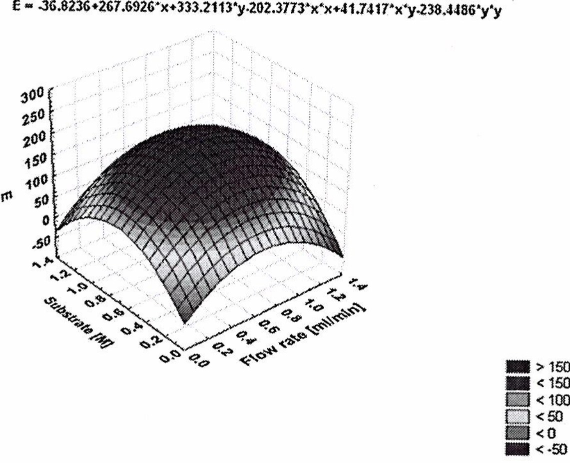


Figure 3. Graphical representation of the enantiomeric ratio E as a function of substrate concentration and flow, in the case of the kinetic resolution of rac-1.

Based on the highest values of E and enantiomeric excess of the product ee_p. obtained by the response surface methodology (RSM), the optimal reaction parameters were set as 0.5 M concentration of the rac-1 substrate, 1,5: 1 vinyl acetate: substrate molar ratio, 50° C reaction temperature, and 0,8 mL/min flow rate.

To validate the results of the experimental model, the acylation reaction of rac-1 in a continuous system was performed using the optimal parameters resulting from this modeling. The results presented in Figure 4 show that for 144 hours of reaction the productivity values remained constant at about 145 $\mu mol \cdot min^{-1} \cdot g^{-1}$, and the enantiomeric ratio was higher than 300, demonstrating the excellent operational stability of the immobilized biocatalyst in a continuous process and the validity of the experimental model.

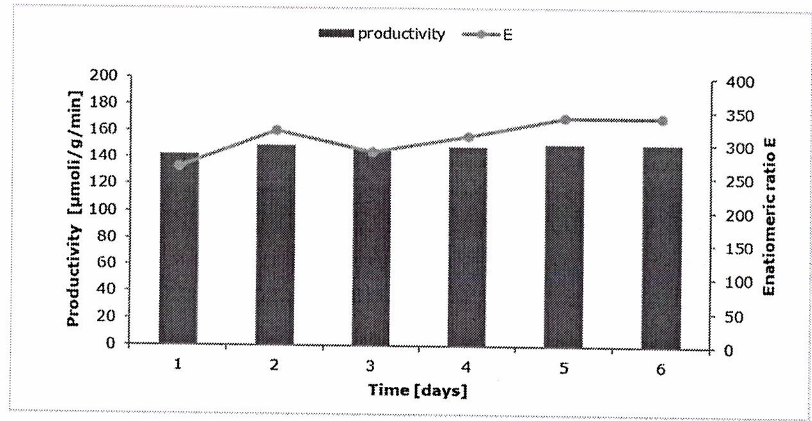


Figure .4. Diagram of productivity and enantioselectivity (E) of Cal B lipase immobilized in sol-gel, for long-term continuous use in a reactor with a fixed layer of biocatalyst, for the kinetic resolution of rac-1, under optimized reaction conditions.

Immobilization of oxidative enzymes by covalent bonding and biotechnological applications

Immobilization of the laccase its evaluation for the synthesis of glucaric acid

Laccase from *Trametes versicolor* was immobilized by covalent bonding using two supports with epoxy functional groups (epoxy / methacrylate and epoxy / butyl methacrylate) and one with amino groups (amino C2 methacrylate), and by adsorption using 3 functionalized supports with different groups: Octadecyl methacrylate, DVB / methacrylate, Macroporous styrene. The supports are commercially available (Purolite).

To compare the two immobilization methods, the activities of the immobilized laccase were represented according to the enzyme / support ratio, expressed as mg protein/100 mg support. Figure 5(a-d) shows the activity values at the same enzyme loading, for all supports used.

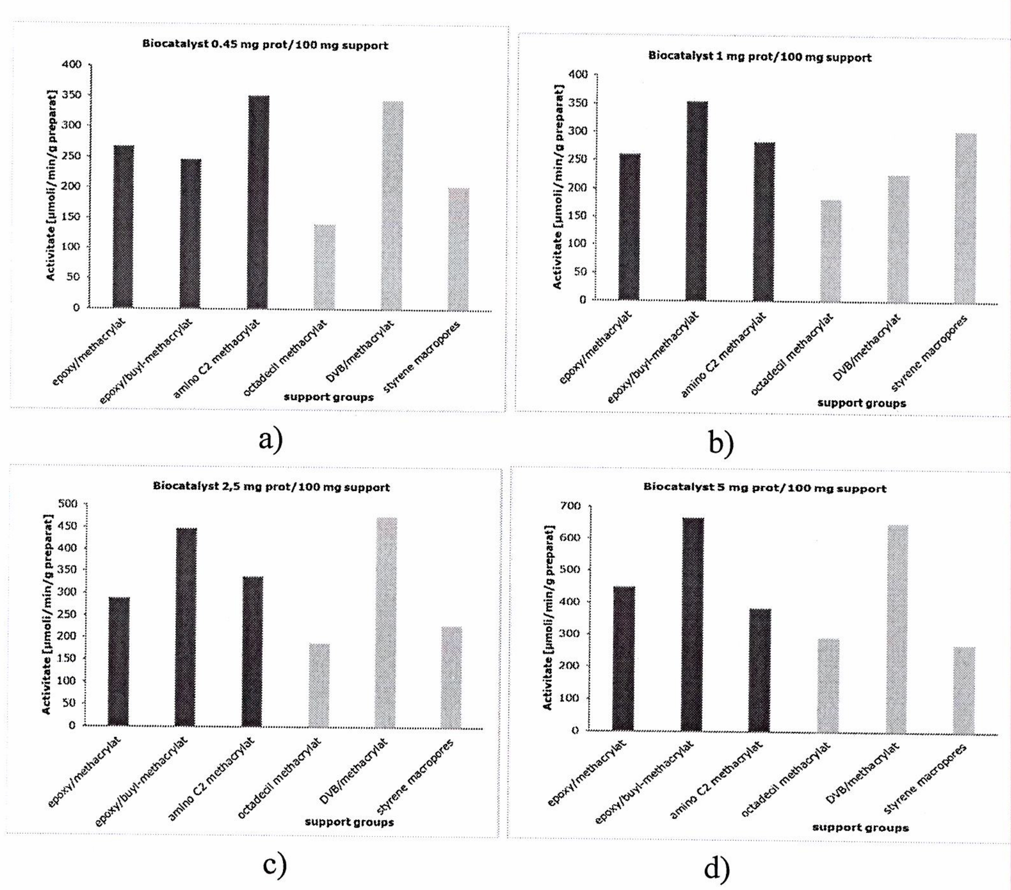


Figure 5. Effect of enzyme / support ratio on the activity of biocatalysts obtained by immobilization of the laccase by covalent binding and adsorption: (a) 0,45 mg protein, (b) 1,0 mg protein, (c) 2,5 mg protein and (d) 5 mg protein, reported in all cases at 100 mg support.

Based on the obtained results, the highest activity values were obtained at the maximum loaded protein amount (5 mg protein / 100 mg support). However, based on the finding that the activities were only double compared to the biocatalyst obtained using the minimum amount of protein (which was 11 times lower) and taking into account that the price of the enzyme is an important element in biocatalytic processes, the optimal load was considered 0,45 mg protein / 100 mg support.

As results from Figure 5, the enzymatic preparations obtained by covalent binding exhibited activity values in the range 245-665 μmol · min^{-1} · g^{-1} preparation, which were generally higher compared to the biocatalysts obtained by adsorption. However, among the adsorbed preparations, those immobilized on divinylbenzene / methacrylate support also showed comparable or even higher activities as obtained by covalent bonding.

Use of immobilized peroxidase to remove of dyes from water

In order to obtain new immobilized biocatalysts from the class of oxidoreductases, the immobilization of peroxidase, another enzyme with applications in various fields, was performed [13].

The selectivity of peroxidase for commercial azo dyes

The selectivity of peroxidase was investigated for six substrates (azo dyes), using the native enzyme at 25°C and two pH values, 3,5 and 6,0.

The results obtained after 24 h reaction time, shown in Figure 6, indicate that at pH 3,5 the conversions were lower compared to those at pH 6,0, except for Neutral Gray dye. Thus, at pH 6, the highest conversion values (up to 80%) were obtained for the dyes Amido Black 10 (AB10) and Acid Orange 7 (AO 7).

Taking these results into account, the dye AB10 was used as a substrate in the following experiments.

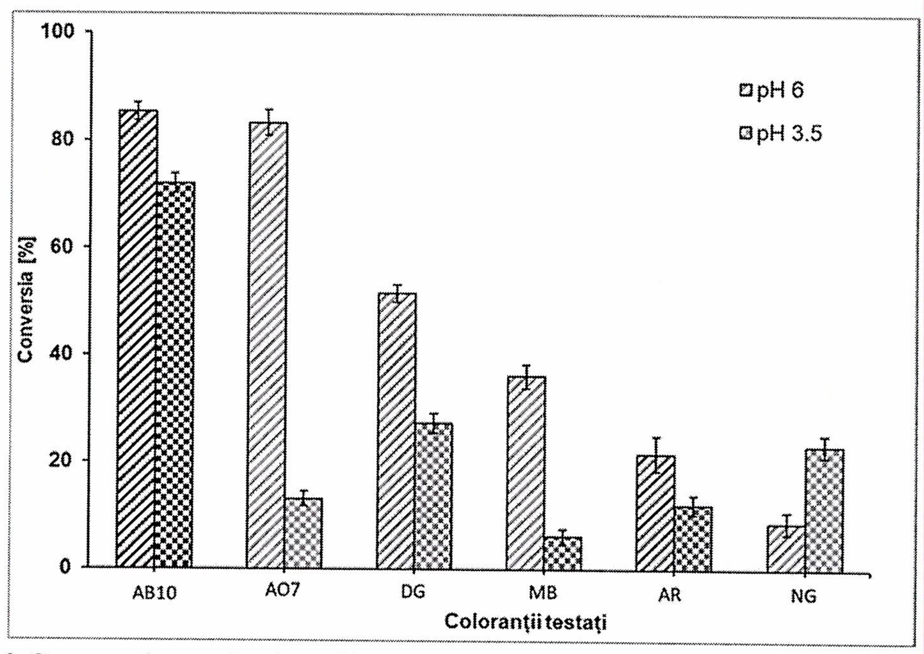


Figure 6. Comparative evaluation of horseradish peroxidase selectivity for degradation of six different dyes (AB 10 - Amido Black 10, AO7 - Acid Orange 7, DG - Direct green; MB - Methylene blue; AR - Acid red; NG - Neutral gray)

Immobilization of peroxidase using ReliZyme ™ supports

From the available immobilization methods, covalent bonding was selected, a quick and easy method. Since the biocatalyst is used in aqueous solutions, covalent binding can also prevent the loss of the enzyme from the support.

Two ReliZyme ™ commercial media were selected, with active epoxy groups and with different linker length and structure. Immobilization was performed in a single step by mixing the enzyme solution with the support for 24 hours. The activity of immobilized peroxidase was

determined by using ABTS as a model substrate, as described in the experimental part. The results presented in Table 2.1 indicate high protein loading values (> 90%) for both types of support. The specific activity and reproducibility were higher when the epoxy-amino support HFA 403 was used. Probably, the longer linker (see the structures shown in Table 2.1) allows a better covalent binding of the enzyme to the epoxy groups of the support. However, taking into account the high immobilization efficiencies, characterization studies were performed for both biocatalysts and the results were compared with the native enzyme.

Table 2.1. Enzyme loading and recovery of immobilized peroxidase on epoxy-ReliZyme media ™

Solid support type	Enzyme loading [%]	Specific activity [U·g·protein^{-1}]
EP 403	93,5	21,24 ± 2,36
HFA 403	99,6	37,24 ± 0,78

The **third chapter** provides an overview of the materials, working and characterization methods, as well as the equipment used in order to carry out the experimental program.

The **fourth chapter** presents the final conclusions for each subchapter and the original contributions.

Following the studies performed, the following conclusions emerged:

a. Immobilization of microbial lipases in sol-gel matrices obtained with a vinyl group precursor

New biocatalysts were obtained by immobilization of lipases from *Pseudomonas fluorescens* (Amano AK), *Burkholderia cepacia* (Amano PS), *Candida antarctica* B (Cal B) and *Candida antarctica* A (Cal A) in sol-gel matrices containing vinyl functional groups. To determine the effect of the presence of vinyl groups in the sol-gel matrix on the activity and enantioselectivity, these biocatalysts were tested in acylation reactions of a series of aliphatic secondary alcohols (2-hexanol, 2-heptanol and 2-octanol), in n- hexane.

In the case of biocatalysts obtained with lipases from *Pseudomonas fluorescens* (Amano AK) and *Burkholderia cepacia* (Amano PS) by the SGE-1 method the highest value of transesterification activity was obtained when the sol-gel matrix was obtained using a ternary system with the ratio molar PhTMOS: VTMOS: TMOS of 1,6: 0,4: 1.

Regarding the enantioselectivity of the biocatalysts obtained by the SGE-1 method, for the preparations obtained with lipases from *Pseudomonas fluorescens* (Amano AK) and *Burkholderia cepacia* (Amano PS) its values were higher compared to the native enzyme.

Binary silane precursor systems were also tested for the combined method. In the case of binary systems for biocatalysts with Amano AK and Amano PS the best values of the activity were obtained at a molar ratio VTMOS: TMOS of 4: 1 and 3: 1, at a higher content of vinyl groups.

In the case of preparations with Cal B, very high values of transesterification activity were obtained for both binary and ternary systems, without being able to make a clear distinction between the different preparations.

Regarding the enantioselectivity, for the preparations with Amano AK high values for the 2-hexanol substrate were obtained, at different molar ratios of precursor silanes. In the case of Amano PS and Cal B lipases, the highest values of the enantiomeric ratio were obtained for 2-hexanol and 2-heptanol substrates, these values being similar regardless of the molar ratios of the precursor silanes.

b. Evaluation of the operational stability of lipase preparations in organic solvents

The solvent stability of lipases after immobilization was excellent, so these biocatalysts can be used as catalysts in reaction media of different polarities, depending on the requirements of the reaction system.

Preparations immobilized with Amano AK showed relative activities higher than 1, after incubation in organic solvents (up to 1.4 in acetonitrile for preparations obtained by the SGE-1 method), demonstrating activating effect.

The enzymatic preparations obtained had high and constant values of the enantiomeric ratio in all the solvents tested, compared to the native enzyme. The values of the enantioselectivity E of the obtained biocatalysts were twice higher for the preparations with Amano AK and Amano PS and even 5 times higher for the preparations with Cal B, compared to the native enzyme.

c. The influence of temperature on the operational stability of the enzymatic preparations obtained.

The immobilized biocatalysts showed excellent thermal stability, the transesterification activity remaining practically constant in the studied temperature range of 40-70°C.

Enantiomeric ratio E values were higher for enzymatic preparations compared to native lipases, regardless of the immobilization technique used.

After incubation of the preparation with Cal B at 80°C in isooctane for 120 h, the catalytic activity values remained unchanged, while the native lipase activity was reduced to less than 25% of the initial activity. The enantioselectivity values of the biocatalysts remained unchanged throughout the incubation period.

d. Evaluation of the operational stability of immobilized lipases by sol-gel technique in several reaction cycles

Immobilization by the sol-gel technique led to biocatalysts with high stability when used in several reaction cycles in a batch system. Excellent results were obtained for the operational stability of Cal B lipase (after 14 reaction cycles over 80% of the activity was found) and good for Amano AK lipase (after 11 reaction cycles over 30% of the activity was found), while lipase from Amano PS had lower operational stability after immobilization than the native enzyme.

e. Optimization of the enzyme kinetic resolution of racemic 2-octanol and 1-phenylethanol in a continuous system, using experimental design

The application of the experimental design for two substrates, 2-octanol and 1-phenylethanol, allowed the optimization of the parameters that influence the kinetic resolution in a continuous reaction system.

By the response surface (RSM) methodology, based on the highest values of E and ee_p, the optimal reaction parameters for the kinetic resolution process were established.

For rac-1 the optimal parameters were: substrate concentration of 0,5 M, vinyl acetate molar ratio: 1,5: 1 substrate, reaction temperature 50°C and flow rate of 0,8 mL / min, and for

rac-2 substrate concentration was 0.2 M, vinyl acetate molar ratio: 1,5: 1 substrate, reaction temperature 50°C and flow rate 0,45 mL / min.

f. Increased stability of the *Trametes versicolor* laccase by covalent immobilization and adsorption

Successful immobilization of *Trametes versicolor* laccase was performed by covalent binding and adsorption, obtaining 24 enzymatic immobilized laccase preparations, whose activity was evaluated in the oxidation reaction of 2,6-dimethoxyphenol as model substrate.

For both immobilization methods tested, the highest activity values were obtained at the highest protein load, 5 mg protein/100 mg support, but the increase of activity was not proportional to the increase of the amount of enzyme subjected to immobilization.

The highest values of enzymatic activity, 665 μmol /min/g prepared, were obtained in the case of covalent bonding on the support with epoxy/butyl active groups.

g. Characterization of the biocatalyst obtained with lacase - immobilization by covalent bonding

After immobilization by covalent bonding on polymethacrylate support with epoxy / butyl groups, the maximum activity value was shifted from 3,5 in the case of the native enzyme to pH 4 at the immobilized enzyme.

Removal of water from the enzyme preparation by drying at 25°C resulted in a 55% decrease in activity after 24 h and an 80% decrease after 48 h.

The immobilized biocatalyst maintained its activity in the temperature range 30-50°C, being observed even a slight increase, while for the native enzyme the activity decreased in the same conditions.

h. Use of immobilized peroxidase to remove dyes from water

Horseradish peroxidase was successfully immobilized by covalent binding on two ReliZyme ™ supports containing active epoxy groups.

Improving stability and activity by immobilization over a wider pH range, especially in the pH range of 5-6, the usual pH value of water contaminated with dyes.

The thermostability of the enzyme has been considerably improved by immobilization. In the case of peroxidase immobilized on HFA 403, 90% of the initial enzymatic activity was recovered after incubation for 30 minutes at 55°C, while the native enzyme lost ~ 60% of the activity under the same conditions.

The selected immobilization method and the favored support obtaining a robust biocatalyst, suitable for several reuse cycles with a high activity. The immobilized enzyme was successfully reused in discontinuous discoloration processes, the activity remaining at 80% of the initial value after 10 cycles of use

ABBREVIATIONS

e.ep.- enantiomeric excess of the reaction product
SGE-1 - immobilization by entrapment in sol-gel by Method 1
SGE-2 - immobilization by entrapment in sol-gel with prepolymer sol by Method 2
SGE-A - immobilization by entrapment in sol-gel combined with adsorption on Celite 545
Conv. - conversion
Atrans – transesterification activity
[Omim]BF4 - 1-Methyl-3-octylimidazolium tetrafluoroborate
PhTMOS - phenyltrimethoxysilane
VTMOS - vinyltrimethoxysilane
TMOS - tetramethoxysilane
MeTMOS - methyltrimethoxysilane
OcTMOS - octiltrimetoxisilan
Amano AK – lipase from *Pseudomonas fluorescens*
Amano PS – lipase from *Burkholderia cepacia*
Cal B - lipase from *Candida antarctica B*
Cal A – lipase from *Candida antarctica A*
SEM - scanning electron microscopy
FITC - fluorescence microscopy
AFM - force atomic microscopy
FT-IR – Fourier transform infrared spectroscopy
rflow - productivity
Rac-1 – 2-octanol
Rac-2 – 1-phenylethanol
E – enantioselectivity coefficient
THF – tetrahydrofuran
t-BuOH – tert-butanol

References

[1] K. Kakaei, M. D. Esrafili, A. Ehsani, Chapter 1 - Introduction to Catalysis, Interface Science and Technology, 2019, 27, 1-21.

[2] P. N. Devine, R. M. Howard, R. Kumar, M. P. Thompson, M. D. Truppo, and N. J. Turner, Extending the application of biocatalysis to meet the challenges of drug development, Nature Reviews Chemistry, 2018, 2, 409–421.

[3] J. Chapman, A. E. Ismail and C. Zoica Dinu, Industrial Applications of Enzymes: Recent Advances, Techniques, and Outlooks, Catalysts 2018, 8, 238.

[4] C. Paul, P. Borza and F. Péter, High thermal stability of sol-gel entrapped lipases, Journal of Agroalimentary Processes and Technologies, 2015, 21(2), 173-180.

[5] A. Ursoiu, P. Borza, C. Paul, F. Péter, Influence of silane precursors system composition on the catalytic efficiency of sol-gel immobilized lipases, Chem. Bull. "POLITEHNICA" Univ. (Timisoara), 2012, 57(71), 1, 42-45.

[6] K. P. Dhake, A. H. Karoyo, M. H. Mohamed, L. D. Wilson and B. M.Bhanage, Enzymatic activity studies of Pseudomonas cepacia lipase adsorbed onto copolymer supports containing β-cyclodextrin, J. Mol. Cat. B: Enzymatic, 2013, 87, 105-112.

[7] A. Ursoiu, C. Paul, T. Kurtan, F. Péter, Sol-gel Entrapped Candida antarctica lipase B — A Biocatalyst with Excellent Stability for Kinetic Resolution of Secondary Alcohols, Molecules, 2012,17, 11, 13045–13061.

[8] C. Paul, P. Borza, A. Marcu, G. Rusu, M. Bîrdeanu, S. Marc Zarcula, F. Péter, Influence of the physico-chemical characteristics of the hybrid matrix on the catalytic properties of sol-gel entrapped Pseudomonas fluorescens lipase, Nanomater. Nanotechno., 2016, 6:0.

[9] P. Borza, F. Péter, I. Hulka, C. Paul, Long-term exposure stability of sol-gel immobilized lipases in organic solvents, Chem. Bull. "POLITEHNICA" Univ. (Timisoara), 2015, 60(74), 1, 25-30.

[10] A. Todea, P. Borza, A. Cimporescu, C. Paul, F. Péter, Continuous kinetic resolution of aliphatic and aromatic secondary alcohols by sol-gel entrapped lipases in packed bed bioreactors, Catal. Today, 2018, 306, 223-232.

[11] J. K. Poppe, C. R. Matte, V. O. de Freitas, R. Fernandez-Lafuente, R. C. Rodrigues, M. A. Z. Ayub, Enzymatic synthesis of ethyl esters from waste oil using mixtures of lipases in a plug-flow packed-bed continuous reactor, Biotechnology Progress, 2018, 34, 4, 952-959.

[12] F. Strniša, M. Bajić, P. Panjan, I. Plazl, A. M. Sesay, P. Žnidaršič-Plazl, Characterization of an Enzymatic Packed-Bed Microreactor: Experiments and Modeling, Chemical Engineering Journal, 2018, 350, 541-550.

[13] 1. P. Borza, I. C. Benea, I. Bîtcan, A. Todea, S. G. Muntean, F. Peter, Enzymatic degradation of azo dyes using peroxidase immobilized onto commercial carriers with epoxy groups, Studia UBB Chemia, 2020, LXV, 1, 291-303.