

# Double Immobilized Lipase for the Kinetic Resolution of Secondary Alcohols

A. Ursoiu, C. Paul, C. Marcu, M. Ungurean, and F. Péter

**Abstract**—Sol-gel immobilization of enzymes, which can improve considerably their properties, is now one of the most used techniques. By deposition of the entrapped lipase on a solid support, a new and improved biocatalyst was obtained, which can be used with excellent results in acylation reactions. In this paper, lipase B from *Candida antarctica* was double immobilized on different adsorbents. These biocatalysts were employed in the kinetic resolution of several aliphatic secondary alcohols in organic medium. High total recovery yields of enzymatic activity, up to 560%, were obtained. For all the studied alcohols the enantiomeric ratios E were over 200. The influence of the reaction medium was studied for the kinetic resolution of 2-pentanol.

**Keywords**—Double immobilization, enantioselectivity, kinetic resolution, lipase, racemates, sol-gel entrapment.

## I. INTRODUCTION

FROM the time enzymes were discovered, they have been a major subject of intense research owing to their spectacular properties. Enzymes, being highly specific and extremely enantio- and regioselective catalysts, are used extensively in the industrial production of bulk chemicals, pharmaceutical and agrochemical intermediates and food ingredients [1]. Resolution of racemates with hydrolases is the most common biocatalytic strategy for the industrial production of enantiomerically pure fine chemicals [2]. The need for enantiopure compounds for the production of pharmaceuticals and fine chemicals can be explained by the fact that all living organisms in nature are chiral. The use of enzymes in synthesis, is very attractive due to the possibility of engaging unnatural substrates in highly selective and efficient reactions catalyzed by these biocatalysts. Moreover, such transformations can be run under mild conditions [3]. Lipases are widely applied as effective biocatalysts for the kinetic hydrolysis, esterification or transesterification of racemic compounds in both aqueous and organic solvents [4-

6].

Enzymes may suffer from instability due to e.g. spontaneous oxidation, self-digestion, or denaturation. The development of biotechnology brings along the immobilization of biomolecules or microorganisms [7]. Most of lipases used in industrial processes were in the immobilized state. Indeed, the immobilization of enzymes to solid support materials is of great interest due to some important advantages such as: improvement of the activity and stability of enzymes, possibility to recover the immobilized enzyme at the end of the reaction and thus its potential reuse and the possibility of a continuous process [8]. A great variety of immobilization methods are available and these can be grouped into categories: immobilization by entrapment, immobilization by non-covalent binding, immobilization by enzyme crystallization and covalent crosslinking, immobilization by covalent binding to properly functionalized carrier materials. Many efforts have been made over the years with the aim of improving catalytic activity and operational stability of industrial enzymes through immobilization. Several factors, including choice of support and selection of an immobilization strategy may affect the activity, recovery and reusability of enzymes in an immobilization process. It is also known that immobilization strategies may influence the catalytic and enantioselective properties of the enzyme. Therefore, use of various immobilization strategies may provide immobilized lipases with different activity/selectivity characteristics [8-12]. The selection of an immobilization strategy is based on effectiveness of enzyme utilization, cost of the immobilization procedure, toxicity of immobilization reagents and the desired final properties of the immobilized biocatalyst. Among the available immobilization methods, entrapment of enzymes in an inorganic polymer matrix is a method that has received considerable attention in the recent years. This approach has several advantages, as mechanical entrapment of enzymes using sol-gel materials allows stabilization of the protein tertiary structure caused by the tight gel network [13]. The sol-gel immobilized lipases can be supported on inert materials such as Celite to improve the diffusion of the substrates or products to and from the enzyme and thus improve the reaction rate [7]. Lipase B from *Candida antarctica* is a globular protein with an  $\alpha/\beta$  hydrolase fold. It consists of 317 amino acids and the molecular weight is 33 kDa [14]. The active site is buried in the core of the enzyme and the binding site has a funnel-like shape [15]. CALB has proven to be a particularly useful biocatalyst. It can catalyze a diverse range of reactions with high regio- and enantioselectivity. In the present work, native and immobilized lipase from *Candida antarctica* B was employed in kinetic resolutions of aliphatic secondary alcohols in

Anca Ursoiu is PhD student of the "Politehnica" University of Timisoara, Faculty of Industrial Chemistry and Environmental Engineering (Corresponding author: phone:+40-256-404234, fax:+40-256-403060, e-mail: anca.ursoiu@chim.upt.ro).

Cristina Paul is Teaching assistant of Industrial Biotechnologies in "Politehnica" University of Timisoara, Faculty of Industrial Chemistry and Environmental Engineering (e-mail: cristina.paul@chim.upt.ro).

Claudiu Marcu is M.Sc. student of the "Politehnica" University of Timisoara, Faculty of Industrial Chemistry and Environmental Engineering (e-mail: marcu.claudiu87@gmail.com).

Mihaela Ungurean is PhD student of the "Politehnica" University of Timisoara, Faculty of Industrial Chemistry and Environmental Engineering (e-mail: mihaela.ungurean@chim.upt.ro).

Francisc Péter is Professor of Industrial Biotechnologies in "Politehnica" University of Timisoara, Faculty of Industrial Chemistry and Environmental Engineering, C. Telbisz 6, 300001 Timisoara, Romania (e-mail: francisc.peter@chim.upt.ro).

different reaction media.

## II. MATERIALS AND METHODS

Lipase from *Candida antarctica* B was produced by C-Lecta (Leipzig, Germany). The silane precursors methyl-(MeTMS) and phenyl-trimethoxysilane (PhTMS) were purchased from Merck and tetramethoxysilane (TMOS) from Fluka. Other materials used: tris-(hydroxymethyl)-aminoethan (Loba Chemie), HCl 1N (Chimopar), 2-propanol (Merck), sodium fluoride (Fluka), Celite 545 (Merck), Celite 521 (Aldrich), Celite C22 (Loba Chemie), CaCO<sub>3</sub> (Loba Chemie), Celulose Avicel (Aldrich), 2-pentanol (Fluka), 2-hexanol (Fluka), 2-heptanol (Fluka), 2-octanol (Merck), 2-nonanol (Merck), vinyl acetate (Merck), *n*-hexane (98%, Merck), acetone (Merck), *iso*-octane 99.5% (Merck), methyl-*tert*-butyl-ether (MTBE) (99.2%, Rompetrol), tetrahydrofuran (THF) (Fluka), toluene (Chimopar), cyclohexane (Fluka), dioxane (Riedel de Haen), dichloromethane (Reactivul), *tert*-butanol (Merck) were of analytical grade and have been used as purchased. Purolite MN200 was a generous gift of Purolite, Romania. Decane (>99%, Aldrich) and dodecane (99%, Merck) were used as internal standards for quantitative gas-chromatographic analysis. Ionic liquid 1-octyl-3-methylimidazolium tetrafluoroborate [Omim]BF<sub>4</sub> was purchased from Fluka at the highest available purity.

### A. General Procedure for Immobilization by Sol-Gel Entrapment and Adsorption

A typical sol-gel immobilization process was used, which involves acid- or base-catalyzed hydrolysis, then polycondensation of alkoxy silane precursors [Si(OR)<sub>4</sub>] and organically modified silanes of the type R'-Si(OR)<sub>3</sub> (6 mmole) in the presence of additives to form a matrix in which the enzyme is encapsulated [10]. When the gelation process started, the solid support was blended with the gelling mixture. The obtained gel was kept for 24 h at room temperature to complete polymerization. The bulk gel was washed with isopropyl alcohol, distilled water and finally hexane, filtered and dried at room temperature. Finally, it was crushed in a mortar and kept in refrigerator.

### B. General Procedure for the Transesterification Studies

Acylation was performed in 4 mL capacity glass vials, charged with a mixture of 2-pentanol, 2-hexanol, 2-heptanol, 2-octanol or 2-nonanol (0.5 mmole), vinyl acetate (1.5 mmole), reaction medium (organic solvent, 1 mL) and free (5 mg) or immobilized lipase (25 mg).

The mixture was incubated using an orbital shaker (MIR-S100, Sanyo, Japan) at 300 strokes/min and 40°C (ILW 115 STD incubator, Pol-Eko-Aparatura, Poland). The conversion and enantiomeric excess of the product were assayed by gas-chromatography, on a Varian 450 instrument (Varian Inc., USA) equipped with flame ionization detector, using a 30 m x 0.25 mm Elite-Cyclosil B chiral column with 0.25 mm film thickness (Perkin-Elmer, USA). The analysis conditions were: oven temperature: 50° to 120°C with 10°C/min heating

rate, injector temperature 240°C, detector temperature 280°C, carrier gas (hydrogen) flow 1.2 mL/min. The reactions were usually run for 24 h. Conversions have been calculated based on the internal standard method. Transesterification activities were calculated at 24 h reaction time and expressed as the average 2-acetoxy-alcohol amount (in micromole) synthesized per hour by 1 mg of free or immobilized enzyme. The control reaction without enzyme did not give any product in the same conditions. To characterize the overall efficiency of the immobilization process, total activity yield was calculated as % of the total enzymatic activity recovered following immobilization, divided by the total activity of the lipase subjected to immobilization. The enantiomeric excess of the resulted ester product (*ee<sub>p</sub>*) was determined from the two enantiomers peak area, and the enantiomeric ratio (*E*) values were calculated based on conversion and *ee<sub>p</sub>* values using the relation [16]:

$$E = \frac{\ln[1 - C(1 + ee_p)]}{\ln[1 - C(1 - ee_p)]}, \text{ where } C \text{ represent the conversion at}$$

24 h.

## III. RESULTS AND DISCUSSION

Sol-gel entrapped enzymes show high operational stability, but some limitations may occur due to inadequate mass transfer through the three-dimensional matrix network. A simple and easy to use technique is adsorption on a solid support, but the weak interactions between the enzyme and support particles may result in reduced operational stability. An interesting method to increase the catalytic efficiency and thermal stability of sol-gel entrapped enzymes can be achieved by deposition of the entrapped biocatalyst on a support material. In this way, the advantages of these methods can be combined. The double immobilization method can be achieved by mixing the colloidal complex formed by the protein-macromer in the initial phase of gelation with the solid material (Fig. 1).

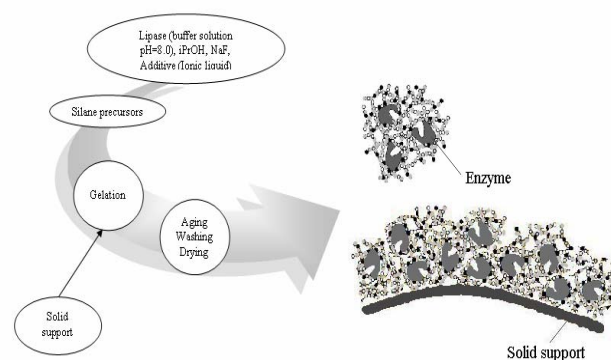


Fig. 1 General scheme for the sol-gel entrapment combined with adsorption

### A. Enantioselective acylation of aliphatic secondary alcohols with double immobilized biocatalysts

In this study, lipase from *Candida antarctica* B (CALB,

C-Lecta) was immobilized by the combined method of sol-gel entrapment with adsorption on solid supports, as described, and activities of the obtained prepartes were tested in the kinetic resolution of several secondary alcohols (from 2-pentanol to 2-nonanol) in organic solvents, at 40°C (Fig. 2).

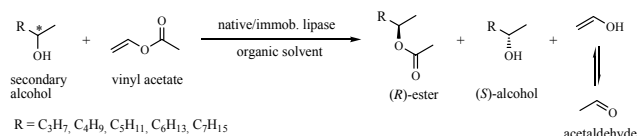


Fig. 2 Reaction scheme of secondary alcohols enzymatic acylation

Enantiomerically pure compounds can be obtained in various ways. One important approach is the kinetic resolution method. This process is based on the difference in reaction rates between enantiomers. For synthetic purposes, the enantioselectivity of the biocatalysts should be as high as possible in order to give the best optical purity and yield. The process of selecting an enzyme and optimizing the enantioselectivity towards the target compound is often laborious. Many alternatives may be considered to overcome

phenyltrimethoxysilane/methyltrimethoxysilane/tetramethoxy silane at 1.6/0.4/1 molar ratio, with 1-octyl-3-methyl-imidazolium tetrafluoroborate as additive [18]. Using different solid supports as adsorbents for the double immobilization of the CALB lipase we obtained enzymatic prepartes with 1.6/0.4/1 molar ratio of the silane precursors and 1-octyl-3-methyl-imidazolium tetrafluoroborate as immobilization additive. Excepting 2-pentanol, the enantiomeric ratios (E) obtained in the acylation reactions of secondary alcohols with immobilized enzymes were between 200 and 458, up to 10 times higher related to the native lipase. In the case of 2-pentanol we observed lower values of the transesterification activity and enantioselectivity (Table I).

TABLE I  
CATALYTIC EFFICIENCY AND ENANTIOSELECTIVITY OF NATIVE AND DOUBLE IMMOBILIZED CALB LIPASE IN ACYLATION OF SECONDARY ALCOHOLS WITH VINYL ACETATE IN *n*-HEXANE

Solid support	2-pentanol		2-hexanol		2-heptanol		2-octanol		2-nonanol	
	Activity <sup>a</sup>	E <sup>b</sup>	Activity <sup>a</sup>	E <sup>b</sup>	Activity <sup>a</sup>	E <sup>b</sup>	Activity <sup>a</sup>	E <sup>b</sup>	Activity <sup>a</sup>	E <sup>b</sup>
-	0.961	277	0.852	35	0.813	125	0.842	21	0.651	238
Celite 545	0.278	114	0.298	125	0.372	1057	0.448	125	0.412	1057
Celite 521	0.266	58	0.301	134	0.372	278	0.433	201	0.400	458
Celite C22	0.266	79	0.308	146	0.367	458	0.429	201	0.419	458
CaCO <sub>3</sub>	0.281	46	0.328	380	0.387	278	0.429	201	0.447	458
Purolite MN200	0.280	56	0.307	113	0.386	458	0.441	380	0.456	1057
Cellulose Avicel	0.210	412	0.204	49	0.228	296	0.220	38	0.253	285

<sup>a</sup> transesterification activity of secondary alcohol, at 24 h reaction time

<sup>b</sup> enantiomeric ratio

this problem and improve the enantioselectivity or the reaction rate. Reactions catalyzed by various types of hydrolases are predominant in biotransformations. Among hydrolytic enzymes, lipases are frequently used because they accept a broad range of substrates and often exhibit high enantioselectivity. Lipase-catalyzed reactions in organic solvents are becoming increasingly important in enantioselective synthetic chemistry, as certain reactions which are sensitive to water can be effectively carried out in organic media. There are several advantages of carrying the process in organic media, such as better solubility of substrates, easier recovery of product and enzyme, and finally, the enantioselectivity in organic solvents is often higher than that of corresponding hydrolytic reaction in water [17]. The biocatalytic properties of the sol-gel immobilized lipases are strongly influenced by the nature and concentration of the nonhydrolyzable alkyl or aryl groups of the silane precursors (PhTMOS and MeTMOS) used in combination with TMOS. Our previous studies showed that the best results regarding activity and enantioselectivity were obtained using

### B. Effect of solid support on the immobilization yield

Lipase B from *Candida antarctica* was immobilized by the combined method of sol-gel entrapment with adsorption on different solid supports, as described. Among the tested adsorbents, best values of the immobilization yield were obtained for the ion-exchange resin Purolite MN200 (94%), CaCO<sub>3</sub> (77%) and for Celite C22 (72%) (Table II). These high values of the immobilization yield were correlated with good transesterification activities of the immobilized prepartes in the studied reactions (Fig. 2).

TABLE II  
IMMOBILIZATION PARAMETERS

Solid support	Immobilized protein <sup>a</sup> (mg)	Immobilization yield <sup>b</sup> (%)	Enzyme loading <sup>c</sup> (%)
Celite 545	4.37	65	60
Celite 521	3.49	52	44
Celite C22	4.82	72	65
CaCO <sub>3</sub>	5.20	77	63
Purolite MN200	6.29	94	106
Cellulose AVICEL	4.56	68	63

<sup>a</sup> (mg initial protein) – (mg protein from washing waters)

<sup>b</sup> (mg immobilized protein) × 100 / (mg initial protein)

<sup>c</sup> (mg immobilized protein) × 100 / (mg obtained enzymatic prepare)

The recovered total activity values, which characterize the overall efficiency of the immobilized lipases, were excellent (from 250 to 560%) for all the obtained biocatalysts (Fig. 2).

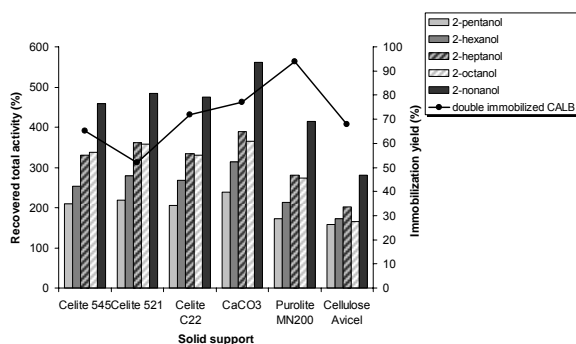


Fig. 2 Influence of solid support on the total activity of double immobilized CALB lipase used as biocatalyst in kinetic resolution of secondary alcohols in *n*-hexane.

### C. Solvent engineering of 2-pentanol kinetic resolution catalyzed by double immobilized *Candida antarctica* B lipase

Among the tested alcohols, lower values of the enantiomeric excess were obtained for 2-pentanol in *n*-hexane, especially when CaCO<sub>3</sub> was used as an immobilization adsorbent (Fig.3).

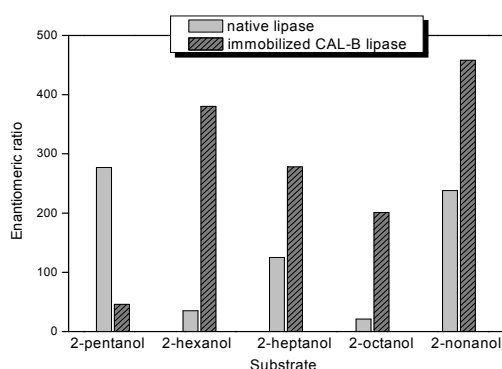


Fig. 3 Influence of substrate on the enantioselectivity of double immobilized lipase from *Candida antarctica* B in matrices with ternary silane precursor systems (PhTMOS, MeTMOS and TMOS at 1.6/0.4/1 molar ratio) and OmimBF<sub>4</sub> as additive and CaCO<sub>3</sub> as adsorbent

To increase the enantioselectivity in the acylation of 2-pentanol with vinyl acetate, we investigated the reaction in various organic solvents, using double immobilized CALB lipase obtained with PhTMOS, MeTMOS and TMOS as silane precursors at 1.6/0.4/1 molar ratio, OmimBF<sub>4</sub> as additive and CaCO<sub>3</sub> as adsorbent.

Selection of a suitable reaction medium is very difficult because some organic solvents can inactivate the enzyme. The highest conversion and activity at 24 h reaction time was achieved in toluene and the lowest in *tert*-butanol (Table III).

When non-polar solvents (hexane, cyclohexane and toluene) were used as reaction medium, we observed higher

TABLE III

INFLUENCE OF ORGANIC SOLVENT NATURE AND POLARITY ON CATALYTIC EFFICIENCY OF DOUBLE IMMOBILIZED CALB LIPASE IN ACYLATION OF 2-PENTANOL. SILANE PRECURSORS FOR IMMOBILIZATION WERE PhTMOS, MeTMOS AND TMOS (1.6/0.4/1 MOLAR RATIO) AND [Omim]BF<sub>4</sub> WAS EMPLOYED AS ADDITIVE

Reaction medium	E <sub>T</sub> <sup>N a</sup>	Conversion (%)	Activity <sup>b</sup>	e.e. <sub>p</sub> (%)	E <sup>c</sup>
Hexane	0.009	52	0.271	84	36
Cyclohexane	0.006	50	0.283	85	33
Toluene	0.099	55	0.290	81	49
1,4-Dioxane	0.164	52	0.257	91	104
MTBE	0.124	52	0.268	82	30
<i>iso</i> -octane	0.012	51	0.265	87	45
Acetone	0.355	52	0.223	95	75
THF	0.207	52	0.274	91	104
Dichlormethane	0.309	49	0.257	95	125
<i>tert</i> -butanol	0.389	44	0.240	93	60

<sup>a</sup> From Reference [19]

<sup>b</sup> transesterification activity of secondary alcohol, at 24 h reaction time

<sup>c</sup> enantiomeric ratio

values of the transesterification activity.

When acetone was employed as reaction medium, the transesterification activity was lower (Table III).

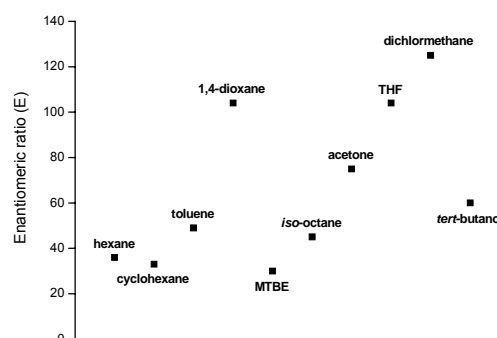


Fig. 4 Influence of the reaction media in acylation of 2-pentanol on enantioselectivity of the double immobilized CALB lipase in matrices with ternary silane precursor systems (PhTMOS, MeTMOS and TMOS at 1.6/0.4/1 molar ratio), OmimBF<sub>4</sub> as additive and CaCO<sub>3</sub> as adsorbent

The enantioselectivity of the immobilized prepare used in kinetic resolution of 2-pentanol was strongly influenced by the nature of the reaction medium. It was demonstrated that

solvent engineering could be a major factor to optimize the catalytic efficiency of lipase. Dichloromethane, dioxane, and THF, solvents with intermediate polarity, proved to be very efficient, leading to enantiomeric ratio values higher than 100, which can be considered an excellent kinetic resolution. Lower values of the enantiomeric ratio were obtained in non-polar solvents like hexane and cyclohexane (Fig. 4).

The enantioselectivity can not be explained only based on the polarity of the solvent, as important differences were observed between acetone and *tert*-butanol, solvents with comparable polarities.

#### IV. CONCLUSIONS

Using different solid supports as adsorbents for double immobilization of the CALB lipase, enzymatic preparates with excellent enantioselectivities towards the studied substrates have been obtained. The recovered total activity values, which characterize the overall efficiency of the immobilized lipases, were excellent for all the biocatalysts. The enantiomeric ratios were very high, over 230 for all tested preparates.

The enantioselectivity of the immobilized preparate used in kinetic resolution of 2-pentanol was strongly influenced by the nature of the reaction medium. High values of the enantiomeric ratio *E* were promoted by using solvents with intermediate polarity.

#### ACKNOWLEDGMENT

Financial support of CNCSIS-EUFISCSU, project number PNII-IDEI 368/2007 is acknowledged. This work was partially supported by the strategic grant ID 50783 POSDRU/88/1.5/S/50783 (fellowships of A. Ursoiu and M. Ungurean), of the Ministry of Labour, Family and Social Protection, and by the grant POSDRU/21/1.5/G/13798, inside POSDRU Romania 2007-2013, co-financed by the European Social Fund - Investing in People.

#### REFERENCES

- [1] T. Raghavendra, D. Sayania, and D. Madamwar, "Synthesis of the "green apple ester" ethyl valerate in organic solvents by *Candida rugosa* lipase immobilized in MBGs in organic solvents: Effects of immobilization and reaction parameters", *Journal of Molecular Catalysis B: Enzymatic*, vol. 63, 2010, pp. 31-38.
- [2] Y. Mine, L. Zhang, K. Fukunaga, and Y. Sugimura, "Enhancement of enzyme activity and enantioselectivity by cyclopentyl methyl ether in the transesterification catalyzed by *Pseudomonas cepacia* lipase co-lyophilized with cyclodextrins", *Biotechnology Letters*, vol. 27, 2005, pp. 383-388.
- [3] A. G. Cunha, A. T. da Silva, A. J. R. da Silva, L. W. Tinoco, R. V. Almeida, R. B. de Alencastro, A. B. C. Simas, and D. M. G. Freire, "Efficient kinetic resolution of ( $\pm$ )-1,2-*O*-benzyl-*myo*-inositol with the lipase B of *Candida antarctica*", *Tetrahedron: Asymmetry*, vol. 21, 2010, pp. 2899-2903.
- [4] J. H. Lee, K. Han, M.-J. Kim, and J. Park, "Chemoenzymatic Kinetic Resolution of Alcohols and Amines", *European Journal of Organic Chemistry*, 2010, pp. 999-1015.
- [5] V. Gotor-Fernandez, R. Brieva, and V. Gotor, "Useful biocatalysts for the preparation of pharmaceuticals", *Journal of Molecular Catalysis B: Enzymatic*, vol. 40, 2006, pp. 111-120.
- [6] H.-M. Chen, P.-Y. Wang, and S.-W. Tsai, "*Carica papaya* lipase-catalyzed transesterification resolution of secondary alcohols in organic solvents", *Journal of the Taiwan Institute of Chemical Engineers*, vol. 40, 2009, pp. 594-554.
- [7] A. Tomin, D. Weiser, G. Hellner, Z. Bata, L. Corici, F. Péter, B. Koczka, and L. Poppe, "Fine-tuning the second generation sol-gel lipase immobilization with ternary alkoxy silane precursor systems", *Process Biochemistry*, vol. 46, 2011, pp. 52-58.
- [8] N. Kharrat, Y. B. Ali, S. Marzouk, Y.-T. Gargouri, and M. Karra-Chaabouni, "immobilization of *Rhizopus oryzae* Lipase on silica aerogels by adsorption: Comparison with free enzyme", *Process Biochemistry*, 2011, doi:10.1016/j.procbio.2011.01.029.
- [9] F. Peter, L. Poppe, C. Kiss, E. Szocs-Biro, G. Preda, C. Zarcu, and A. Olteanu, "Influence of precursors and additives on microbial lipases stabilized by sol-gel entrapment", *Biocat. Biotrans.*, vol. 23, nr. 3/4, 2005, pp. 251-260.
- [10] C. Zarcu, L. Corici, R. Croitoru, A. Ursoiu, and F. Péter, "Preparation and properties of xerogels obtained by ionic liquid incorporation during the immobilization of lipase by the sol-gel method", *Journal of Molecular Catalysis B: Enzymatic*, vol. 65, 2010, pp. 79-86.
- [11] A. Uyanik, N. Sen, and M. Yilmaz, "Improvement of catalytic activity of lipase from *Candida rugosa* via sol-gel encapsulation in the presence of calix(aza)crown", *Bioresource Technology*, 2011, doi: 10.1016/j.biortech.2010.12.105.
- [12] F. Péter, C. Paul, and A. Ursoiu, "Application of ionic liquids to increase the efficiency of lipase biocatalysis", in "Ionic Liquids: Applications and Perspectives", edited by Alexander Kokorin, 2011, ISBN: 978-953-307-248-7, under publishing.
- [13] C. Mateo, J. M. Palomo, G. Fernandez-Lorente, J. M. Guisan, and R. Fernandez-Lafuente, "Improvement of enzyme activity, stability and selectivity via immobilization techniques", *Enzyme and Microbial Technology*, vol. 40, 2007, pp. 1451-1463.
- [14] H. P. Heldt-Hansen, M. Ishii, S. A. Patkar, T. T. Hansen, and P. Eigtved, "A new immobilized positional nonspecific lipase for fat modification and ester synthesis", *ACS Symposium Series*, vol. 389, 1989, pp. 158-172.
- [15] L. Brady, A. M. Brzozowski, Z. S. Derewenda, E. Dodson, G. Dodson, S. Tolley, J.P. Turkenburg, L. Christiansen, B. Høge-Jensen, L. Nørskov, L. Thim, and U. Menge, "A serine protease triad forms the catalytic centre of a triacylglycerol lipase", *Nature*, vol. 343, 1990, pp. 767-770.
- [16] C.-S. Chen, Y. Fujimoto, G. Girdeas, and C. J. Sih, "Quantitative analyses of biochemical kinetic resolutions of enantiomers", *Journal of the American Chemical Society*, vol. 104, 1982, pp. 7294-7299.
- [17] D. Zelazczyk, and K. Kiec-Konowicz, "Biocatalytic Approaches to Optically Active  $\beta$ -Blockers", *Current Medicinal Chemistry*, vol. 14, 2007, pp. 53-65
- [18] A. Ursoiu, C. Paul, C. Marcu, T. Péter, and F. Péter, "Binary and ternary silane precursor systems for immobilization of lipases", *Annals of West University of Timisoara*, vol. 16, no. 2, 2010, pp. 17-22.
- [19] C. Reichardt, "Solvatochromic Dyes as Solvent Polarity Indicators", *Chem. Rev.*, vol. 94, 1994, pp. 2319-2358.