STUDIA UBB CHEMIA, LVII, 2, 2012 (p. 129 - 137)

MULTI-SUBSTRATE KINETIC RESOLUTION SCREENING METHOD FOR LIPASE BIOCATALYSTS

MARTON ŐSZE[a], DIÁNA WEISER[a], GÁBOR HORNYÁNSZKY[a], LÁSZLÓ POPPE[a, b,*]

ABSTRACT. Development of efficient screening methods has increasing significance in rapid evaluation of novel biocatalysts. Our study reveals the scopes and limitations of a novel GC-based multi-substrate screening method for initial characterization of the activity and selectivity of lipase biocatalysts. The multi-substrate kinetic resolution of four different racemic alcohols **1-4a** by native and immobilized biocatalysts were analyzed by GC using enantiomer selective stationary phase.

Keywords: *lipase, immobilization, biocatalysis, multi-substrate screening*

INTRODUCTION

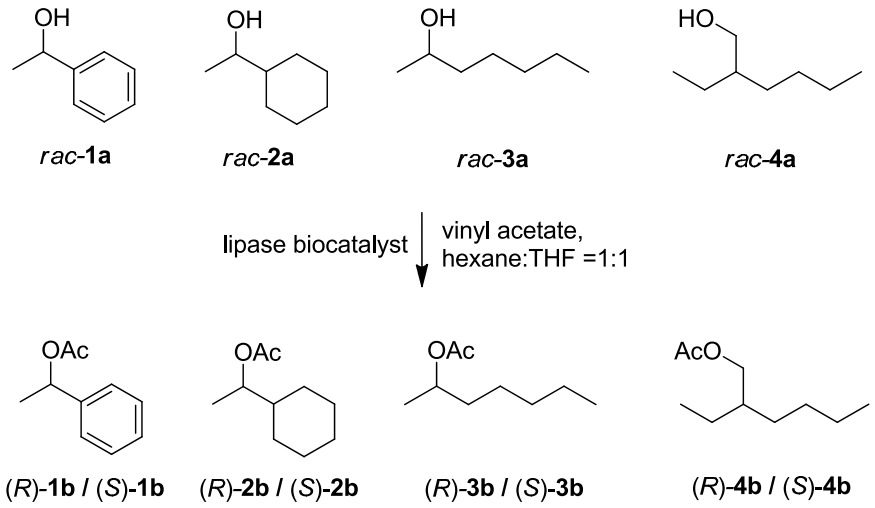
The use of enzymes in industrial processes is of growing interest due to its potential for the straightforward selective synthesis of many complex chemicals [1]. Discovery of novel biocatalysts or further development and optimization of the already known ones require efficient and robust screening methods. Our goal was to develop a rapid and efficient method for screening various lipases in their native or immobilized form to select the most promising biocatalyst candidates for enantioselective biotransformations.

An example was found for HPLC-based multi-substrate screening of lipase-catalyzed kinetic resolution of arylalkylethanols with succinic anhydride as acylating agent [2]. In our hands, a GC-based multi-substrate kinetic resolution method proved to be efficient in testing the bioimprinting effect in sol-gel immobilization of lipases [3]. The multi-substrate mixture consisted of a series of racemic, aliphatic alcohols of various chain lengths. In this study our goal was to explore the generality, the scopes and limitations of the GC-based multi-substrate kinetic resolution screening method to characterize the biocatalytic performance of various forms of lipases.

[a] Budapest University of Technology and Economics, Department of Organic Chemistry and Technology, and Research Group for Alkaloid Chemistry of HAS, Műegyetem rkp 3, H-1111, Budapest, Hungary, * poppe@mail.bme.hu
[b] SynBiocat Ltd., Lázár Deák u. 4/1, H-1173 Budapest, Hungary

RESULTS AND DISCUSSION

Four different racemic alcohols of (*rac*-**1-4a**) were selected as components of a multi-substrate system for rapid screening the biocatalytic behavior of various lipase biocatalysts (*Scheme 1*).



Scheme 1. The multi-substrate screen for lipases

The test system for screening lipase biocatalysts was based on acylation reaction of a mixture of four racemic alcohols, namely 1-phenylethanol (*rac*-**1a**), 1-cyclohexylethanol (*rac*-**2a**), 2-heptanol (*rac*-**3a**) and 2-ethyl-1-hexylethanol (*rac*-**4a**). Importantly, all enantiomers of the test substrates **1-4a** and all enantiomers of the product acetates **1-4b** proved to be separable by GC in reasonably short time (*Figure 1*).

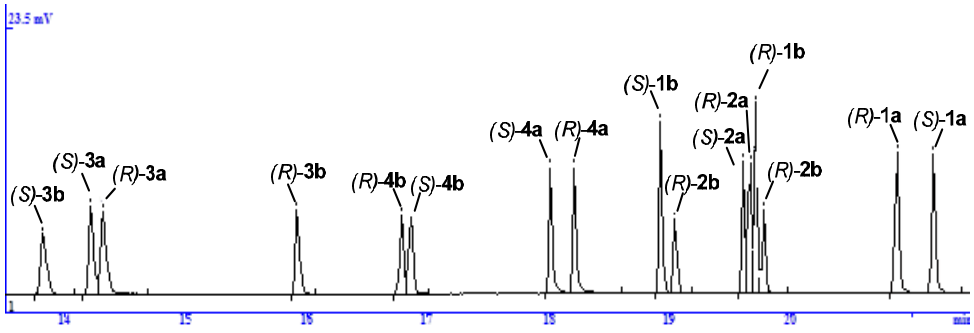


Figure 1. A chromatogram of rac-**1-4a** and rac-**1-4b** by enantiomer selective GC

After adjusting the GC method by racemic substrates, we demonstrated the performance of this method by using commercial and in-house-made lipase biocatalysts.

First, the method for was validated by comparing single-substrate (*1SS*) and the two-substrate screening (*2SS*) results. The kinetic resolution of 1-phenylethanol (rac-**1a**) and 2-heptanol (rac-**3a**) with lipase AK as biocatalyst were carried out in hexane:tetrahydrofuran solvent mixture using vinyl acetate as acylating agent. Good accordance were found in the conversion (*Figure 2*) and the enantiomeric excess (*Figure 3*) of single-substrate and two-substrate systems.

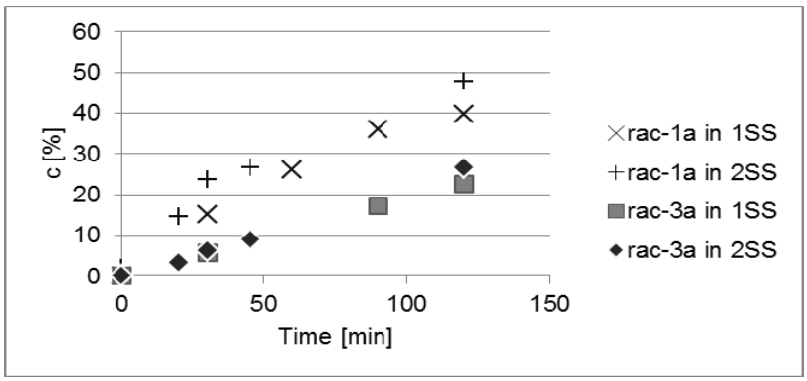


Figure 2. Comparison of the conversion course of the single (1SS) and the two-substrate (2SS) reactions by lipase AK catalyst

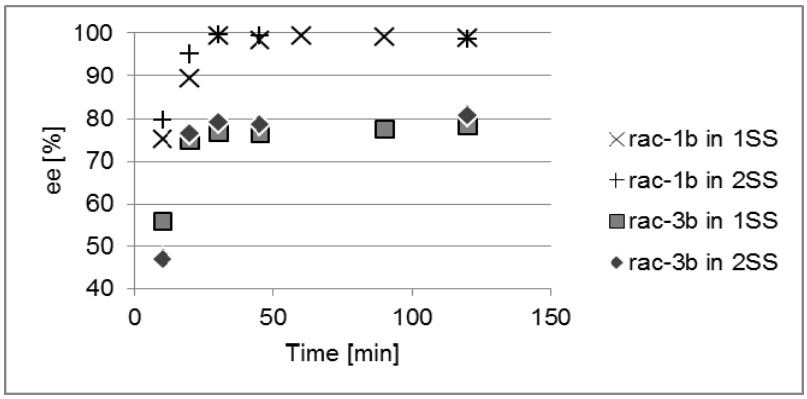


Figure 3. Comparison of the enantiomeric excess of products **1b** and **3b** of the single- (1SS) and the two-substrate (2SS) reactions by lipase AK catalyst

Next, a hydrolysis test with **1b** and **3b** was performed to determine its influence on the screening method (*Figure 4*). The hydrolysis test indicated that *rac*-**1b** hydrolyzed rapidly: more than 10% of the racemic acetate was transformed into its alcohol form (*R*)-**1a** after 8 hours due to the sensitivity of the forming ester (*R*)-**1b** to hydrolysis (i.e. the reverse process of the screening reaction). Consequently, only the results from the first 4 hours period were considered as reliable in our further investigations.

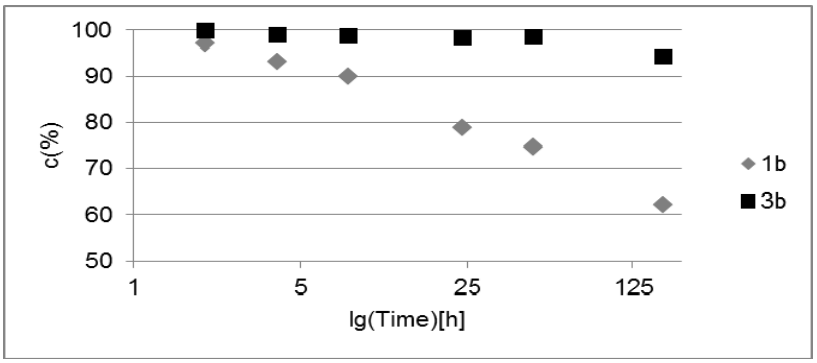


Figure 4. Hydrolysis of **1b** and **3b** catalyzed by lipase AK

All tested lipases proved to be active but non-selective towards *rac*-**4a** (0% ee for both **4a** and **4b**). Therefore, for sake of better transparency, the results with **4a** will not be shown in the following diagrams. Our multi-substrate test after 4 hours revealed that four lipases BUTE 3A, BUTE 3B, AK or NOV435 were significantly more active than other lipases (AYS, CCL, M, PPL, PS, R and TL IM) (*Figure 5*).

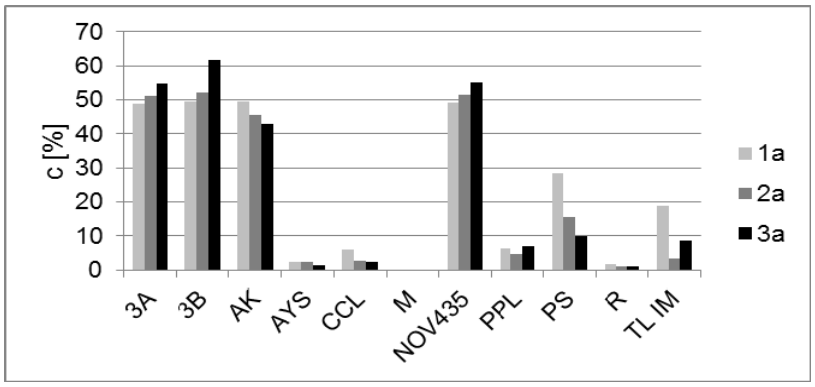


Figure 5. Conversions of **1-3a** with in-house-made and commercial lipases (4 h)

Most of the lipases were quite selective towards *rac*-**1a** and *rac*-**2a**, and less selective towards the acyclic alcohol *rac*-**3a** (*Figure 6*). Among the tested lipases, AYS, CCL, M, PPL and R showed significantly lower selectivity than the others (<80% ee for **1-3b** after 4 h) (*Figure 6*).

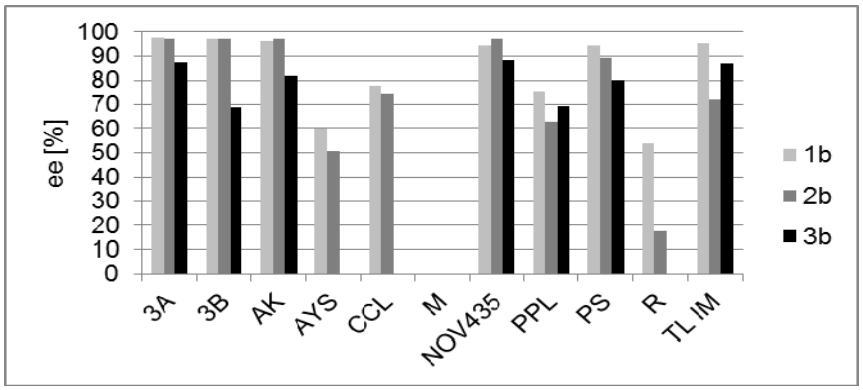


Figure 6. Enantiomeric excess of **1-3b** using in-house-made and commercial lipases (4 h)

Finally we demonstrated the performance of our method by characterization of eight various sol-gel immobilized lipase AK preparations (*Table 1*) by their productivity (*Figure 7*) and selectivity (*Figure 8*).

Table 1. Composition of the sol-gel immobilized lipase AK enzymes

Code	Composition		
173	Octyltriethoxy-silane:Phenyltriethoxy-silane:Tetraethoxy-silane preadsorbed on Celite	=	0.7:0.3:1
179	Octyltriethoxy-silane:Phenyltriethoxy-silane:Tetraethoxy-silane	=	0.7:0.3:1
188	Octyltriethoxy-silane:Phenyltriethoxy-silane preadsorbed on Celite	=	1:1
250	Octyltriethoxy-silane:Phenyltriethoxy-silane:Tetraethoxy-silane preadsorbed on Celite	=	0.6:0.4:1
251	Octyltriethoxy-silane:Phenyltriethoxy-silane:Tetraethoxy-silane preadsorbed on Celite	=	0.5:0.5:1
260	Propyltriethoxy-silane:Phenyltriethoxy-silane:Tetraethoxy-silane preadsorbed on Celite	=	0.5:0.5:1
261	Propyltriethoxy-silane:Phenyltriethoxy-silane:Tetraethoxy-silane preadsorbed on Celite	=	0.4:0.6:1
263	Propyltriethoxy-silane:Phenyltriethoxy-silane:Tetraethoxy-silane preadsorbed on Celite	=	0.2:0.8:1

The native lipase AK showed the highest activity but with moderate selectivity. The decrease in specific activity of the sol-gel biocatalysts is quite understandable as they contain considerably lower amount of enzyme entrapped in the sol-gel matrix than that present in the native form.

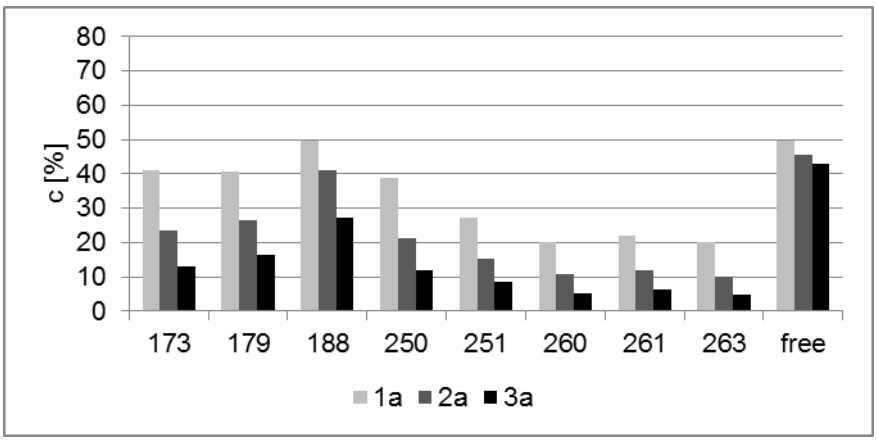


Figure 7. Conversions of **1-3a** with sol-gel immobilized and native lipase AK (4 h)

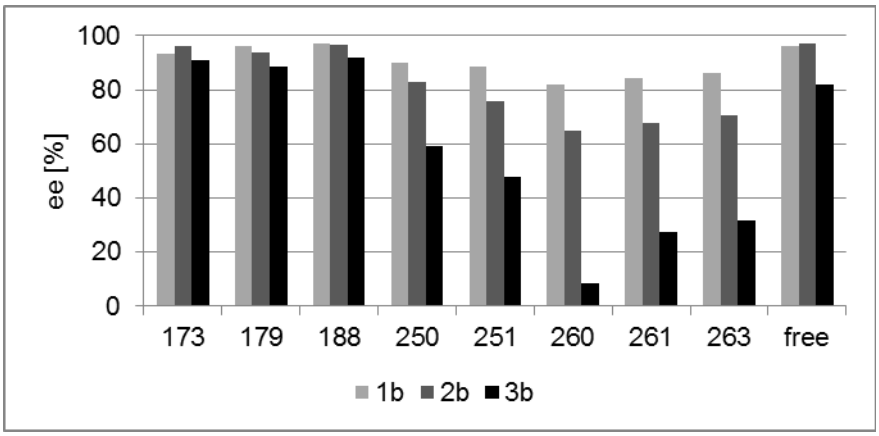


Figure 8. Enantiomeric excess of **1-3b** with sol-gel immobilized and native lipase AK (4 h)

In accordance with previous results [4,5], binary or ternary mixtures of silane precursors enabled the fine-tuning of matrix hydrophobicity of the forming sol-gel matrices. The most hydrophobic preparations (173, 179 and 188) were the most active and most selective forms on substrates *rac*-**1-3a** (*Figure 7* and *8*). Celite support allowed to omit the less hydrophobic tetraethoxy-silane precursor and to prepare the most hydrophobic matrix (188) which in fact resulted in the most productive and selective biocatalyst (*Figure 7* and *8*).

Decreasing the aliphatic character of the forming matrix (250, 251 and especially the propyl group containing systems 260, 261, 263) resulted in diminished activity and decreased selectivity towards aliphatic substrates **2a** and **3a** (*Figure 7* and *8*).

CONCLUSIONS

A rapid multi-substrate screening method was developed to characterize lipase biocatalyst based on enantiomer selective GC analysis of a reaction system including four racemic alcohols. Thorough comparison of the results from multi-substrate screening with single and two-substrate reactions revealed that the method was appropriate for rapid screening of the productivity and enantiomer selectivity of the lipases. This method, however was not adequate for exact determination of activity, selectivity, particularly at longer reaction times (over 4 h, due to reversible reaction of the forming ester **1b**). The robustness of the method was demonstrated by analysis of nine commercial and two in-house-made lipases. The fine-tunability of sol-gel biocatalysts was demonstrated also by the multi-substrate screening of lipase AK biocatalysts entrapped in eight various sol-gel matrices.

EXPERIMENTAL SECTION

Chemicals and enzymes

Racemic 1-phenylethanol *rac*-**1a**, racemic 1-cyclohexylethanol *rac*-**2a**, racemic 2-heptanol *rac*-**3a**, racemic 2-ethyl-1-hexanol *rac*-**4a**, vinyl acetate, acetyl chloride, triethylamine, tetrahydrofuran, hexane, toluene, acetone were products of Sigma Aldrich or Fluka. Lipase AK, Lipase PS, Lipase AYS, Lipase M and Lipase R were obtained from Amano Europe. Novozym 435 and Lipozyme TL IM were products of Novozymes, Denmark. *Mucor miehei* lipase, Lipase PPL, Lipase CcL were purchased from Sigma. Immobilized lipase from *Candida rugosa* (CRL T2-150) was obtained from Chiral Vision. Immobilized lipase PS was a kind gift of Iris Biotech GmbH. Lipases from a thermophilic fungi (BUTE 3A, BUTE 3B) were obtained from Fermentia Ltd [6]. The sol-gel immobilized lipases were prepared from native lipase AK as described earlier [4,7] (compositions are shown in Table 1).

Analytical methods

TLC was carried out on Kieselgel $60F_{254}$ (Merck) sheets. Spots were visualized under UV light (Vilber Lourmat VL-6.LC, 254 nm and 365 nm) or by treatment with 5% ethanolic phosphomolybdic acid solution and heating of the dried plates. GC analyses were carried out on Younglin ACME 6100 or

Agilent 4890D instruments equipped with FID detector and Hydrodex-β-TBDA column (50 m × 0.25 mm × 0.25 μm film with heptakis-(2,3-di-*O*-acetyl-6-*O*-*t*-butyldimethylsilyl)-β-cyclodextrin; Macherey&Nagel) using H_2 carrier gas (injector: 250°C, detector: 250°C, head pressure: 10 psi, 50:1 split ratio, column oven: 60°C/10 min, 60-130°C 7°C/min, 130°C/2 min). Retention times of the enantiomers of the four substrates **1-4a** and for the enantiomers of the four esters **1-4b** are listed in Table 2.

Table 2. Retention times of substrates and products

Compound	(*S*)-**3b**	(*S*)-**3a**	(*R*)-**3a**	(*R*)-**3b**	(*R*)-**4b**	(*S*)-**4b**	(*S*)-**4a**	(*R*)-**4a**
t_R (min)	13.81	14.20	14.30	15.91	16.78	16.86	18.00	18.20

Compound	(*S*)-**1b**	(*S*)-**2b**	(*S*)-**3a**	(*R*)-**3a**	(*R*)-**4b**	(*R*)-**2b**	(*R*)-**1a**	(*S*)-**1a**
t_R (min)	18.91	19.03	19.60	19.65	19.70	19.77	20.87	21.17

Preparation of the racemic acetates (rac-*1-4b*)

Acetyl chloride (6.25 mmol) was added dropwise to a solution of the racemic alcohol (*rac*-**1-4a**, 5 mmol) and triethylamine (7.5 mmol) in dry dichloromethane (10 mL) under argon atmosphere at 0°C and the reaction mixture was stirred for 2 h at this temperature. Then the resulting mixture was extracted with 5% HCl (35 mL), saturated $NaHCO_3$ (35 mL) and 40 mL saturated NaCl (40 mL), dried on Na_2SO_4. After evaporation of the solvent in vacuum, the residue was purified by column chromatography over silica gel (hexane:acetone 50:1) to give the corresponding product (*rac*-**1-4b**) as colorless oil.

Hydrolysis test for the acetates rac-*1b* and rac-*3b*

The racemic acetate (*rac*-**1b** or *rac*-**3b**, 50 μL) and lipase AK (50 mg) were added to a mixture of water saturated hexane and tetrahydrofuran (in 1:1 ratio; 2 mL) and mixture was shaken in screw-capped glass vial at 1000 rpm and 30°C. Samples (50 μL) were taken regularly (at 1, 2, 4, 8, 16 and 24 h). The diluted samples (with hexane, 500 μL) were analyzed by GC on Hydrodex-β-TBDA column.

Single-substrate tests with alcohols rac-*1-4a*

The racemic alcohol (*rac*-**1a**, *rac*-**2a**, *rac*-**3a** or *rac*-**4a;** 50 μL, each), vinyl acetate (100 μL) and the lipase biocatalyst (50 mg, in each case) were added to a mixture of hexane:tetrahydrofuran (in 1:1 ratio; 2 mL) and the resulting mixture was shaken in screw-capped glass vial at 1000 rpm and

30°C. Samples (50 µL) were taken regularly (at 1, 2, 4, 8, 16 and 24 h). The diluted samples (with hexane, 500 µL) were analyzed by GC on Hydrodex-β-TBDA column.

Multi-substrate screening of lipases with a mixture of alcohols rac-*1-4a*

An equimolar mixture of the racemic alcohols (*rac*-**1a**, *rac*-**2a**, *rac*-**3a** and *rac*-**4a**; 50 µL), vinyl acetate (100 µL) and the lipase biocatalyst (50 mg, in each case) were added to a mixture of hexane:tetrahydrofuran (in 1:1 ratio; 2 mL) and the resulting mixture was shaken in screw-capped glass vial at 1000 rpm and 30°C. Samples (50 µL) were taken regularly (at 1, 2, 4, 8, 16 and 24 h). The diluted samples (with hexane, 500 µL) were analyzed by GC on Hydrodex-β-TBDA column.

ACKNOWLEDGEMENTS

This research work was supported by the Hungarian National Office for Research and Technology (NKFP-07-A2 FLOWREAC) and is related also to the scientific program of "*Development of quality-oriented and harmonized R+D+I strategy and functional model at BME*" project (TÁMOP-4.2.1/B-09/1/KMR-2010-0002), supported by the New Hungary Development Plan. The authors thank Dr. György Szakács (Budapest University of Technology and Economics, Hungary) and Dr. Balázs Erdélyi (Fermentia Ltd, Budapest, Hungary) for the BUTE 3 lipase preparations.

REFERENCES

1. Tholey, A.; Heinzle, E., *Advances in Biochemical Engineering / Biotechnology,* **2002**, *74*, 1-19.
2. Debbeche, H.; Toffano, M.; Fiaud, J.-C.; Aribi-Zouioueche, L., *Journal of Molecular Catalysis B: Enzymatic,* **2010**, *66*, 319-324.
3. Hellner, G.; Boros, Z.; Tomin, A.; Poppe, L., *Advanced Synthesis & Catalysis,* **2011**, *353*, 2481-2491.
4. Tomin, A.; Weiser, D.; Hellner, G.; Bata, Z.; Corici, L.; Péter, F.; Koczka, B.; Poppe, L., *Process Biochemistry,* **2011**, *46*, 52-58.
5. Weiser, D.; Boros, Z.; Hornyánszky, G.; Tóth, A.; Poppe, L., *Process Biochemistry,* **2012**, *47*, 428-434.
6. Bódai, V.; Peredi, R.; Bálint, J.; Egri, G.; Novák, L.; Szakacs, G.; Poppe, L., *Advanced Synthesis & Catalysis,* **2003**, *345*, 811-818.
7. Weiser, D.; Tomin, A.; Poppe, L., *Studia UBB Chemia,* **2010**, *55*, 283-288.