RSC Advances

ROYAL SOCIETY OF CHEMISTRY

www.rsc.org/advances

Lipase Immobilization Towards Improved Productivity on Kinetic Resolutions by Continuous-Flow Process

Marcus V.M. Silva, [a] Jonathan Bassut, [a] Ivaldo I. Junior, [b] Stefania P. de Souza, [a] Melissa L. G. Estrada, [b,c] Leandro S. M. Miranda, [a] Rodrigo O. M. A. de Souza [a*]

a- Biocatalysis and Organic Synthesis Group, Federal University of Rio de Janeiro, Chemistry Institute, Rio de Janeiro, Brazil. b- School of Chemistry, Federal University of Rio de Janeiro, Rio de Janeiro, Brazil. c- Campus Xerém, Federal University of Rio de Janeiro, Rio de Janeiro, Brazil

Keywords: Biocatalysis, Lipases, Continuous-Flow, Kinetic resolution, Immobilization

Abstract: Development of asymmetric transformations for the synthesis of chiral molecules has achieved great results in the last decade. Besides the great evolution achieved during the recent years into the biotechnology and biocatalysis field, industrial process using enzymatic approach are few and a more broad application of such technology towards the synthesis of chiral molecules is still under development. Here in we report our results on the immobilization of lipase B from *Candida antarctica* on five different commercial supports for the development of a continuous-flow kinetic resolution of alcohol. Immobilization on Accurel MP1000 gave the best results arriving on productivities around 140g/h.g of protein and the immobilized biocatalyst could be recycled for 5 times without significant loss of activity.

Introduction

Chirality is well recognized as an important feature for organic molecules but looking backward, until 1990, 90% of the active pharmaceutical ingredients (API's) available in the market were still racemic mixtures. [1] Fifteen years later the scenario was completely different and 39% of the commercial API's were a single-enantiomer drug (75 billion dollars market) with a prediction of 10.2% of increase per year reaching more then 100 billion dollars by 2015.[2,3]

Development of asymmetric transformations for the synthesis of chiral molecules has achieved great results in the last decade. Among the different transformations developed so far, successful strategies towards the synthesis of chiral alcohols are very important for organic chemistry. The synthesis of chiral alcohols can be done by different asymmetric strategies, being the most important, the aldol reaction, [4-7] epoxide opening, [7-10] ketone hydrogenation, [11-14] kinetic / dynamic resolution, [15-17] among others (Figure 1). It is important to note that most of the asymmetric chemical catalysts used to afford the desired chiral alcohol can find an equivalent on enzyme catalysis.

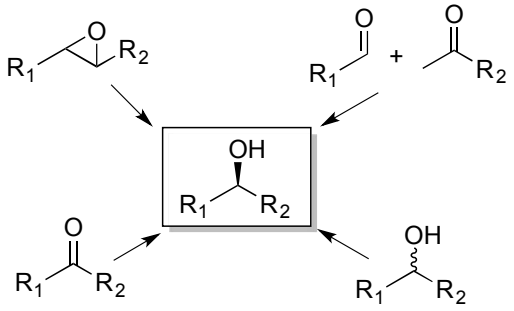


Figure 1: Different strategies for asymmetric synthesis of chiral alcohols.

Besides the great evolution achieved during the recent years into the biotechnology and biocatalysis field, industrial process using enzymatic approach are few and a more broad application of such technology towards the synthesis of chiral

molecules is still under development. Besides the benefits of working with biocatalysts the cost associate with such technology are still high, making recyclability and productivity important issues on the development of a biocatalytic process.

In order to improve recyclability, immobilization of enzymes is mandatory and different techniques can be found over literature being adsorption, when the protein is linked to the support by hydrophobic interactions, the easiest, cheapest and most common way of producing an immobilized lipase. [18-23] To improve productivity, one of the technologies that have gained attention recently is continuous-flow process [24,25] by the use of packed bed reactors. Mass transfer can be enhanced by continuous-flow protocols leading to an increase on productivity of biocatalyzed continuous-flow processes. [4,14,22,26-29]

During the recent years our group has focus attention on the development of continuous-flow protocols for biocatalyzed reactions aiming to improve recyclability and productivity of different process. Here we report our results on the immobilization of lipase B from *Candida antarctica* in five different hydrophobic commercial supports for the development of a continuous-flow kinetic resolution process. The developed process was compared to the traditional batch reactors in terms of productivity and also with a commercial immobilized enzyme in order to verify the efficiency of the new biocatalyst.

Materials and Methods

Materials. Novozym 435 (*Candida antarctica* lipase B immobilized on macroporous acrylic resin type), purchased from Novozymes S/A, Amano Lipase AK and Lipase PS-C Amano I purchased from Sigma-Aldrich. IB-EC1 and IB-S861 purchased from Immobead, ECR1091F and ECR8806F purchased from Purolite. All other materials were at least reagent-grade. Free *Candida antarctica* lipase B phosphate buffer solution was purchased from Novozymes S/A.

Experimental Design. An experimental design was used aiming to evaluate the influence of temperature, enzyme concentration and the proportion of acyl donor/receptor on the kinetic resolution of 1-phenylethanol (real and coded values on table 1). The experimental design and results analysis were carried out using the software Statistica 6.0 (Statsoft, Inc., USA), according with the significance level established to obtain the mathematical model. The significance of the regression coefficients and the associated probabilities, p(t), were determined by Student's t test; the model equation significance was determined by Fisher's F test. The variance is given by the multiple determination coefficients, R^2.

Table 1- Real and coded values for the independent variables.

Variables	-1	0	+1
temperature (^{0}C)	50	60	70
[E] (%)	5	10	15
acylating agent proportion / alcohol (mol/mol)	1/1	2/1	3/1

* ethyl acetate, isopropenyl acetate and vinyl acetate.

**1-phenylethanol

Kinetic resolution. **Batch Reaction:** *rac*-1-Phenylethanol (122mg, 0,12ml), acyl donor acetate (1-3 mol. eq.), and 5 - 15 % w/w of the corresponding immobilized enzyme were reacted in cyclohexane (3 mL) for 2 h at 50 - 70°C. **Continuous flow reaction:** An equimolar stock solution of 1-phenylethanol and vinyl acetate in cyclohexane was prepared (0.33M final concentration). The starting mixture was stirred for 5 min while the instrument Asia Flow Reactor was equipped with Omnifit (6.6mm x 100mm with 0.3421cm^2 base and height 3cm column) containing the immobilized lipase (1g). The reaction parameters were selected on the flow reactor, and processing was started, where by only pure solvent was umped through the system until the instrument had achieved the desired reaction parameters and stable processing was assured. Enantiomeric excess values (ee) were determined by chiral GC analysis (chiral column Betadex-325).

Immobilization conditions. Before immobilization, supports were kindly washed with ethanol and water. 6 mL of the enzyme solution (20mg of protein per gram of support) was dissolved in 29 mL of 0.02 M phosphate buffer pH 7.0, and added to 1 g, support (Accurel MP1000, IB-EC1, IB-S861, ECR1091F and ECR8806F). The mixture was stirred for 30 h, at room using a stirrer Roller type, during the appropriate time. New biocatalysts were filtered and dried under vacuum and drying over night at room temperature. Samples were collected during immobilization process and submitted do lipase activity by *p*-nitrophenylbutyrate assay. Immobilization efficiency was evaluated by the difference between initial amount of enzyme added and that in the supernatant after filtration of the immobilized enzyme.

Esterification reactions. The immobilized lipase (10 mg of support in 1 mL of reaction media) was evaluated in an esterification reaction between oleic acid and

ethanol (1:1 – 100 mM in n-heptane) at different temperatures. The reactions were performed in cryotubes under 200 rpm of agitation on a shaker. Samples (10 μL) were collected after 30, 60 and 120min. All quantifications were done by GC-MS analysis.

GC analysis. All GC-MS measurements were carried out in duplicate using a DB 5 (Agilent, J&W. Scientific®, USA) capillary column (30 m × 0.25 mm × 0.25 μm). The injector and detector temperatures were 250°C, and the oven temperature was constant at 60°C for 1 min, and then increased by 10 °C/min to 250°C, where it was held constant for 3 min. The percentages of conversion and selectivity were analyzed by the area on the chromatograms. GC-FID: chiral column Betadex-325 capillary column 1μL samples were injected at 100ºC. The oven was heated at 15ºC/min to 150 ºC, at 8ºC/min to 200ºC, at 2ºC/min to 240ºC, and then maintained for 4min. After this, the oven was heated at 15ºC/min to 300ºC.

Thermo gravimetric analysis. The TG curves were obtained in a thermogravimetric module, coupled in a thermal analyzer, both manufactured by Netzsch®. Thermogravimetric measurements were performed using a platinum sample holder containing about 10 mg of each immobilized enzyme. Each sample was heated from 35 to 600 °C at 10 °C min^{-1}, under atmosphere of synthetic air and N2, both with a flow rate of 60 mL min^{-1}.

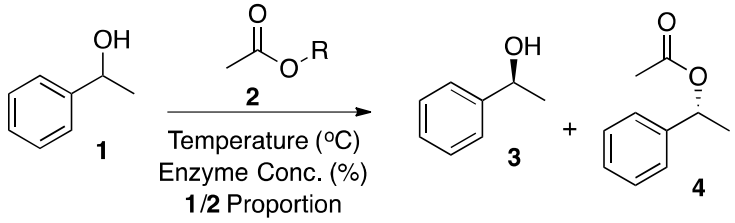
Infrared analysis. Analysis by infrared spectroscopy used a Shimadzu 8300 FTIR spectrophotometer. The spectrum was obtained with 32 scans and with 4 cm−1 of resolution. For the analysis, 10 mg of sample was placed in sample collector to form tablets of approximately 2 mm of thick and 5 mm in diameter, without KBr addition.

Scanning electron microscopy (SEM) analysis. The supported had their structure analysed by scanning electron microscopy (SEM) using a Zeiss EVO® 50H

microscope. All micrographs were obtained from the fractured surfaces of SECs coated with gold, prepared using a Shimadzu® sputter equipment.

Results and Discussion

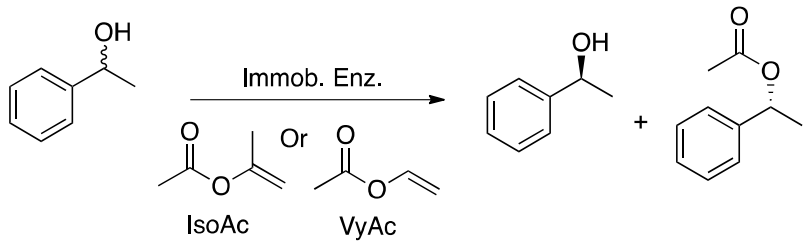
We began our work on the optimization of reaction parameters, by the use of a three level full factorial design, in order to tune reaction temperature (50-70 ^{o}C), enzyme concentration (5-10%) and 1-phenylethanol / acylating agent proportion (1:1 to 1:3) under batch conditions for the immobilized commercial enzymes Novozyme 435, Amano Lipase AK, Lipase PS-C Amano I, which will be used as a positive standard for our immobilized enzymes (Scheme 1). The optimization mentioned above was performed for three different acyl donors: ethyl acetate, isopropenyl acetate and vinyl acetate.



Scheme 1: General scheme for kinetic resolution

Results obtained from this reaction optimization have shown that ethyl acetate leads to good conversion but very low enantiomeric ratios (*E*), even when Novozyme 435 was used. Changing the acyl donor to isopropenyl acetate (IsoAc) or vinyl acetate (VyAc), better results of conversion and enantiomeric ratio, can be obtained for all immobilized enzymes as shown on Table 2.

Table 2: Selected results for reaction optimization with Novozyme 435 (N435), Amano Lipase AK (AK) and Lipase PS-C Amano I (PSC).



Entry	Enzyme	Acyl Donor	Temp. (^{0}C)	[E] (%)	Ratio acyl donor/alcohol	Conversion (%)	*E*
1	PSC	IsoAc	70(+1)	5(-1)	1/1(-1)	52	46
2	PSC	VyAc	70(+1)	15(+1)	3/1(+1)	64	25
3	N435	IsoAc	50(-1)	15(+1)	1/1(-1)	50	175
4	N435	VyAc	50(-1)	15(+1)	1/1(-1)	51	194
5	AK	IsoAc	70(+1)	15(+1)	3/1(+1)	25	2
6	AK	VyAc	50(-1)	15(+1)	1/1(-1)	33	62

Reaction conditions: *rac*-1-Phenylethanol (122mg, 0,12ml), acyl donor acetate (1-3 mol. eq.), and 5 - 15 % w/w of the corresponding immobilized enzyme were reacted in cyclohexane (3 mL) for 2 h at 50 - 70°C.

Initial screening shown on Table 2 revel that both Amano Lipase AK and Lipase PS-C Amano I do not present satisfactory results arriving at very poor conversions and enantiomeric ratios (*E*) while Novozyme 435 can lead to the formation of the desired product in high yields and selectivity. The best reaction condition were similar to vinyl and isopropenyl acetate where 50^{o}C, 15% immobilized enzyme (w/w) and 1:1 proportion between substrate (**1**) and acylating agent (**2**) gave the best results.

With these results in hands we decided to start the immobilization of Lipase B from *Candida Antarctica* (Cal-B) into 5 different commercial supports (Accurel MP1000, IB-EC1, IB-S861, ECR1091F and ECR8806F) by hydrophobic interaction (See supporting information for further details).

Immobilization procedure was followed by 30 hours in order to define the reaction time needed for maximum protein incorporation into the support. The behavior of each enzyme during the immobilization procedure is given in Figure 2.

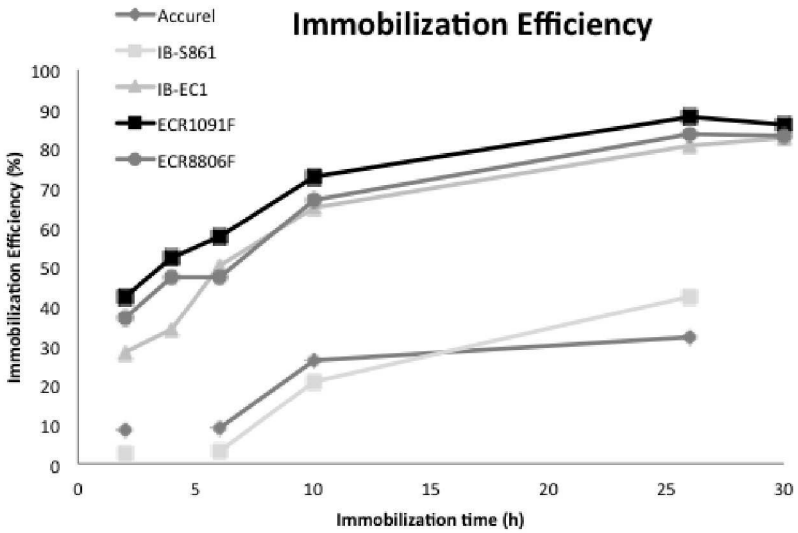


Figure 2: Immobilization efficiency during 30h.

From the data presented on Figure 2 is possible to observe that macroporous styrene (ECR1091F), polystyrene (IB-EC1) and octadecyl methacrylate (ECR8806F) have the highest amount of protein incorporation, around 80% after 26 hours. For both ECR1091F and ECR8806F the explanation for this high efficiency can be related to the hydrophobicity and also to the support porous diameter of each material that can be on the range of 950-1220 and 500-700 (Å), respectively. Unfortunately, Accurel MP1000 and IB-S861 did not lead to a high immobilization efficiency reaching 32 and 42% of protein incorporation respectively. All immobilized biocatalysts were characterized by infrared spectroscopy, thermogavimetric analysis (TG) and scanning electron microscopy (SEM). Table 3 presents an additional data for the immobilization procedure showing the amount of protein loaded into each support

Support	Immobilization Efficiency (%)	mg of protein /g of support
Accurel MP1000	32	6.4
IB-EC1	80	16.1
IB-S861	42	8.5
ECR1091F	88	17.6
ECR8806F	83	16.7

Immobilization conditions: 20mg of protein per gram of support in phosphate buffer, pH 7.0, 20mM for 26 hours at room temperature

Table 3: Protein loading after 26 hours of immobilization procedure.

Characterization of the immobilized biocatalysts was performed in order to enable reproducibility of the immobilization procedure. Infrared (IR) analysis shows the presence of protein on the support but thermogravimetric (TG) results do not show significant changes between sampels before and after immobilization, probably due to small amount of protein loaded into the support (See supporting information for further details). On the other hand, scanning electron microscopy (SEM) revels some differences between the immobilized enzyme and the parent support (Figure 3). For example, IB-EC1 (1a and 1b) and ECR1091F (2a and 2b), clearly shows changes on the surface after immobilization, as well as, Accurel MP1000 (5a and 5b) where the macrosporous structure is changed after the immobilization procedure.

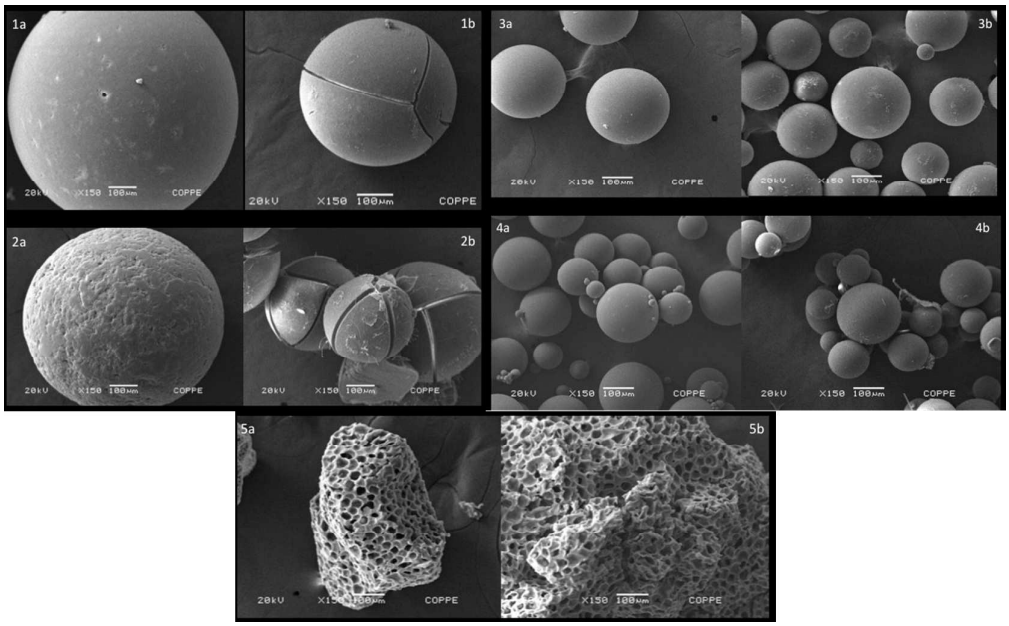


Figure 3: Scanning electron microscopy images of supports used in this work before (**a**) and after (**b**) immobilization (1-IB-EC1, 2-IB-S861, 3- ECR1091F, 4- ECR8806F and 5-Accurel MP1000)

Before starting to use this immobilized biocatalysts on the kinetic resolution of 1-phenylethanol, we have performed some experiments in order to identify the stability of the biocatalysts at different temperatures such as: 50°C, 60°C and 70°C. For this purpose we have used, as a standard experiment, the esterification reaction between oleic acid and ethanol in cyclohexane at different reaction temperatures, as shown in Table 4.

Table 4: Esterification reaction between oleic acid and ethanol in cyclohexane at different reaction temperatures.

Biocatalyst	Time (min)	Conversion (%)		
		50°C	60°C	70°C
Accurel MP1000	30	74	70	60
IB-EC1		34	60	47

IB-S861		76	73	58
ECR1091F		60	76	60
ECR8806F		71	74	60
Accurel MP1000		75	77	62
IB-EC1		46	67	54
IB-S861	60	74	71	57
ECR1091F		69	75	59
ECR8806F		73	70	59
Accurel MP1000		75	75	62
IB-EC1		55	68	54
IB-S861	120	72	70	57
ECR1091F		68	71	57
ECR8806F		74	70	58

Reaction conditions: 10 mg of biocatalyst in 1 mL solution of oleic acid/ethanol 1:1, 100mM in n-heptane at different temperatures for 60 minutes

As shown in Table 4, the immobilized biocatalysts present good behavior at different temperatures being 50 and 60^{o}C the best temperatures for these biocatalysts. Even at higher temperatures (70^{o}C), the immobilized biocatalysts prepared in this work could lead to moderate conversion towards the desired product.

Moving forward to the use of the prepared immobilized biocatalysts on the kinetic resolution of 1-phenylethanol (**1**) we decided to use the reaction conditions already optimized to commercial immobilized biocatalysts in order to have them as a reference to our work, in combination with the results obtained about temperature behavior presented on Table 4. The comparative results between the commercial immobilized enzyme (Novozyme 435 – N435) and the prepared immobilized

biocatalysts are shown in Table 5. It is important to note that in order to have a true comparison between all immobilized biocatalysts, productivity should be used in place of conversion because it takes into account the amount of protein presented in each biocatalyst.

Table 5: Kinetic resolution of 1-phenylethanol mediated by different immobilized catalysts under batch conditions.

Entry	Enzyme	mg Cal-B/g support	Conv. (%)	E	Productivity (g product/h.g ptn)
1	N435	30*	49	>200	7.4
2	Accurel MP1000	6.4	24	>200	17.0
3	IB-EC1	16.1	48	>200	13.5
4	IB-S861	8.5	50	>200	26.9
5	ECR1091F	17.6	20	>200	5.1
6	ECR8806F	16.7	20	>200	1.9

Reaction conditions: 0.33M 1-phenylethanol, vinyl acetate 0.33M in 3 mL of cyclohexane with 15% of biocatalyst for 2 hours at 50°C. [30]

As presented on Table 5, all immobilized biocatalysts could lead to the desired product with very high enantiomeric ratios (*E*) but the conversions were slightly different between them. A first look at conversion we will say that Novozyme 435, IB-EC1 and IB-S861 were the best biocatalysts for this transformation. But besides the good conversion of Novozyme 435 the productivity is very low when compared to IB-EC1 and IB-S861 since these immobilized biocatalysts have respectively 46 and 71% less protein then the commercial one. Accurel MP1000 was also effective on performing the kinetic resolution with high productivities, comparable to those

obtained by IB-EC1 and IB-S861, but conversions were low which can difficult downstream process with more laborious purification steps.

In order to increase the productivity of the immobilized biocatalysts we decided to evaluate the kinetic resolution of 1-phenylethanol under continuous-flow conditions, by the use of packed bed reactors. The packed bed reactors were packed with the same amount of immobilized biocatalyst and the reaction screened at different residence times. The results are presented on Table 6.

Table 6: Continuous-flow kinetic resolution of 1-phenylethanol mediated by different immobilized lipases.

Entry	Enzyme	mg Cal-B /g support	Res.Time (min)	Conv. (%)	E	Productivity (g product/h.g ptn)
1	N435	30*	7	31	>200	13.9
			15	46	>200	9.7
			20	50	>200	7.8
2	Accurel MP1000	6.4	7	15	>200	90.2
			15	50	>200	141.1
			20	50	>200	104.8
3	IB-EC1	16.1	7	21	>200	13.9
			15	30	>200	9.3
			20	37	>200	8.5
4	IB-S861	8.5	7	14	>200	20.4
			15	26	>200	17.8
			20	34	>200	17.2
5	ECR1091F	17.6	7	15	>200	7.6
			15	21	>200	5.0

			20	24	>200	4.2
			7	8	>200	3.2
6	ECR8806F	16.7	15	14	>200	2.6
			20	42	>200	4.8

Reaction conditions: 0.33M 1-phenylethanol and 0.33M vinyl acetate in cyclohexane.

Results presented on Table 6 shows that in general continuous-flow protocol can reduce reaction time from hours to a few minutes without loosing productivity towards the desired product. Novozyme 435 can improve by 2x the productivity under continuous flow conditions while IB-EC1 and IB-S861 did not show great improvements on productivity but could reduce reaction time by more then 1 hour and 30 minutes. ECR1091F and ECR8806F presented a slightly improvement on both conversion and productivity under such conditions.

Accurel MP1000 was the one to have a huge enhancement on conversion and productivity arriving on the desired product after 15 minutes with 50% of conversion (E>200) and productivity around 140g of product/h.g ptn. This great productivity is observed because under continuous-flow conditions this immobilized lipase can be very efficient with a small amount of protein, probably due to the enhanced mass transfer obtained under such conditions.

Another important feature of this immobilized biocatalyst is the fact that recyclability was better under continuous-flow conditions compared to the batch reactors probably due to mechanical damage of the support, which can lead to protein leakage. The results obtained for recycling are shown in Figure 4.

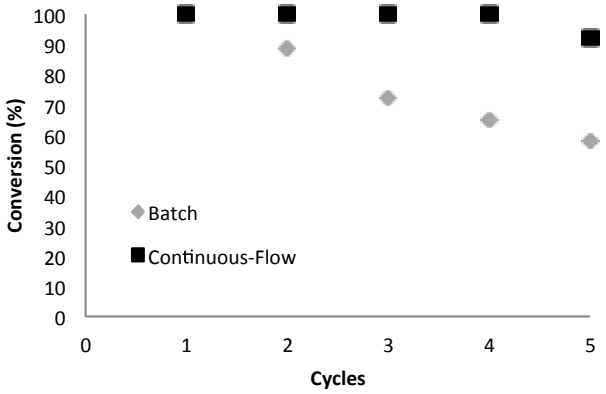


Figure 4: Reciclability of lipase B from *Candida antarctica* immobilized on Accurel MP1000 resin: Continuous-Flow X Batch reactors.

Conclusion

In conclusion, in this work we have immobilized lipase B from *Candida antarctica* in five different polymeric supports by hydrophobic interaction with moderated to good immobilization efficiencies and thermo stability. The prepared immobilized biocatalysts were used as catalysts for kinetic resolutions under batch and continuous-flow conditions. Comparatively, the use of continuous flow system enables the reduction of reaction time from hours to a few minutes and improved the productivity being Accurel MP1000 the most productivity biocatalyst for continuous-flow process leading to the desired product in 50% of conversion after 15 minutes (141.1 g product/h.g ptn). The immobilized biocatalyst could also be recycled for 5 times without significant lose of activity in continuous-flow process.

Acknowledgment

Authors thank CAPES, FAPERJ, CNPq and Petrobras for financial support.

References

(1) Noyori, R. *Adv. Synth. Cat.* **2003**, *345*, 1.

(2) Stinson, S. C. *Chem. Eng. News* **1998**, *76*, 83.

(3) Rouhi, A. M. *Chem. Eng. News* **2003**, *81*, 45.

(4) Finelli, F. G.; Miranda, L. S. M.; de Souza, R. O. M. A. *Chem. Comm.* **2015**, *51*, 3708.

(5) Guan, Z.; Li, L.-Y.; He, Y.-H. *Rsc Advances* **2015**, *5*, 16801.

(6) Kumar, M.; Kumar, A.; Rizvi, M. A.; Shah, B. A. *Rsc Advances* **2015**, *5*, 55926.

(7) Vilotijevic, I.; Jamison, T. F. *Angew. Chem. Int. Ed.* **2009**, *48*, 5250.

(8) Lin, H.; Liu, J.-Y.; Wang, H.-B.; Ahmed, A. A. Q.; Wu, Z.-L. *J. Mol.r Cat. Enz.* **2011**, *72*, 77.

(9) Ogawa, C.; Kokubo, M.; Kobayashi, S. *J. Synth. Org. Chem. Japan* **2010**, *68*, 718.

(10) Pineschi, M.; Bertolini, F.; Di Bussolo, V.; Crotti, P. *Curr. Org. Synth.* **2009**, *6*, 290.

(11) Jolley, K. E.; Zanotti-Gerosa, A.; Hancock, F.; Dyke, A.; Grainger, D. M.; Medlock, J. A.; Nedden, H. G.; Le Paih, J. J. M.; Roseblade, S. J.; Seger, A.; Sivakumar, V.; Prokes, I.; Morris, D. J.; Wills, M. *Adv. Synth. Cat.* **2012**, *354*, 2545.

(12) Ni, Y.; Xu, J.-H. *Biotech. Adv.* **2012**, *30*, 1279.

(13) Chen, Q.-A.; Ye, Z.-S.; Duan, Y.; Zhou, Y.-G. *Chem. Soc. Rev.* **2013**, *42*, 497.

(14) Cossar, P. J.; Hizartzidis, L.; Simone, M. I.; McCluskey, A.; Gordon, C. P. *Org. Biom. Chem.* **2015**, *13*, 7119.

(15) de Miranda, A. S.; Miranda, L. S. M.; de Souza, R. O. M. A. *Biotech. Adv.* **2015**, *33*, 372.

(16) Fernandez-Salas, J. A.; Manzini, S.; Nolan, S. P. *Chem. Eur. J.* **2014**, *20*, 13132.

(17) Verho, O.; Backvall, J.-E. *J. Am. Chem. Soc.* **2015**, *137*, 3996.

(18) Cantone, S.; Ferrario, V.; Corici, L.; Ebert, C.; Fattor, D.; Spizzo, P.; Gardossi, L. *Chem. Soc. Rev.* **2013**, *42*, 6262.

(19) DiCosimo, R.; McAuliffe, J.; Poulose, A. J.; Bohlmann, G. *Chem. Soc. Rev.* **2013**, *42*, 6437.

(20) Garcia-Galan, C.; Berenguer-Murcia, A.; Fernandez-Lafuente, R.; Rodrigues, R. C. *Adv. Synth. & Cat.* **2011**, *353*, 2885.

(21) Sheldon, R. A.; van Pelt, S. *Chem. Soc. Rev.* **2013**, *42*, 6223.

(22) Sutili, F. K.; Nogueira, D. d. O.; Leite, S. G. F.; Miranda, L. S. M.; de Souza, R. O. M. A. *Rsc Advances* **2015**, *5*, 37287.

(23) Zoumpanioti, M.; Stamatis, H.; Xenakis, A. *Biotech. Adv.* **2010**, *28*, 395.

(24) Gutmann, B.; Cantillo, D.; Kappe, C. O. *Angew. Chem. Int. Ed.* **2015**, *54*, 6688.

(25) Vaccaro, L.; Lanari, D.; Marrocchi, A.; Strappaveccia, G. *Green Chem.* **2014**, *16*, 3680.

(26) Itabaiana, I., Jr.; de Mariz e Miranda, L. S.; Mendonca Alves de Souza, R. O. *J. Mol. Cat. Enz.* **2013**, *85-86*, 1.

(27) Lopes, R. O.; Grimm, S.; Ribeiro, J. B.; Leal, I. C. R.; Miranda, L. S. M.; de Souza, R. O. M. A. *J. Braz. Chem. Soc.* **2015**, *26*, 550.

(28) Manoel, E. A.; Pais, K. C.; Flores, M. C.; Miranda, L. S. d. M. e.; Zarur Coelho, M. A.; Simas, A. B. C.; Freire, D. M. G.; de Souza, R. O. M. A. *J. Mol. Cat. Enz.* **2013**, *87*, 139.

(29) Sutili, F. K.; Ruela, H. S.; Leite, S. G. F.; Miranda, L. S. d. M.; Leal, I. C. R.; de Souza, R. O. M. A. *J. Mol. Cat. Enz.* **2013**, *85-86*, 37.

(30) Cabrera, Z., Fernandez-Lorente, G., Fernandez-Lafuente, R., Palomo, J. M., & Guisan, J. M. *J. Mol. Cat. Enz.* **2009**, *57*, 171-176.