Received: 19 July 2017 | Revised: 13 September 2017 | Accepted: 17 September 2017

DOI: 10.1002/pat.4189

WILEY polymers advanced technologies

SHORT COMMUNICATION

Simple and economical CALB/polyethylene/aluminum biocatalyst for fatty acid esterification

Valeria Cavallaro[1] | Daniel Ricardo Ercoli[2] | Gabriela Marta Tonetto[2] | María Luján Ferreira[2]

[1] PLAPIQUI, Chemistry Department, Universidad Nacional del Sur (UNS), CONICET, Camino La Carrindanga km 7, 8000 Bahía Blanca, Argentina

[2] PLAPIQUI, Chemical Engineering Department, Universidad Nacional del Sur (UNS), CONICET, Camino La Carrindanga km 7, 8000 Bahía Blanca, Argentina

Correspondence
Valeria Cavallaro, PLAPIQUI, Chemistry Department, Universidad Nacional del Sur (UNS), CONICET, Camino La Carrindanga km 7, 8000 Bahía Blanca, Argentina.
Email: valeria.cavallaro@uns.edu.ar

Funding information
National Council for Scientific and Technological Research, CONICET, Argentina; National Agency of Scientific and Technological Promotion, Argentina

Candida antarctica lipase B was immobilized for the first time (at the author's knowledge) onto linear low density polyethylene (LLDPE) films. Polymer films were previously bonded to a commercial aluminum sheet using a simple support preparation method. Biocatalyst performance was evaluated in penthyl oleate synthesis at room temperature. Two different catalyst geometries were tested and compared: one aluminum-polyethylene 50 mm × 50 mm foil (50CAT) or near 5 mm × 5 mm aluminum-polyethylene foils (5CAT). The obtained results demonstrate that the biocatalyst obtained with 50 mm × 50 mm aluminum-polyethylene foil or 50CAT is reusable in up to 7 cycles, easy to separate from reaction products, and economical in comparison with commercial Novozym 435. Novel and economical CALB/LLDPE/Al biocatalyst is an attractive alternative for possible applications in a continuous monolithic reactor and future industrial scaling up.

KEYWORDS

aluminum, CALB, enzyme immobilization, esterification, polyethylene film

1 | INTRODUCTION

The main issue regarding industrial enzymatic processes is the relatively high cost of the catalyst. It is well known that the immobilization of enzymes on suitable support materials is an effective way to improve the catalytic properties of the enzyme and enables multiple uses and the easy separation of the enzyme from reaction products, reducing economic impact of enzyme costs.[1]

Among suitable supports tested for lipases, porous materials are preferred because they allow the effective dispersion of enzyme molecules on a large surface, thus providing a higher number of enzyme molecules to deliver their catalytic potential.[2] Synthetic polymer matrices are widely used for enzyme adsorption, as they can be adapted to suite the specific enzyme and the conditions of a particular process. Moreover, their production is relatively simple, and also, they show high thermal and chemical resistance to many solvents.[3] Polyethylene and polypropylene powders have been extensively used as lipase carriers, displaying good results in the synthesis of several fine chemicals.[4-8] However, these preparations required laborious filtration steps to recover the biocatalyst after use. Significant catalyst loss has been reported due to particle size reduction during long stirring hours.[9] Reuse of these catalysts is limited and poorly reported in scientific bibliography. In this work, the use of a polyethylene film as a support of lipase is explored as a reusable alternative to polymer powder.

Another way to significantly reduce the impact of catalyst costs on the product is to use immobilized lipases in continuous reactors. Studies have shown that these systems present the advantage of easy operation, scaling up and lowering the shear stress on the immobilized enzymes, generally leading to long-term enzyme stability.[10]

The aim of this work is to develop a novel biocatalyst with future possible applications in a continuous monolithic reactor, using low-cost materials and an easy-to-prepare support. To do this, linear low density polyethylene (LLDPE) films were thermally bonded to commercial aluminum sheets and they were used as support for the adsorption of *Candida antarctica* lipase B (CALB). The efficiency and reuse of prepared biocatalyst in penthyl oleate synthesis were studied and compared with an immobilized commercial lipase preparation (Novozym 435). In addition, costs analysis of prepared biocatalyst is included.

2 | EXPERIMENTAL

2.1 | Materials

C. antarctica lipase B (batch LCN02103) and Novozym 435 were kindly donated by Novo Nordisk (Brazil). Oleic acid (OA), phenolphthalein,

and potassium hydroxide were obtained from Anedra. Absolute ethanol, ethylic ether, and *n*-heptane were supplied by Dorwill (Argentina); 1-penthanol was purchased from Sigma. All products used were of analytical grade.

A commercial injection molding grade LLDPE supplied by Dow Chemical under the trade name IP20 was used to prepare the support films in a hydraulic press.

2.2 | Preparation of LLDPE/Al supports

Supports were prepared using a commercial laminated pure aluminum as metallic substrate with 0.2 mm width. The aluminum sheets were cut in pieces of 50 mm × 50 mm. After cutting, and to increase the roughness of the original sheet, aluminum surface was softly scratched with a stainless-steel sponge repeatedly. Later, sheets were washed with water and soup, and they were dried at 50°C. LLDPE films were obtained in a hydraulic press from LLDPE pellets by applying 30 kg/cm^2 at 150°C for 60 seconds and then 50 kg/cm^2 for 60 seconds at the same temperature. Next, films were thermally bonded to both sides of the aluminum sheet applying 10 kg/cm^2 at 160°C for 120 seconds and then 30 kg/cm^2 for 60 seconds.

Two different support geometries were used, and consequently, two different biocatalysts were prepared: 5CAT was obtained using an aluminum support of 5 mm × 5 mm, meanwhile 50CAT was obtained using a support of 50 mm × 50 mm.

2.3 | Immobilization procedure

To completely cover with solvent, the aluminum foil covered with polymer during lipase immobilization and the biocatalyst surface in the esterification reaction, different glass flasks, and solvent volumes were used (15 mL for 5CAT and 40 mL for 50CAT).

LLDPE/Al supports were pretreated with ethanol 10 minutes at room temperature. Then, 200 μL of commercial CALB solution was diluted up to 15 or 40 mL with distilled water and contacted with 5CAT or 50CAT at room temperature for 2 hours with 300 to 400 rpm magnetic stirring. After this time, the biocatalyst was removed from solution, washed with 15 or 40 mL distilled water for 10 minutes and dried to constant weight at 45°C.

2.4 | Support and biocatalyst characterization

LLDPE IP20 has a melt flow index of 20 g/10 min (190°C, 16 kg) and is recommended for applications requiring good mechanical properties even at low temperatures. Average molecular weights (Mw, Mn) of LLDPE IP20 were measured using size exclusion chromatography with a Viscotek 350 system from Malvern at 135°C and 1,2,4-trichlorobenzene as a solvent, giving Mw = 52200 and Mn = 14430.

Support and biocatalyst were characterized by means of scanning electron microscopy (SEM) and Fourier transform infrared spectroscopy–diffuse reflectance infrared Fourier transform spectroscopy (FTIR-DRIFTS). Scanning electron microphotographs were obtained using a SEM LEO EVO 40-XVP microscope from CCT, Bahía Blanca, Argentina. Diffuse reflectance infrared Fourier transform spectroscopy was performed using a Thermo Scientific Nicolet 6700 spectrometer (4000-400 cm^{-1}).

2.5 | Lipase quantification

Catalyst enzyme loading was determined by measuring the sulfur content using inductively coupled plasma atomic emission spectroscopy (AE-ICP Shimadzu 9000) in protein solutions before and after immobilization and in washing residues after immobilization. *C. antarctica* lipase B content was calculated considering 12 sulfur atoms and a molecular weight of 33 kDa per each lipase molecule.[11] Sulfur content in the support was determined following immobilization procedure without protein available and used as blank for enzyme loading calculations. Results of sulfur content were reproducible in different, thrice repeated lipase immobilization steps. The lipase content was calculated based on the initial and final sulfur concentrations.

2.6 | Synthesis of penthyl oleate via enzymatic esterification

A total of 150 mg of OA (0.53 mmol) and 46.8 mg of 1-penthanol (0.53 mmol) were dissolved in 15 mL (5CAT) or 40 mL (50CAT) *n*-heptane in hermetically sealed glass flask. Reactions were started by the addition of the biocatalyst to reaction mixture, kept at 25°C, and stirred during 9 hours at an average of 900 or 1400 rpm. At defined time intervals, 0.5 mL samples were withdrawn from reaction media, dissolved in 5 mL of ether:ethanol (1:1), and titrated with KOH solution in the presence of phenolphthalein. The conversion percentage of the fatty acid was defined as follows:

$$\text{OA Conversion}\% = 100 - \left(\frac{\textit{titrated acid after reaction}}{\textit{inicial amount of acid}}\right) \times 100.$$

Novozym 435 activity was tested following same procedure described before using 5 mg of commercial biocatalyst in 40 mL of solvent at 1400 rpm.

2.7 | Storage stability test

For storage stability studies, 50CAT was kept at 4°C for 3 months. Oleic acid conversion of the biocatalyst was determined according to the method described above.

3 | RESULTS AND DISCUSSION

3.1 | Characterization of support and biocatalyst

SEM or Scanning electron microscopy images clearly show the differences in morphology between support and biocatalyst (Figure 1). After LLDPE/Al was in contact with CALB commercial solution, the appearance of protein aggregates is evident. As seen in Figure 1C, aggregates are not uniformly distributed on the support. It might seem that support-protein interactions are not the same along the polymer surface. This effect could be associated with the different superficial additive distribution during polymer heating and film formation. Cross-sectional image of support (Figure 1E) showed polymer film grafted to the aluminum sheet. LLDPE film is visualized as a uniform layer with an average of 16.5 μm width.

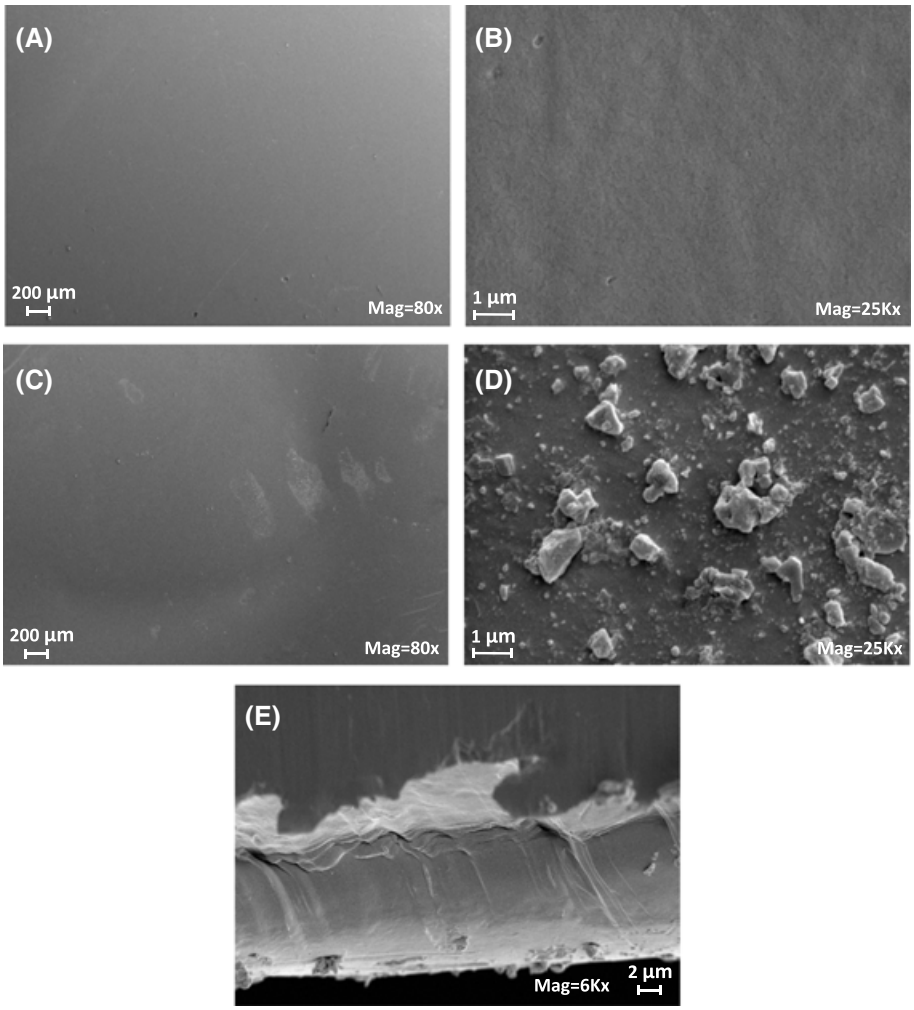

FIGURE 1 A, B, Scanning electron microscope images of LLDPE/Al support. C, D, CALB immobilized on LLDPE/Al support (50CAT). E, Cross-section of LLDPE/Al support. CALB, *Candida antarctica* lipase B; LLDPE, linear low density polyethylene

FTIR-DRIFTS of support and biocatalyst were also obtained (data not shown). Even though slight spectra differences were observed in the regions around 3200 to 3400 cm^{-1} (associated with NH amide groups) and 1720 to 590 cm^{-1} (associated with carbonyl group) after CALB incorporation, these were not significant since LLDPE absorption bands were much more intense and masked lipase ones.

3.2 | Determination of the amount of CALB immobilized on LLDPE/Al support

Lipase quantification was performed determining S content in the CALB immobilization solution. A contribution of S from the support was verified by ICP perhaps coming from some resin's additives such as specific antioxidants (ie, 2-mercaptobenzimidazole or thioesters).[12,13] To overcome this interference, supports with exact known LLDPE mass were stirred separately in distilled water under the same immobilization protocol but with no protein addition. Using AE-ICP, a significant and average amount of 0.27 µg of sulfur released in aqueous solution per milligram of LLDPE was determined and considered as "blank" correction in the following determinations. It was then determined that between 1.94 and 2.50 mg of CALB were attached to the support after 2-hour immobilization. Two different geometries of biocatalyst were obtain, fragments of 5 mm × 5 mm (5CAT) and one piece of 50 mm × 50 mm (50CAT).

3.3 | Catalytic performance of CALB/LLDPE/Al

Prepared biocatalysts were used in the esterification of OA with 1-penthanol at 25°C. Figure 2A shows OA conversion vs time for the 5CAT and 50CAT catalysts at different magnetic stirring.

At lower agitation rate (900 rpm), 5CAT showed a higher OA conversion than 50CAT (29% and 18.4%, respectively, at 9-h reaction time). The lower activity presented by 50CAT might be due to mass transfer limitations, since as agitation speed increased, a conversion increase to 34% was observed. In the case of 5CAT, higher agitation rate produced an improvement in mass transfer during the first reaction hours. Later, it was observed a rapid enzyme deactivation and, consequently, a lower OA conversion at 9-hour reaction time (16.7%). Considering the goal of future application of this biocatalyst in a continuous monolithic reactor, it is clear that 50CAT sample is a better approximation to that system, because of the lower mechanical impact on the immobilized enzyme. Comparatively, Novozym 435 was

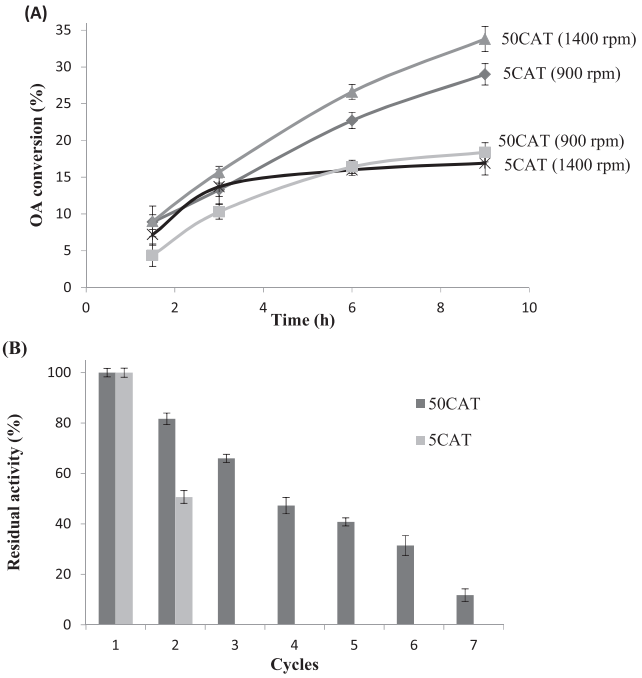

FIGURE 2 A, Performance of immobilized *Candida antarctica* lipase B on 5 mm × 5 mm (5CAT) or 50 mm × 50 mm (50CAT) polyethylene-aluminum support at different agitation speed. B, Stability during successive oleic acid (OA) esterification (reaction time: 9 h. Agitation speed: 900 rpm for 5CAT, 1400 rpm for 50CAT)

tested under the same experimental conditions. The commercial preparation reached a conversion of 72% after 9 hours of reaction at 1400 rpm.

3.4 | Biocatalyst reuse and storage stability

To investigate the reusability of the samples, biocatalysts were repeatedly used in the esterification of OA and 1-penthanol. At the end of each batch (reaction time: 9 h), the biocatalyst was separated from reaction mixture, washed to remove any substrate or product retained in the matrix, and dried at room temperature overnight to be introduced in fresh reaction media the following day. Conversion of OA was calculated after each batch. In Figure 2B, the residual relative activity for the successive OA esterification is presented (reaction time: 9 h. Agitation speed: 900 rpm for 5CAT, 1400 rpm for 50CAT).

There are two main factors to be considered to reuse these biocatalysts, ie, the geometry of the biocatalyst and the washing conditions used between batches. Regarding biocatalyst geometry, it was observed that 50CAT was able to be used during 7 cycles, retaining 40% of its activity up to the fifth batch. In contrast, 5CAT showed a rapid decrease in OA conversion, completely loosing activity after the second use. Activity loss might be explained mainly due to mechanical inactivation caused by the long time stirring. The 5CAT agitation results with many fragments continuously hitting each other and also with the walls of the reactor leading to a rapid enzyme deactivation. The mechanical shear probably induced protein denaturation.

Inhibition by substrates or products is a common catalyst deactivation mechanism. To avoid this, two different washing conditions were tested: (1) using *n*-heptane for 10 or 15 minutes and (2) using *n*-heptane:acetone (1:1) as solvent for 10 or 15 minutes. It was observed, when using acetone, that catalyst deactivation was complete and no reuse was possible. Therefore, *n*-heptane was chosen as the most appropriate washing solvent and 10 minutes as the best washing time. Longer washing time (15 min or more) showed no improvement in activity.

In addition, lipase leaching also affects immobilized biocatalysts. Especially for weak lipase-support interactions like the ones involved in adsorbed lipases, desorption is a common limitation for biocatalyst reuse.[14] After the first reaction cycle, biocatalyst was removed and reaction medium was stored at room temperature for 24 hours. Next, enzyme activity was measured by OA titration. Results demonstrated that there was no enzyme leaching during reaction or at least no active enzyme was detached from the biocatalyst.

The storage stability of the biocatalysts was also studied. It was determined that after 3 months, 50CAT retained 70% of its original activity. Performance decrease could be explained by the modification in the 3D structure of the enzyme as a result of the interactions with the support, which leads to a conformational change in the active center.[15]

Besides biocatalyst reuse and storage stability, product contamination is also an important issue to be considered. Even though commercial Novozym shows high conversion in penthyl oleate synthesis, as seen before in the text, no reuse was possible as a result of support dissolution in the reaction media. Several authors have shown that Novozym resin support dissolves in contact with organic solvents, mainly ethanol and other alcohols, leading to the migration of the polymer and other substances towards the liquid phase.[16,17] After the fifth use, 50CAT only showed small support physical deterioration due to the mechanical impact of the stirrer.

In summary, our catalyst has proven not only to be reusable but also to facilitate an easy separation of immobilized lipase from reaction mixture.

3.5 | Economic aspects of the novel biocatalyst

Despite the broad range of possibilities and academic interest, applications of lipases in industry, and in particular CALB, are relatively limited. It would seem that the primary factor involved is the high price of the immobilized enzyme combined with its limited stability.[18] Several factors have to be considered to estimate the cost of a biocatalyst, including the cost of the support, the cost of the enzyme, and the operating costs.[1] Taking into account these considerations, a value of 6.1 USD/g was estimated for prepared catalyst 50CAT, substantially lower than the 71.8 USD/g of commercial Novozym 435 (Sigma-Aldrich).

4 | CONCLUSIONS

A novel biocatalyst was obtained by simple physical adsorption of CALB on polyethylene/aluminum support. Enzyme mechanical deactivation was observed. An easy separation from reaction media was found. The best biocatalyst tolerated up to 7 cycles of reaction.

Furthermore, unlike commercial preparations, obtained biocatalyst did not dissolve in alcohols.

This work demonstrates that immobilization of CALB on a polymer film is an interesting alternative and has the potential to provide a cost-efficient biocatalyst preparation method for esterification reactions. Further studies for the application of CALB/LLDPE/Al in a monolithic continuous reactor are in progress.

ACKNOWLEDGEMENTS

The authors thank the National Agency of Scientific and Technological Promotion, Argentina, and the National Council for Scientific and Technological Research, CONICET, Argentina, for the financial support.

ORCID

Valeria Cavallaro http://orcid.org/0000-0002-1566-7575

REFERENCES

1. Séverac E, Galy O, Turon F, et al. Selection of CalB immobilization method to be used in continuous oil transesterification: Analysis of the economical impact. *Enzyme Microb Technol*. 2011;48:61-70.
2. Cesarini S, Infanzón F, Pastor FIJ, Diaz P. Fast and economic methods described for non-commercial *Pseudomonas* lipases. *BMC Biotechnol*. 2014;14:27-36.
3. Jesionowski T, Zdarta J, Krajewska B. Enzyme immobilization by adsorption: A review. *Adsorption*. 2014;20:801-821.
4. Foresti ML, Ferreira ML. Solvent free ethyl oleate synthesis mediated by lipase from *Candida antarctica* B adsorbed on polypropylene powder. *Catal Today*. 2005;107-108:23-30.
5. Gitlesen T, Bauer M, Adlercreutz P. Adsorption of lipase on polypropylene powder. *Biochim Biophys Acta*. 1997;1345:188-196.
6. Loftabad TB, Roostaazad R. Immobilization of *Rhizomucor miehei* lipase on high density polyethylene. *Iran J Chem Eng*. 2004;1(2):29-37.
7. Minovska V, Winkelhausen E, Slobodanka K. Lipase immobilized by different techniques on various support materials applied in oil hydrolysis. *J Serb Chem Soc*. 2005;70(4):609-624.
8. Watanabe T, Suzuki Y, Sagesaka Y, Kohashi M. Immobilization of lipases on polyethylene and application to perilla oil hydrolysis for production of α-linolenic acid. *J Nutr Sci Vitaminol*. 1994;41:307-312.
9. Foresti ML, Ferreira ML. Ethanol pretreatment effect and particle diameter issues on the adsorption of *Candida rugosa* lipase onto polypropylene powder. *Appl Surf Sci*. 2004;238:86-90.
10. Hama S, Tamalampudi S, Yoshida A, et al. Enzymatic packed-bed reactor integrated with glicerol-separating system for solvent-free production of biodiesel fuel. *Biochem Eng J*. 2011;55:66-71.
11. Uppenberg J, Hansen MT, Patkar S, Jones A. The sequence, crystal structure determination and refinement of two crystal forms of lipase B from *Candida Antarctica*. *Structure*. 1994;2:293-308.
12. Fries E, Dekiff JH, Willmeyer J, Nuelle MT, Ebert M, Remy D. Identification of polymer types and additives in marine microplastic particles using pyrolysis-GC/MS and scanning electron microscopy. *Environ Sci: Processes Impacts*. 2013;5:1949-1956.
13. Zhou LY. Quantitative analysis of additives in low density polyethylene using on-line supercritical fluid extraction/supercritical fluid chromatography. Ph.D. Thesis, Virginia Polytechnic Institute, Virginia, USA, 1998.
14. Foresti ML, Ferreira ML. Analysis of the interaction of lipases with polypropylene powder of different structure and polypropylene-modified glass surface. *Colloid Surface A*. 2007;294:147-155.
15. Li S, Chen J, Wu W. Electrospun polyacrylonitrile nanofibrous membranes for lipase immobilization. *J Mol Catal B-Enzym*. 2007;41:117-124.
16. José C, Bonetto RD, Gambaro LA, et al. Investigation of the causes of deactivation-degradation of the commercial biocatalyst Novozym® 435 in ethanol and ethanol-aqueous media. *J Mol Catal B-Enzym*. 2011;71:95-107.
17. Villalba M, Verdasco-Martín CM, dos Santos JC, Fernandez-Lafuente R, Otero C. Operational stabilities of different chemical derivatives of Novozym 435 in an alcoholysis reaction. *Enzyme Microb Technol*. 2016;90:35-44.
18. Tufvesson P, Törnvall U, Carvalho J, Karlsson AJ, Hatti-Kaul R. Towards a cost-effective immobilized lipase for the synthesis of specialty chemicals. *J Mol Catal B-Enzym*. 2011;68:200-205.

How to cite this article: Cavallaro V, Ercoli DR, Tonetto GM, Ferreira ML. Simple and economical CALB/polyethylene/aluminum biocatalyst for fatty acid esterification. *Polym Adv Technol*. 2017;1-5. https://doi.org/10.1002/pat.4189