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Laísa Quadros Barsé

Síntese de palmitato de isopropila catalisada por lipase B de *Candida antarctica* imobilizada em partículas core-shell

Porto Alegre
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Trabalho de conclusão de curso de graduação apresentado ao Instituto de Biociências da Universidade Federal do Rio Grande do Sul como requisito parcial e obrigatório para a obtenção do título de Bacharela em Biotecnologia.

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Coorientadora: Ms. Natália Guilherme Graebin

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BANCA EXAMINADORA

_____

Prof. Dra. Manuela Poletto Klein – UFCSPA

_____

Prof. Dr. Plinho Francisco Hertz – ICTA/UFRGS

_____

Prof. Dr. Rafael Costa Rodrigues – ICTA/UFRGS

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RESUMO

O palmitato de isopropila é um éster amplamente utilizado nas indústrias farmacêutica e de cosméticos. Sua síntese por via biotecnológica apresenta as vantagens de ser mais sustentável e por utilizar condições de reação menos severas. Lipases são utilizadas, tanto em sua forma livre ou imobilizada, sendo essa de maior interesse industrial, visto que o reuso é facilitado e reações paralelas são diminuídas. Dentre as inúmeras técnicas de imobilização, tem-se o sistema núcleo-casca (core-shell), no qual os suportes são suspensos e emulsificados em etapas simultâneas a fim de produzir o núcleo e a casca da partícula. Baixo custo de produção e condições sintéticas menos severas estão entre as vantagens desse método. Assim, esse trabalho teve como objetivo estudar a síntese de palmitato de isopropila utilizando lipase B de *Candida antarctica* imobilizada em partículas core-shell. O potencial dos imobilizados PMMA/PMMA (poli (metil-metacrilato)), PMMA-*co*-DVB/PMMA-*co*-DVB (poli (metil-metacrilato) copolimerizado com divinilbenzeno) e Ps-*co*-DVB/Ps-*co*-DVB (poliestireno copolimerizado com divinilbenzeno) foi avaliado na reação de síntese do éster. Além disso, os solventes orgânicos hexano, ciclohexano, isoctano e heptano foram estudados, assim como as condições ótimas de temperatura, razão molar de ácido:álcool e quantidade de enzima, através de um delineamento composto central. A estabilidade operacional do biocatalisador e a utilização de peneiras moleculares também foram investigadas. A lipase imobilizada em Ps-*co*-DVB/Ps-*co*-DVB foi aquela que apresentou maior rendimento da reação. Dentre os solventes, o isoctano apresentou a maior produção de éster, atingindo rendimento de 51,54 %. As condições ótimas de reação foram: 55 ºC, razão molar de substrato 1:1,42 (ácido:álcool) e 24 % de enzima (m/m). Em relação ao reuso, 23 % de atividade residual foi observada após 7 ciclos de reação. O uso de peneiras moleculares não promoveu maiores rendimentos na reação. Como conclusão, tem-se que a lipase B de *C. antarctida* imobilizada em Ps-*co*-DVB/Ps-*co*-DVB mostrou-se promissora na produção biotecnológica de palmitato de isopropila. Além disso, os conhecimentos aqui descritos agregam à literatura, por ser esse, o primeiro trabalho a descrever a produção do palmitato de isopropila com esse tipo de imobilizado.

Palavras-chave: Palmitato de isopropila, esterificação, lipase, imobilização, core-shell.

ABSTRACT

Isopropyl palmitate is an ester widely used in the pharmaceutical and cosmetic industries. Its synthesis through biotechnology presents the advantages of being more sustainable and by using less severe reaction conditions. Lipases are used, either in their free or immobilized form, which is of greater industrial interest, since reuse is facilitated and parallel reactions are diminished. Among the many techniques of immobilization, we have the core-shell system, in which the supports are suspended and emulsified in simultaneous steps in order to produce the core and shell of the particle. Low cost of production and less stringent synthetic conditions are among the advantages of this method. Thus, this work aimed to study the synthesis of isopropyl palmitate using *Candida antarctica* lipase B immobilized on core-shell particles. The potential of PMMA/PMMA (poly (methyl methacrylate)), PMMA-co-DVB/PMMA-co-DVB (poly (methyl methacrylate) copolymerized with divinylbenzene) and Ps-co-DVB/Ps-co-DVB (polystyrene copolymer with divinylbenzene) was evaluated in the reaction of ester synthesis. In addition, the organic solvents hexane, cyclohexane, isoctane and heptane were studied, as well as the optimal conditions of temperature, acid:alcohol molar ratio and amount of enzyme, through a central composite design. The operational stability of the biocatalyst and the use of molecular sieves were also investigated. Lipase immobilized in Ps-co-DVB/Ps-co-DVB was the one that presented the highest yield of the reaction. Among the solvents, isoctane presented the highest ester production, reaching yield of 51.54%. The optimum reaction conditions were: 55 ° C, molar ratio of substrate 1: 1.42 (acid: alcohol) and 24% of enzyme (w/w). Regarding reuse, 23% of residual activity was observed after 7 cycles of reaction. The use of molecular sieves did not promote higher yields in the reaction. As a conclusion, it has been found that *C. antarctida* lipase B immobilized on Ps-co-DVB/Ps-co-DVB has shown promise in the biotechnological production of isopropyl palmitate. In addition, the knowledge described here adds to the literature, as this is the first work to describe the production of isopropyl palmitate with this type of immobilized.

Keywords: Isopropyl palmitate, esterification, lipase, immobilization, core-shell.

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1 INTRODUÇÃO GERAL

As lipases (EC 3.1.1.3) são enzimas versáteis e de grande interesse biotecnológico por apresentarem quimiosseletividade, regiosseletividade e estereosseletividade. Essas características, muitas vezes, estão relacionadas com maior eficiência no processo de síntese e com menores operações de purificação, uma vez que reações paralelas são diminuídas ou eliminadas (KLIBANOV, 2001). Além disso, na maioria desses processos, essas enzimas não requerem cofatores (JAEGER; EGGERT, 2002). Uma das lipases mais amplamente estudadas é a lipase B de *Candida antarctida* (CALB). Essa é uma proteína globular do tipo α/β hidrolase com dimensões aproximadas de 30 Å x 40 Å x 50 Å, que contém uma tríade catalítica similar a encontrada em serinas-proteinases. Por ser estereoespecífica tanto em reações de hidrólise quanto em sínteses orgânicas (MARTINELLE; HULT, 1995; UPPENBERG *et al.*, 1994), é aplicada na produção dos mais diversos compostos, como por exemplo hidrólise de óleos, biodiesel, detergentes, medicamentos, e alguns ésteres que são utilizados em indústria de alimentos e de cosméticos (ANSORGE-SCHUMACHER; THUM, 2013a; FJERBAEK; CHRISTENSEN; NORDDAHL, 2009; HO *et al.*, 2010; SIÓDMIAK *et al.*, 2015; XIAO *et al.*, 2015). Especificamente em reações de esterificação, a CALB age por um mecanismo de acilação e desacilação, no qual a acilação ocorre na fração imidazólica da histidina, removendo um próton da serina e assim, aumentando seu poder de ataque nucleofílico na carbonila do ácido carboxílico do substrato, o que forma um complexo intermediário tetraedral conhecido como acil-enzima (TSAI, 2016).

Devido aos avanços na área da biotecnologia e de engenharia de proteínas, boa parte das enzimas já podem ser adquiridas no mercado com preços competitivos e com boas propriedades, tais como especificidade e estabilidade térmica (SHELDON; PELT, VAN, 2013). Além disso, a técnica de imobilização de enzimas faz com que os biocatalisadores possam ser reutilizados por vários ciclos, o que também acarreta em uma menor oneração de processos *downstream*. Atualmente, existem algumas empresas, como a Novozymes, que realizam a produção da lipase B de forma comercial, conhecida pelo nome de Novozym 435. Sua produção é feita por cultivo de uma cepa de *Aspergillus* geneticamente modificado e é imobilizada por adsorção física na resina macroporosa de poli(ácido

metacrílico) reticulado com divinilbenzeno (DVB), Lewatit VP OC 1600. Tal enzima possui excelente atividade e estabilidade em solventes orgânicos hidrofóbicos (SIÓDMIAK *et al.*, 2015). Por outro lado, busca-se a produção de CALB em outros organismos geneticamente modificados e também a obtenção de novos tipos de suportes que se assemelhem ao bom desempenho do suporte utilizado na produção da Novozym 435 e que difiram daqueles suportes hidrofóbicos convencionais utilizados na imobilização da lipase, com a finalidade de obter um menor custo de produção da enzima (MATEO *et al.*, 2007). Uma alternativa de metodologia para a imobilização enzimática é o uso da técnica de núcleo-casca (core-shell), onde uma polimerização combinada por emulsão de compostos hidrofóbicos é feita. Como vantagem, essa técnica de imobilização demonstra melhores propriedades de transporte de substrato no meio, baixo custo e maior integridade das enzimas (KARIMI *et al.*, 2013; KUWAHARA *et al.*, 2012; TAQIEDDIN; AMIJI, 2004). Dentre as possibilidades de polímeros que podem ser utilizados na produção de partículas core-shell, tem-se a utilização de metil-metacrilato e de poliestireno, por exemplo, no núcleo e na casca do suporte. Também pode ser aplicado a polimerização com o mesmo agente reticulante da Novozym 435 (divinilbenzeno) (MANOEL, E. A.; ROBERT; *et al.*, 2016a).

As lipases são utilizadas em hidrólise de óleos e gorduras e, em condições específicas, também atuam em reações de síntese, como transesterificação, interesterificação e esterificação. Sua aplicação em reações de esterificação é de grande interesse industrial, uma vez que os produtos formados por via enzimática, tem sua síntese denominada como “natural” (HASAN; SHAH; HAMEED, 2006). Alguns ésteres produzidos por lipases são descritos como aromatizantes, e tem-se como exemplos o acetato de isoamila (aroma de banana), acetato de geranilo (aroma de rosa floral), acetato de butila (aroma de maçã ou banana), butirato de etila (aroma de abacaxi), entre outros (FRIEDRICH *et al.*, 2013; HARI KRISHNA; SATTUR; KARANTH, 2001; LARIOS *et al.*, 2004; MARTINS *et al.*, 2013; OGUNTIMEIN; ANDERSON, W. A.; MOO-YOUNG, M., 1995). Outros ésteres de cadeia mais longa têm propriedades emulsificantes e lubrificantes. Particularmente, o palmitato de isopropila é produzido a partir da esterificação do ácido palmítico com o álcool isopropílico, e esse é um emulsificante bastante utilizado nas indústrias cosmética e farmacêutica, porém pouco relatado na literatura. Esse éster é

empregado em produtos de higiene pessoal, como xampus, condicionadores, cremes e géis de banho (FU *et al.*, 2015), além de ser descrito como veículo de uso tópico de fármacos contra inflamações de pele e no transporte transdermal de drogas (BOONME, 2007; JAMES; THERAPY; GOLDBERG, 1989; ORGANOGEL, 1992).

Sendo assim, faz-se importante o estudo dos parâmetros da síntese de ésteres a fim de otimizar o processo enzimático. Dentre os mais investigados na literatura estão: temperatura, pH, razão molar de substratos, tempo de reação, quantidade de água inicial na reação e concentração de enzima (HARI KRISHNA; SATTUR; KARANTH, 2001; WU *et al.*, 1999). Diferentes substratos já foram estudados, tais como butanol (MARTINS *et al.*, 2011), álcool isopropílico (YAHYA; ANDERSON, William A.; MOO-YOUNG, Murray, 1998), ácido mirístico (SRIVASTAVA; MADRAS; MODAK, 2003) e ácido láurico (REETZ; JAEGER, 1998). Além disso, a utilização de peneiras moleculares tem sido utilizada em reações que produzem água como um subproduto e tem como objetivo aumentar o rendimento, uma vez que desloca o equilíbrio químico da reação de esterificação, favorecendo a síntese dos produtos. Tal tecnologia vem sendo relatada na literatura com sucesso, como nas sínteses de ésteres com ácidos graxos de tamanhos variados com álcoois de cadeia curta (ALVES *et al.*, 2015; WEHTJE *et al.*, 1997) e alguns ésteres de açúcar (CHAMOULEAU *et al.*, 2001).

Dentro desse contexto, o objetivo desse trabalho foi estudar a síntese de palmitato de isopropila catalisada pela lipase B de *Candida antarctica* imobilizada em partículas core-shell. Durante o trabalho, foram avaliados três tipos diferentes de imobilizados enzimáticos, diferentes solventes orgânicos, a concentração molar dos substratos na reação, assim como a otimização das condições da síntese de palmitato de isopropila, feita através de um delineamento composto central (DCC). Além disso, um estudo sobre a utilização de peneiras moleculares e a estabilidade operacional do biocatalisador na síntese do éster foi realizado.

2 ARTIGO CIENTÍFICO

Neste capítulo está apresentando o estudo da síntese do palmitato de isopropila em forma de artigo científico a ser submetido no periódico Molecular Catalysis. Nesse trabalho, foram determinados o melhor catalisador e solvente orgânico, a concentração molar ótima de substrato e as condições ótimas de síntese de palmitato de isopropila. Também foram avaliadas a estabilidade operacional da enzima e a utilização de peneiras moleculares na reação.

SYNTHESIS OF ISOPROPYL PALMITATE CATALYZED BY LIPASE B FROM *CANDIDA ANTARCTICA* IMMOBILIZED ON THE CORE-SHELL SYSTEM WITH POLY(STYRENE-*CO*-DIVINYLBENZENE)

Laísa Quadros Barsé,[1] Natália Guilherme Graebin,[1] Eliane Pereira Cipolatti,[2] Denise Maria Guimarães Freire,[2] Rafael C. Rodrigues*[1]

[1] Biotechnology, Bioprocess, and Biocatalysis Group, Food Science and Technology Institute, Federal University of Rio Grande do Sul, Av. Bento Gonçalves 9500, PO Box 15090, ZC 91501-970 Porto Alegre, RS, Brazil.
[2] Biochemical Department, Federal University of Rio de Janeiro, Av. Athos da Silveira Ramos, 149, ZC 21949-909, Rio de Janeiro, RJ, Brazil

*Corresponding author:
Tel.: +55 51 3308 7793; site: fax: +55 51 3308 7048
E-mail address: rafaelcrodrigues@ufrgs.br (R. C. Rodrigues).

ABSTRACT

In this work, we studied the application of lipase B from *Candida Antarctica* (CALB) immobilized on the core-shell system in the synthesis of isopropyl palmitate. Firstly, CALB was immobilized in three different combinations of core and shell particles and tested in the esterification reaction, being the CALB-Ps-*co*-DVB/Ps-*co*-DVB (polystyrene-*co*-divinylbenzene in the core and in the shell) the most suitable immobilized biocatalyst for this purpose. The effect of organic solvents – n-hexane, isoctane, n-heptane and cyclohexane – in the ester conversion was evaluated, and the best solvent was isoctane. The optimal conditions, obtained with a central composite design, for isopropyl palmitate synthesis were: temperature, 55 °C; substrate molar ratio, 1:1.42 palmitic acid:isopropyl alcohol, and enzyme content, 24 % (w/w substrate). Besides, the best acid concentration for the reaction was determined to be 1 M at molar ratio 1:1.42 (acid:alcohol). A conversion of, approximately, 78 % were obtained under these conditions in 4 h. The use of molecular sieves did not enhance the ester yield. Enzyme reuse was tested washing the enzyme with isoctane and the relative activity remained at 23 % for up to 7 cycles. The results show a promising application of CALB-Ps-*co*-DVB/Ps-*co*-DVB for isopropyl palmitate synthesis.

Keywords: Isopropyl palmitate, esterification, lipase, core-shell, polystyrene-*co*-divinylbenzene;

2.1 Introduction

Lipases (EC 3.1.1.3) are an important group of biocatalysts for biotechnological applications, such as for the synthesis of biopolymers and biodiesel, the production of pharmaceuticals, agrochemicals, flavor compounds and compounds for use in the cosmetics industry [1–5]. The main activity of lipases is the hydrolysis of fats and oils, but under specific conditions they catalyze esterification reactions between carboxylic acids and alcohols [6–9]. Depending of the substrates chain length, such ester molecules present different properties, as emulsifiers, lubricants or flavor characteristics [10–13].

Among these esters, isopropyl palmitate is largely used in skincare products like moisturizers, sunscreens, and conditioners [4]. The chemical synthesis of this compound was extensively reported using several catalysts, such as sulfonic acid type ion exchange resin [14], zinc acetate complex supported over functionalized silica [15], and solid superacids [16]. However, replacing the conventional chemical pathway by enzymatic processing offers environmental advantages and low energy costs, and the product can be considered as “green” [17]. Otherwise, due to advances in biotechnology and protein engineering, most enzymes can be already purchased competitively and with good properties, like thermal stability [2].

In the ester synthesis, the lipases can be used in free or immobilized forms, being the immobilized form with major industrial interest [3,18]. The enzyme immobilization presents as advantages a reduction in downstream processes cost, because the enzyme can be easily removed from the reaction medium, allowing its reuse for several cycles or in continuous processes, and, in some cases, the increase of the enzyme thermal stability [19]. Activated agarose, cellulose, Sepharose® and magnetic nanoparticles are some examples of supports that have been used for the immobilization of lipases [20–23] and the use of core-shell appears as an alternative to conventional hydrophobic supports normally used in lipase immobilization. As advantaged to other immobilization methods, core-shell shows a better transport properties of the substrate in the media, low cost, integrity of enzymes [24–26]. In this methodology, a combined emulsion polymerization of hydrophobic compounds is carried out [27–29]. There are, in the literature, some examples of immobilization in core-shell system, as alginate-chitosan [25],

poly(methyl methacrylate) (knows as PMMA) [28], and silica with nanoparticles [22]. The use of hydrophobic core-shell system has been reported for the esterification reaction using oleic acid and ethanol as substrates [29,30] and there is no literature showing the use of core-shell for ester synthesis.

In this sense, in this work, we tested the homemade preparation of the recombinant lipase B from *Candida antarctida* (CALB) immobilized in core-shell system in the synthesis of isopropyl palmitate. CALB was immobilized in three different combinations of core-shell polymers (CALB-PMMA-PMMA, CALB-PMMA-*co*-DVB/PMMA-*co*-DVB and CALB-PS-*co*-DVB/PS-*co*-DVB) and applied in the esterification reaction. The effect of organic solvent, the reaction conditions, substrate concentration, the use of molecular sieves, and the operational stability were evaluated.

2.2 Materials and methods

2.2.1 Materials

The agents used in the supports preparation, like styrene (S), was supplied by Nitriflex Resinas S/A. Methyl methacrylate (MMA), the crosslinking agent divinyl benzene (DVB) and molecular sieves (3 Å) were purchased from Sigma-Aldrich (St. Louis, USA). Palmitic acid, isopropyl alcohol and other chemicals were of analytical grade.

2.2.2 Lipase production and immobilization

A constitutive expression vector pPGKD3_PRO_LIPB constructed from a synthetic gene of *Candida antarctica* lipase B (sequencing LIPB) was inserted to transform the X-33 wild strain of *Pichia pastoris*. Lipase production was followed as described by Manoel *et al*. [31]. The core-shell polymer particles (CALB-PMMA/PMMA, CALB-PMMA-c*o*-DVB/ PMMA-*co*-DVB and CALB-PS-*co*-DVB/PS-*co*-DVB) were prepared as described elsewhere [29,32]. The core-shell supports were produced by a combined suspension and emulsion polymerization process. A

suspension polymerization was carried out for the synthesis of the cores and after two hours, the emulsion constituents were added to the reactor for the synthesis of the porous shell. The reaction conditions were determined based on previous studies [30]. Particles of PMMA/PMMA; PS-*co*-DVB/PS-*co*-DVB (25 wt. % of DVB) and PMMA-*co*-DVB/PMMA-*co*-DVB (25 wt. % of DVB) were synthesized.

After that, the support was pretreated to facilitate the wetting. 1 g of support was suspended in 10 mL of ethanol under gentle stirring for 30 min. Afterwards, the support particles were filtrated, and suspended in 10 mL of distilled water. The suspension was kept under stirring for additional 30 min. Finally, the particles were filtrated and washed with abundant distilled water. The wetted support was used for immobilization of LipB, mixing 1 g of support with 10 mL of enzyme solution previously (24.9 mg of protein, 199.5 U of activity) diluted in 5 mM phosphate buffer pH 7 at 25 °C. At the end of immobilization, the support was filtered and washed abundantly with distilled water and storage at 4 °C before use.

2.2.3 Screening of immobilized lipases in the esterification reaction and quantification of ester amount

Three core-shell particles (CALB-PMMA/PMMA, CALB-PMMA-c*o*-DVB/ PMMA-*co*-DVB and CALB-PS-*co*-DVB/PS-*co*-DVB) were evaluated to isopropyl palmitate production. The esterification reaction was carried out into Erlenmeyer flasks using n-hexane as solvent and 0.1 M of palmitic acid and isopropyl alcohol (1:1 molar ratio) in an orbital shaker with controlled agitation and temperature (180 rpm at 40 °C) for 4 h. Enzyme content used in this reaction was 15 % (w/w by mass of substrate). The progress of esterification was monitored by the determination of the residual acid content by titration of 0.5 mL of sample against NaOH 5 mM in ethanol using phenolphthalein and 1:1 ethanol:acetone, as indicator and quenching agent, respectively. The amount of ester was calculated as being equivalent to consumed acid according to Equation 1 where $[acid]_i$ is the initial concentration of palmitic acid and $[acid]_f$ is the final concentration of palmitic acid.

$$Conversion = \left[\frac{[acid]i - [acid]f}{[acid]i}\right] \times 100 \qquad \text{(Eq. 1)}$$

2.2.4 Effect of the organic solvents

After evaluating the production of ester by the immobilized lipases, the effect of the organic solvent was investigated for the best lipase preparation. The organic solvents (n-hexane, isoctane, n-heptane and cyclohexane) were tested in Erlenmeyer flasks, using 0.1 M of palmitate acid and isopropyl alcohol (1:1 molar ratio) in an orbital shaker with controlled agitation and temperature (180 rpm at 40 °C) for 5 h. Enzyme content used in this reaction was 15 % (w/w by mass of substrate).

2.2.5 Experimental design – Central Composite Design (CCD)

A central composite design with three variables (temperature, substrate molar ratio and enzyme concentration) was carried out in order to obtain the optimal conditions for the synthesis of isopropyl palmitate for 5 h. The variables and their coded and uncoded values are presented in Table 1. Table 2 shows 17 treatments of the three variables, each at five levels. The design was constructed by 8 factorial points, 6 axial points (two axial points on the axis of design variable), and 3 replications at the central point. In each case, the ester conversion was determined by Equation 2.

$$Y = \beta_0 + \sum \beta_i \, X_i + \sum \beta_{ij} \, X_i X_j + \sum \beta_{ii} \, {X_i}^2 \qquad \text{(Eq. 2)}$$

Where Y is the response variable, β_0 the constant, β_i, β_{ii}, β_{ij} were the coefficients for the linear, quadratic, and for the interaction effects, respectively, and X_i and X_j the coded level of variables x_i and x_j. The above quadratic equation was used to obtain the contour plots for all variables.

Table 1. Process variables and their levels used in CCD

Variables	Parameter	Levels				
		-1.68	-1	0	1	1.68

X_1	Temperature (°C)	30	38.1	50	61.9	70
X_2	Substrate molar ratio[a]	2:1	1.62:1	1:1	1:1.62	1:2
X_3	Enzyme content[b]	10	14	20	26	30

[a]: palmitic acid:isopropyl alcohol;

[b]: % by mass of substrate;

Table 2. Experimental design and results of the CCD

Run	X_1	X_2	X_3	Conversion (%)
1	-1	-1	-1	20.12
2	-1	-1	1	41.23
3	-1	1	-1	38.89
4	-1	1	1	59.25
5	1	-1	-1	11.69
6	1	-1	1	42.57
7	1	1	-1	56.51
8	1	1	1	54.75
9	-1.68	0	0	45.19
10	1.68	0	0	65.28
11	0	-1.68	0	31.15
12	0	1.68	0	61.71
13	0	0	-1.68	36.82

14	0	0	1.68	70.25
15	0	0	0	58.56
16	0	0	0	60.40
17	0	0	0	57.09

2.2.6 Use of molecular sieves

Two different concentrations of molecular sieves (30 and 90 mg of molecular sieves per mmol of palmitic acid) were evaluated in order to remove the produced water and improve the isopropyl palmitate synthesis. The experiments were carried out under optimum conditions determined in the CCD.

2.2.7 Effect of palmitic acid concentration

The effect of palmitic acid concentration was studied varying the acid concentration from 0.2 to 1 M. The experiments were carried out under optimum conditions determined in the CCD.

2.2.8 Operational stability

The reuse of the immobilized biocatalyst was tested in the esterification reaction under the optimal conditions determined in the CCD. At the end of each batch, the immobilized enzyme was separated from the reaction medium by a simple filtration, and it was washed with isoctane at 55 ºC to remove possible residues of substrate and/or product. The biocatalyst was dried at room temperature for 24 h. Then, the dried immobilized lipase was reused in a new fresh reaction.

2.2.9 Statistical analysis

The experimental design and analysis of results were carried out using Statistica 7 (Statsoft, USA). The statistical analysis of the model was performed using the analysis of variance (ANOVA). The significance of the regression coefficients and

the associated probabilities, p(t), were determined by Student's t-test; the second order model equation significance was determined by Fisher's F-test. The variance explained by the model is given by the multiple determination coefficients, R^2. For each variable, the quadratic models were represented as contour plots (2D).

2.3 Results and discussion

2.3.1 Biocatalyst screening

Lipase B from *C*. *antarctica* was immobilized in three different core-shell polymers (CALB-PMMA/PMMA, CALB-PMMA-*co*-DVB/PMMA-*co*-DVB and CALB-Ps-*co*-DVB/Ps-*co*-DVB), and the results for the synthesis of the isopropyl palmitate are shown in Fig. 1. The biocatalyst immobilized on CALB-Ps-*co*-DVB/Ps-*co*-DVB, presented the highest conversion reaching to 27.83 %. CALB-PMMA/PMMA showed the lowest conversion of ester, with only 14.28 %. In the esterification reaction, using oleic acid and ethanol as substrates in solvent-free medium, the lipase immobilized on CALB-Ps-*co*-DVB/Ps-*co*-DVB also presented the highest yield [30]. The differences found in the ester yields using these three enzymes may be associated with the distinct conformation of each immobilized biocatalyst. CALB-Ps-*co*-DVB/Ps-*co*-DVB shows a better contact angle compared to the others immobilized lipases (87.75 º degree to 70.81 º degree in PMMA-PMMA) which may help in mass transfer [29]. Divinylbenzene is known as a hydrophobic support that can hold the open form of lid structure in lipases and stabilizes it in esterification reactions [21,33,34]. The use of divinylbenzene as crosslinking agent may change the structure of the core-shell system, increasing the specific area, which implies in a higher catalytic activity, as already described [30].

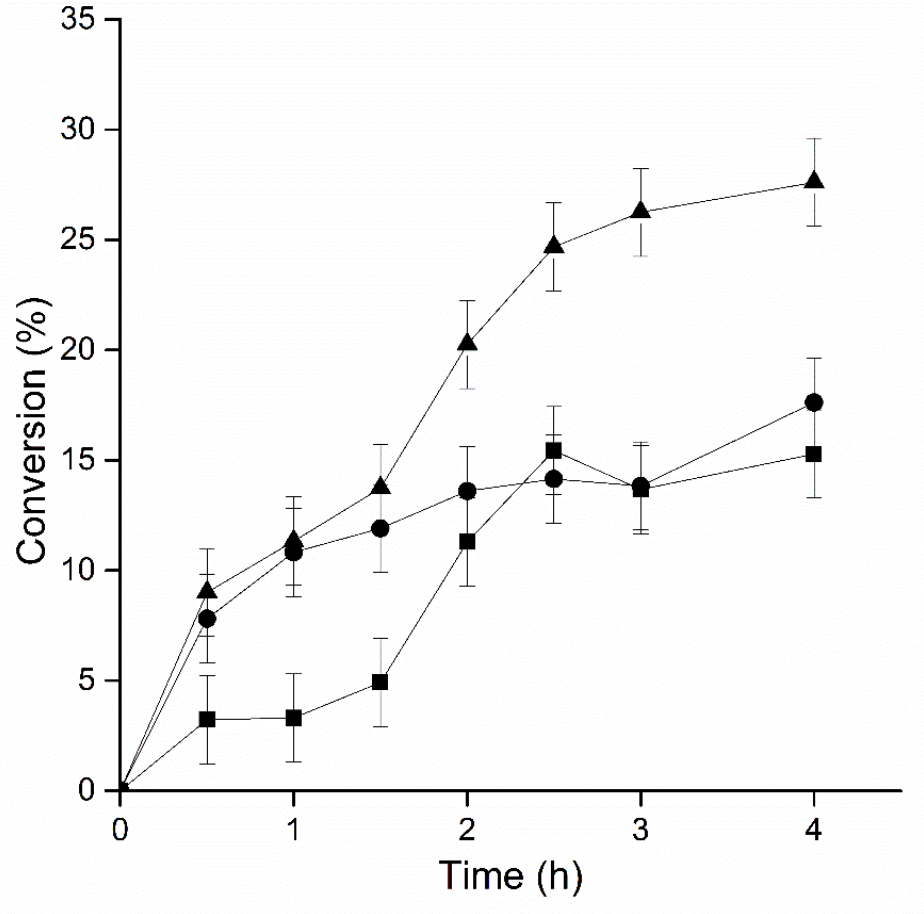

Fig. 1. Isopropyl palmitate conversion using lipase B from C. antarctica immobilized on (■) CALB-PMMA-PMMA, (●) CALB-PMMA-co-DVB/PMMA-co-DVB and (▲) CALB-Ps-co-DVB/Ps-co-DVB. Reaction conditions: 0.1 M of palmitate acid and isopropyl alcohol (molar ratio 1:1), 15 % of enzyme (w/w), 40 °C, 180 rpm, 4 h.

2.3.2 Effect of the organic solvents on the isopropyl palmitate synthesis

Four different organic solvents were tested for the esterification of isopropyl palmitate catalyzed by CALB-Ps-*co*-DVB/Ps-*co*-DVB (Table 3). An explanation for the good activity of CALB-Ps-*co*-DVB/Ps-*co*-DVB using isoctane as a solvent in the reaction is its higher log *P* compared to the other organic solvents [35–37]. This higher log *P* of isoctane (4.5) in comparison to cyclohexane (3.2), for example, indicates an increased hydrophobicity of the reaction medium, probably holding a favorable conformation of the lipase to catalyze the conversion of the substrates to isopropyl palmitate [38,39]. Laane *et al*. (1987) described that enzymatic activity is moderate in solvents with log *P* between 2 and 4 and that is high in nonpolar solvents where log $P > 4$.

Table 3. Effect of the organic solvent on the esterification reaction of isopropyl palmitate catalyzed by CALB-Ps-co-DVB/Ps-co-DVB.

Solvent	Log P	Boiling point (°C)	Conversion (%)
Cyclohexane	3.2	81.0	20.01 ± 1.82 [c]
n-Hexane	3.5	69.0	35.68 ± 1.17 [b]
n-Heptane	4.0	98.0	42.13 ± 0.94 [a,b]
Isoctane	4.5	99.0	51.54 ± 1.03 [a]

2.3.3 Experimental design, model fitting and ANOVA

The CCD was carried out to evaluate the optimal reaction conditions for isopropyl palmitate synthesis and the data results are shown in Table 2. The best results were obtained in treatment 14 (70.25 % of conversion) in which the conditions were 50 °C, molar ratio 1:1 (acid:alcohol) and 30 % of enzyme content (w/w). The less effective conversion was found in the treatment 5 (11.69 %) with the following conditions: 61.9 °C, molar ratio 1.62:1 (acid:alcohol), 14 % of enzyme content. Most of treatments presented conversion yields higher than 50 % within 5 h of reaction, showing that CALB-Ps-*co*-DVB showed good activity for isopropyl palmitate synthesis.

In order to check the model fitness, Fisher's statistical test for analysis of variance (ANOVA) was performed and showed a computed F-value of 4.61, which is significant ($p < 0.05$). The determination coefficient (R^2) was 0.85 and implies that the variation of 85 % for isopropyl palmitate synthesis is attributed to the independent variables, and can be explained by the model. Linear, quadratic, and interaction terms were significant at the 5 % level. Therefore, the second-order polynomial model is given by:

$$Y = 59.39 + 2.92\ X_1 + 10.63\ X_2 + 9.28\ X_3 - 3.60\ X_1^2 - 6.72\ X_2^2 - 4.20\ X_3^2 + 2.52\ X_1X_2 - 4.17\ X_2X_3$$

(Eq. 3)

Where Y is the percentage conversion, and X_1, X_2 and X_3 are the coded values of temperature, substrate molar ratio and enzyme content, respectively.

The linear, quadratic, and the interaction effects of the temperature (X_1), substrate molar ratio (X_2) and enzyme content (X_3) on the enzymatic reaction are shown in Table 4. All the linear effects were positives and statistically significant for the synthesis of isopropyl palmitate. The relationship between reaction variables and response can be better understood by examining the contour plots presented in Fig. 2.

Table 4. Statistical analysis of CCD

Variable	Effect	*p*-Value
Mean	59.39	0.0002
Linear		
X_1[a]	5.83	0.0023
X_2[a]	21.27	0.0018
X_3[a]	18.58	0.0023
Quadratic		
X_1X_1[a]	- 7.20	0.0183
X_2X_2[a]	- 13.45	0.0054
X_3X_3[a]	- 8.41	0.0136
Interactions		
X_1X_2[a]	5.05	0.0499
X_1X_3	-3.09	0.1191
X_2X_3[a]	- 8.35	0.0192

[a] Statistically significant at the 95 % confidence level.

As can be seen in Fig. 2a and Fig. 2b, the substrate molar ratio has a strong impact in the ester conversion. Our results demonstrate that excess of palmitic acid in relation of isopropyl alcohol led to small ester production. The substrates are competitive inhibitors of the enzyme, but a hypothesis, for the case of isopropyl

palmitate synthesis, is that the acid binds first to the enzyme, forming an acyl-enzyme, already reported to lipase B, causing an effect of inhibition over the alcohol [40,41]. The lipase also can present an inhibition originated by mass transfer diffusional limitation when the acid is in excess on reaction media [42]. On the other hand, when the isopropyl alcohol is the major substrate, it was possible to obtain a good ester conversion and this may suggest that excess alcohol does not promote the same toxicity as acid in the microenvironment and the immobilization may help in enzyme stabilization, as also suggested in the production of butyl acetate, isoamyl isobutyrate and ethyl ester [7,33,43].

The temperature is a parameter that influences the isopropyl palmitate conversion, as exhibited in Figures 2b and 2c. This variable is still important for this particular esterification in which long-chain carboxylic acids are used, since palmitic acid is found in crystalline form at room temperature, and a total homogenization is required for good conversion [15]. As already reported in the literature, the temperature is also fundamental for better conversions of pineapple and apple flavors by lipases [44–46]. Our results demonstrate that a higher reaction temperature is required and may be to the fact that the temperature may affect thermodynamically the acid and alcohol binding in the immobilized lipase and the acid solubility in the microenvironment [7,47].

As expected, an enhance in the enzyme content contributed to higher isopropyl palmitate synthesis. Moreover, less biocatalyst content can be used when higher isopropyl alcohol content (Fig. 2a) and temperature (Fig. 2c) were applied.

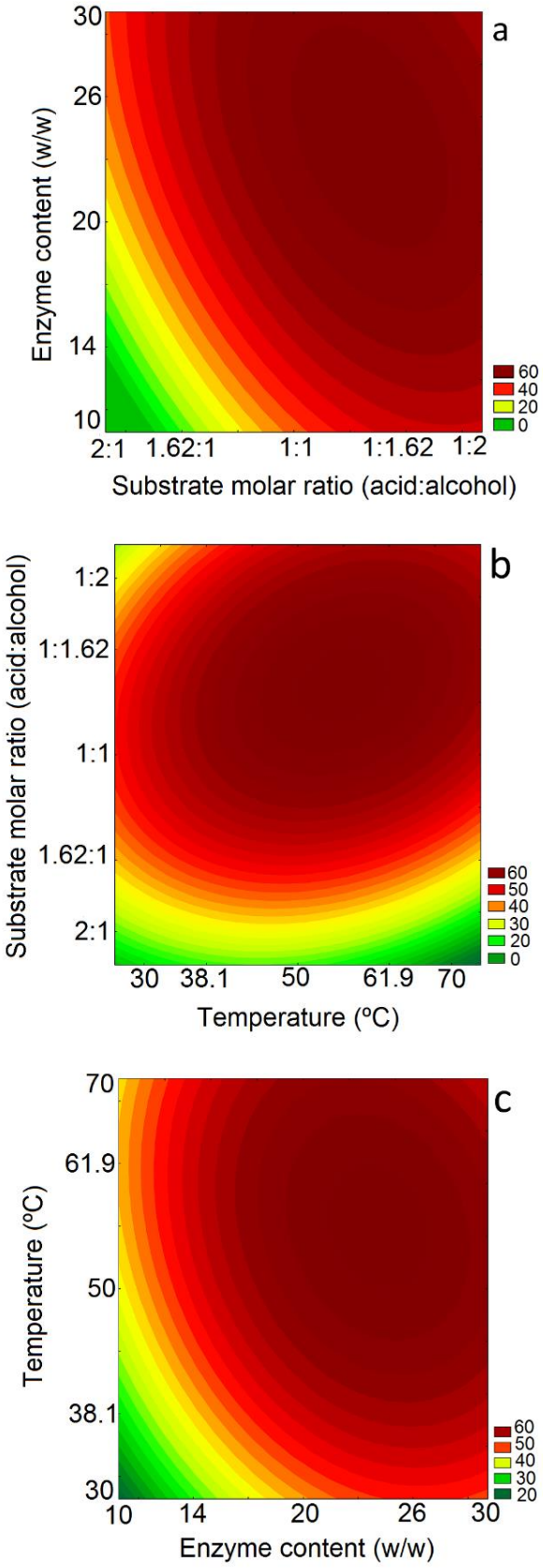

Fig. 2. Contour plots for the synthesis of isopropyl palmitate catalyzed by CALB-Ps-co-DVB/Ps-co-DVB. (a) Temperature was fixed at level 0.504 (55 ºC); (b) Enzyme

content was fixed at level 0.672 (24 % w/w); (c) Substrate molar ratio (acid:alcohol) was fixed at level 0.672 (1:1.42).

2.3.4 Model validation and optimal conditions

The optimal conditions for isopropyl palmitate synthesis were determined by the response desirability profile in the Statistica software. The optimal values were: temperature of 55 ºC; substrate molar ratio of 1:1.42 (acid:alcohol) and enzyme content of 24 %.

In order to validate the predicted model, experiments were carried out in duplicate at the optimal condition for up to 7 h and the time-course of the isopropyl palmitate catalyzed by the CALB-Ps-*co*-DVB/ Ps-*co*-DVB is presented in Fig 3. The predicted value of conversion was 66.85 %, whereas the experimental value was 77.71 %. There is no significative enhance – only 5 % – in ester production after 4 h of reaction since from this point the linear increase in the synthesis of isopropyl palmitate reduces. At the end of reaction, it was found a value of 83 % of isopropyl palmitate conversion. In conclusion, the results obtained for palmitate isopropyl synthesis indicated that the observed value matched to the predicted value proposed in Eq. (2).

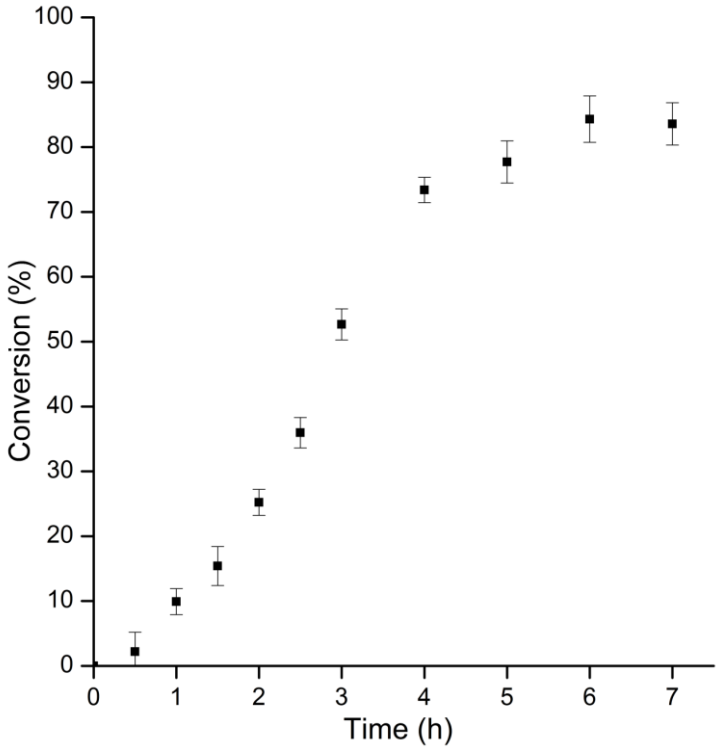

Fig. 3. Model validation of isopropyl palmitate synthesis under optimal conditions (0.1 M of palmitic acid and 0.14 of isopropyl alcohol, 24 % of enzyme (w/w), 55 °C, 180 rpm, 7 h).

2.3.5 Effect of addition of molecular sieves

The use of molecular sieves, in esterification reactions, is described as efficient method to shift the reaction to produce esters, since water is a by-product, and it is removed by the sieves. As shown in Fig. 4, the addition of molecular sieves in the isopropyl palmitate did not change the ester yield in the end of reaction. When 30 mg of molecular sieves per mmol of palmitic acid was used, the initial rate of reaction was increased. Using high amounts of molecular sieves (90 mg per mmol of palmitic acid), it was observed a decrease rate of conversion in the beginning of reaction, possibly due to the role of water in the biocatalyst function microenvironment [48]. As already reported, water contributes in the polarity, stability and the structural integrity of the protein when immobilized, so the total removal of the water helps to contribute to the loss of three-dimensional conformation state [23,43,48]. For the synthesis of butyric acid and several alcohols [8], butyl acetate [6] and sugar esters [49], the addition of molecular sieves had a positive effect.

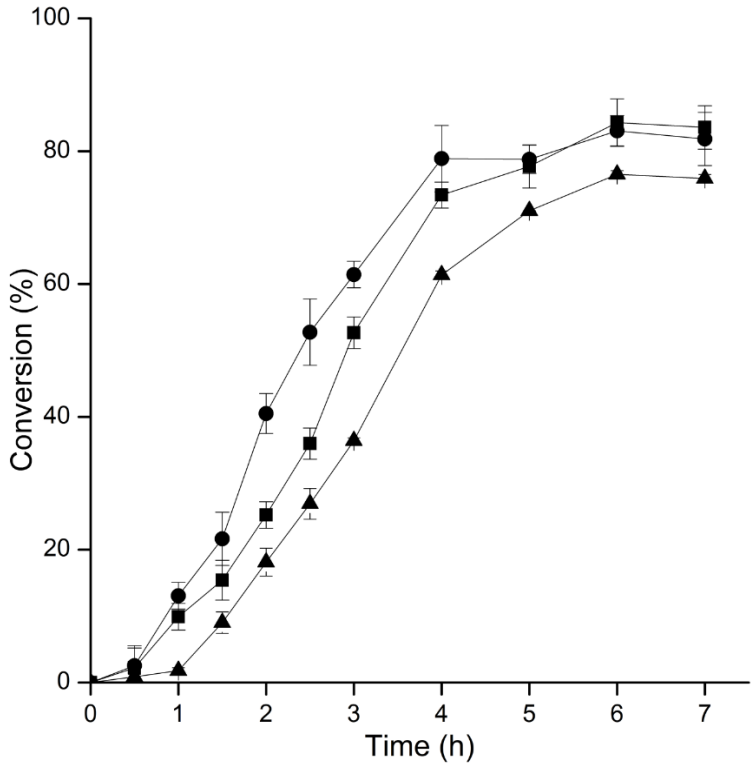

Fig. 4. Isopropyl palmitate synthesis using molecular sieves. (●) 30 mg of molecular sieves per mmol of palmitic acid; (▲) 90 mg of molecular sieves per mmol of palmitic acid. As a control, the reaction was also carried out without molecular sieves (■). Reaction conditions: 0.1 M of palmitic acid, 0.14 M of isopropyl alcohol, 24 % of enzyme (w/w), 55 °C, 180 rpm, 7 h.

2.3.6 Effect of palmitic acid concentration on the isopropyl palmitate synthesis

The effect of substrate concentration was tested on isopropyl palmitate synthesis using CALB-Ps-*co*-DVB/Ps-*co*-DVB with the molar ratio of 1:1.42 (acid:alcohol). As demonstrated in Fig. 7, a linear behavior is observed in the reaction rate up to 0.6 M of palmitic acid concentration. When the acid concentration was increased to 1 M, the reaction rate enhanced only 5 %. Therefore, the best molar concentration of substrates found for the isopropyl palmitate synthesis using CALB-Ps-*co*-DVB/Ps-*co*-DVB was 1 M: 1.42 M (acid:alcohol) (Fig. 5).

As already discussed, it is interesting to carry out the esterification reactions with high concentration of substrates in order to obtain high quantity of products. However, the use of higher acid concentration for the production of esters can affect negatively the biocatalyst due to its toxicity. Immobilized enzymes are, generally, more resistant to changes in the reaction environment [36,43]. The cross-linker agent divinylbenzene, as used for the CALB-Ps-*co*-DVB/Ps-*co*-DVB particles, can help to create a hydrophobic layer around the enzyme, avoiding the acid accumulation in the system as previously described [18,21,30].

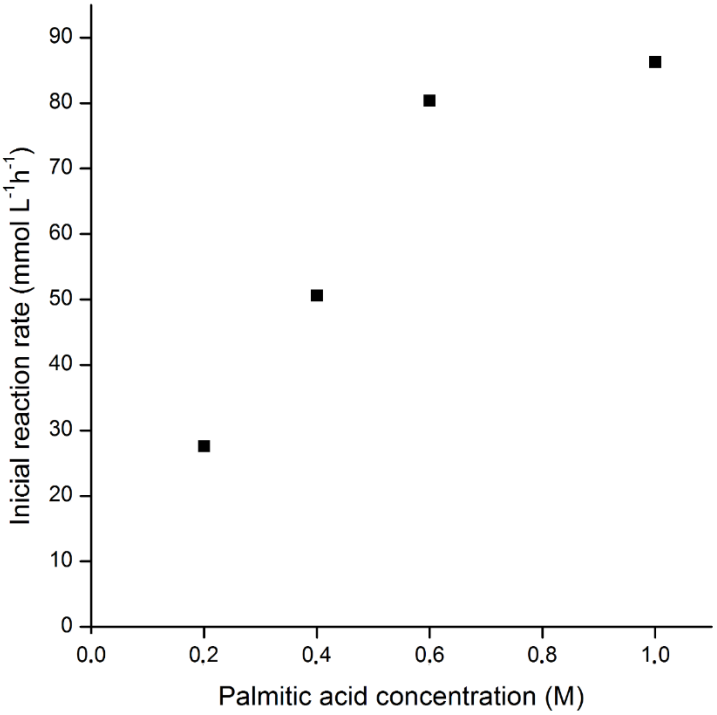

Fig. 5. Effect of palmitate acid concentration in isopropyl palmitate synthesis. Reaction conditions were performed in optimal condition obtained in CCD.

2.3.7 Operational stability

One of the advantages of the enzyme immobilization is the possibility of its reuse. The biocatalyst can be recovered and used in new reactions, which is desirable for industrial application. The operational stability of CALB-Ps-*co*-DVB/Ps-*co*-DVB was tested under the best conditions for the synthesis of isopropyl palmitate. CALB-Ps-*co*-DVB/Ps-*co*-DVB used for the synthesis of isopropyl palmitate showed a good result, since it was possible to reuse the immobilized biocatalyst for up to 7 with 23.2 % of remained activity (Fig. 6). Comparatively, when CALB-Ps-*co*-DVB/Ps-*co*-DVB was reused in solvent-free medium [30], the activity remained after 12 reaction cycles, but the used temperature was 45 ºC, 10 ºC less than used in our work, probably explaining the higher stability. Besides, the immobilized enzyme can lose its activity due to non-reacted substrates or products attached to the microenvironment support/enzyme. Other explanation for the reduced activity after each new cycle could be the formation of acid crystals accumulated when the enzyme was dried at room temperature.

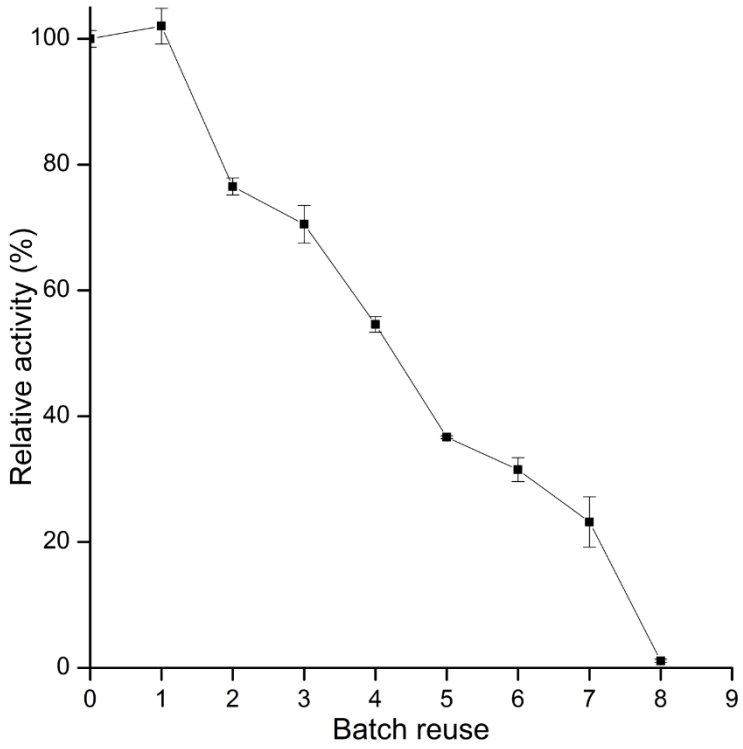

Fig. 6. Operational stability of CALB-Ps-co-DVB/Ps-co-DVB in isopropyl palmitate synthesis under optimal conditions.

2.4 Conclusion

In the present study, lipase B from *C. antarctica* immobilized in three core-shell systems was applied for the synthesis of isopropyl palmitate. CALB-PS-*co*-DVB/PS-*co*-DVB presented the best activity for the isopropyl palmitate synthesis, being the isoctane the most suitable organic solvent. The optimization of the reaction conditions was determined and led to maximum conversion of the ester, also fitting to the predicted model. The reuse of immobilized lipase for the ester production was satisfactory, but the addition of molecular sieves in the reaction medium did not enhance the esterification yield. Besides, this new immobilized lipase also presents an advantage compared to the commercial support used for Novozym 435 preparation, since it is more hydrophobic reducing the adhesion to the biocatalyst in the production of hydrophilic compounds. Moreover, in the literature, the chemical synthesis to produce isopropyl palmitate is well established and few studies deals with the enzymatic route. Thus, our work represents new lights in this field where the

CALB-PS-*co*-DVB/PS-*co*-DVB was never used before for this ester synthesis, being a promising biotechnological application for this biocatalyst.

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2.5 References

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3 DISCUSSÃO GERAL

Esse trabalho foi desenvolvido a fim de estudar a síntese do palmitato de isopropila catalisado por uma lipase B de *C. antarctida* imobilizada. Em um primeiro momento, três tipos de partículas core-shell de lipase B de *C. antarctida* foram avaliadas para identificar aquele que apresentava maior potencial para a produção do palmitato de isopropila, sendo essa a CALB-Ps-*co*-DVB/Ps-*co*-DVB. Uma possível explicação para tal resultado é o uso dos agentes poliestireno (Ps) e divinilbenzeno (DVB), que podem alterar a estrutura dos core-shells, melhorando sua área específica no caso do Ps e aumentando a espessura no caso do DVB. Acredita-se que essa característica auxilia na transferência de massa do substrato para ser convertido em produto. Além disso, no caso dos imobilizados usando PMMA, o ângulo de contato é menor (70,81 º) em comparação com a partícula com Ps (87,75 º), dado a sua hidrofilicidade, o que acarreta em uma menor transferência de massa, prejudicando na produção do produto de interesse (CUNHA *et al.*, 2014; MANOEL, E. A. *et al.*, 2015; MANOEL, E. A.; PINTO, M.; *et al.*, 2016; MANOEL, E. A.; ROBERT; *et al.*, 2016a).

Em relação à avaliação dos solventes orgânicos, quatro solventes com diferentes valores de log *P* foram estudados. O uso de solventes com maiores valores de log *P*, como no caso do isoctano, levou a uma maior conversão do palmitato de isopropila. Esse resultado corrobora com a literatura, onde se relata que a atividade de lipases tende a ter maior atividade de conversão em solventes com log *P* maiores do que 4, visto que a correlação entre a polaridade e a atividade enzimática estão ligadas, pois isso determina a capacidade de distorção em relação a camada de água que fica ao redor do biocatalisador (LAANE *et al.*, 1987; SAREEN; MISHRA, 2008).

No estudo do planejamento composto central, foram investigados três parâmetros de reação: temperatura, razão molar de substrato e quantidade de enzima. Tal investigação é necessária para que haja viabilidade técnica e econômica, a fim de se utilizar menos energia, a quantidade ótima de biocatalisador e se alcançar rendimento máximo de produto. Em temperaturas mais altas, maiores conversões de esterificação foram obtidas, o que já era esperado, visto que a lipase B de *C. antarctida* tem melhor capacidade catalítica em temperaturas um pouco mais elevadas (ANDERSON, E. M.; LARSSON; KIRK, 1998; GOTOR-FERNÁNDEZ;

BUSTO; GOTOR, 2006; JAEGER; EGGERT, 2002). Já na avaliação da razão molar de substrato, o excesso de álcool em relação ao ácido foi observado como a melhor opção por causar o deslocamento do equilíbrio da reação, e além disso, demonstrou menor efeito tóxico. Alguns trabalhos na literatura reforçam essa observação de que maiores quantidades de álcool tendem a ter melhores efeitos no rendimento de ésteres (ANSORGE-SCHUMACHER; THUM, 2013b; BARROS, DE *et al.*, 2012; FU *et al.*, 2015; GARCIA *et al.*, 1999; J; PH; RAMON, 2009; XIAO *et al.*, 2015). Quanto à quantidade de biocatalisador, o valor ótimo encontrado foi de 24 %. O modelo matemático gerado demonstrou boa correlação, uma vez que, sob condições ótimas de reação, obteve-se 78 % de conversão de palmitato de isopropila experimentalmente, enquanto que o valor predito foi de 67 %.

A estabilidade operacional do biocatalisador também foi avaliada e observou-se 23 % de atividade residual após 7 ciclos reacionais. Esse é um resultado satisfatório, visto que a imobilização enzimática visa justamente a reutilização do biocatalisador, e esse é um parâmetro muito importante para escalonamento em processos industriais. Já em relação ao uso de peneiras moleculares, a utilização de 30 mg de peneiras moléculas apresentou um aumento na taxa inicial de reação. No final de reação, o uso das peneiras não demonstrou diferença em relação a reação que não foi adicionada as peneiras moleculares. Mesmo que o objetivo seja a remoção da água, a qual é um subproduto de reações de esterificação, para que haja deslocamento do equilíbrio no sentido dos produtos, uma possível explicação para uma menor conversão de produto utilizando concentrações maiores de peneiras moleculares (90 mg) é que a água também é necessária para a estabilização da conformação tridimensional da enzima. Sobre o estudo da concentração molar de ácido palmítico, foi visto que quanto maior a concentração, maior velocidade inicial da enzima, sendo o valor ótimo de 1 M, na razão molar 1:1,42 ótimo.

Após o desenvolvimento desse trabalho, pode-se concluir que o biocatalisador CALB-Ps-*co*-DVB/Ps-*co*-DVB possui potencial biotecnológico para ser aplicado na produção de ésteres. Esse tipo de imobilizado, até o presente momento, nunca foi reportado na literatura na produção de palmitato de isopropila. Além disso, o suporte utilizado na imobilização da lipase B de *C. antartida* apresenta algumas características mais interessantes em relação ao suporte comercial (Novozym 435), atualmente utilizado na indústria, como maior hidrofobicidade e maior área

específica, o que pode influenciar nas mais diversas aplicações. Como perspectiva desse estudo, pode-se avaliar a utilização do CALB-Ps-*co*-DVB/Ps-*co*-DVB em reatores enzimáticos na tentativa de se obter maiores conversões de ésteres, assim como a própria purificação do produto e avaliação do seu grau de pureza para a utilização em indústria.

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