

Isopropyl myristate continuous synthesis in a packed-bed reactor using lipase immobilized on magnetic polymer matrix

Mateus V. C. Silva¹, Caio A. P. de Souza², Pedro C. de Oliveira³, Heizer F. de Castro⁴, Larissa Freitas⁵

Department of Chemical Engineering, Engineering School of Lorena, University of São Paulo, Lorena, SP 12602-810, Brazil.

Abstract— The aim of this study was to synthesize isopropyl myristate, an emollient ester, in a continuous-flow packed-bed reactor using *Candida antarctica* lipase immobilized on poly(styrene-co-divinylbenzene) matrix prepared by suspension polymerization and magnetized by co-precipitation of Fe^{2+} and Fe^{3+} in alkaline medium. To determine the best esterification conditions, we investigated the effects of acid/alcohol molar ratio (1:5, 1:10, and 1:15) on reaction yield in shake flasks. The three tested conditions provided similar results, esterification yields of approximately 80%. An acid/alcohol molar ratio of 1:15 was chosen for further experiments because it allowed for better operability of the bioreactor. Subsequently, we compared the reactor performance in up flow and down flow modes. This experiment showed that greater ease of operation was achieved with down flow operation. We also evaluated the influence of space time (8 and 20 h) on reaction yield and productivity. A space time of 8 h provided better results. An experimental system consisting of two bioreactors and a molecular sieve packed column was used to remove the water formed during esterification and thus increase the yield of isopropyl myristate. There was a significant improvement in performance with the use of the two-stage system, which resulted in almost complete conversion of reagents, an increase of about 150% in biocatalyst half-life, and an isopropyl myristate productivity of $25 \text{ g L}^{-1} \text{ h}^{-1}$, confirming the beneficial effect of adding a water extraction column to the experimental system.

Keywords— esterification, isopropyl myristate, lipase immobilized, magnetic particles, packed-bed reactor.

I. INTRODUCTION

Isopropyl myristate is an emollient ester widely used in cosmetic preparations, especially in skin care products, because of its excellent spreading properties, non-toxicity, great biocompatibility, and high skin permeation ability [1-2]. In the pharmaceutical industry, isopropyl myristate is used as a skin penetration enhancer in topical formulations for transdermal drug delivery[3].

Currently, most industrial processes for the synthesis of isopropyl myristate use conventional chemical catalysis at elevated temperatures, which affords a low-quality product with residual color and odor, demanding expensive purification steps before the product can be marketed [1]. Efforts have been intensified to replace industrial chemical processes with eco-friendly methods. A major problem in chemical industries is the use of chemical catalysts, as these compounds generate waste, have high environmental impact, and increase purification costs [4]. Bioprocesses can be a sustainable alternative to a wide variety of conventional chemical processes.

Enzymes have advantages over chemical catalysts, not only in terms of environmental impact but also in terms of productivity, specificity, toxicity, and temperature and pressure reaction conditions. Among the enzymes that are used industrially, lipases (EC 3.1.1.3) are notable for catalyzing reactions in aqueous and organic media, such as esterification and hydrolysis reactions. This class of enzymes has applications in the manufacture of pharmaceutical products, surfactants, and cosmetics [5]. Industrial biocatalysis can benefit from enzyme immobilization techniques to increase the biocatalyst's thermal stability and pH stability and allow its recovery and reuse both in batch and continuous reactors [6].

Several materials have been researched as enzyme supports. Magnetic materials are outstanding for this application because they can be easily recovered from the reaction medium, which obviates the need for centrifugation, filtration, or column separation steps [7]. When lipase is immobilized on a highly hydrophobic support, such as a poly (styrene-co-divinylbenzene) matrix, the hydrophobic lid, which controls access to the catalytic site, interacts with the support, exposing the enzyme's active site and increasing its affinity for the substrate, a mechanism known as interfacial activation. Another advantageous characteristic of hydrophobic supports is that they absorb less water from the reaction medium, which is desirable in esterification reactions [8].

The present work aimed to study the continuous enzymatic synthesis of isopropyl myristate in a fixed-bed reactor using lipase immobilized on a magnetic matrix as biocatalyst. The influence of the molar ratio of starting materials, feed flow direction, space time, and use of a water extraction column on reaction yield and productivity was investigated with the aim of developing a stable enzymatic process with potential industrial application.

II. MATERIALS AND METHODS

2.1 Materials

Polyvinyl alcohol (PVA) (MW 78,000, 88% hydrolyzed; Polysciences Inc.), azobisisobutyronitrile (AIBN) (MIG Química), divinylbenzene (80%; Sigma-Aldrich[®]), styrene (99%; Sigma-Aldrich[®]), heptane (95%; Cromoline), methanol (99.8%; Cromoline), and toluol (99.5%; Cromoline) were used for the synthesis of the magnetic poly(styrene-*co*-divinylbenzene) matrix. Ethyl acetate (99.5%; Cromoline), ultrapure water, sodium hydroxide (99%; Synth), iron(II) chloride tetrahydrate (99%; Sigma-Aldrich[®]), iron(III) chloride hexahydrate (97%; Sigma-Aldrich[®]), oleic acid (p.a.; Cromoline), and ethanol (96%; Cromoline) were used for the synthesis and modification of magnetite. The following materials were used for enzyme immobilization: *Candida antarctica* lipase (Sigma-Aldrich[®]), polyethylene glycol (PEG) (MW 1,500; Synth), and heptane (95%; Cromoline). Methyl butyrate (99%; Sigma-Aldrich[®]), potassium dihydrogen phosphate (99%; Cromoline), ethanol (96%; Synth), sodium hydrogen phosphate (99%; Synth), and potassium hydroxide (85%; Synth) were used for determination of enzyme activity. Pure myristic acid (Cromoline), isopropyl alcohol (99.5%; Cromoline), heptane (95%; Cromoline), and 3A molecular sieve (8–12 mesh beads; Sigma-Aldrich[®]) were used for the synthesis of isopropyl myristate.

2.2 Methods

2.2.1 Determination of enzyme activity

Enzyme activity was quantified by measuring the hydrolysis of methyl butyrate in 25 mmol L⁻¹ phosphate buffer medium, pH 7.0, at 200 rpm and 45 °C in shake flasks, according to the modified method described by Fidalgo et al. (2016) [9]. Briefly, 300 µL of methyl butyrate and 30 mL of phosphate buffer were added to Erlenmeyer flasks. Then, 0.05 g of immobilized *C.antarctica* lipase was added to each flask. After 10 min of reaction, 10 mL of ethanol was added, and samples were titrated with 0.04 mol L⁻¹ KOH. The mean activity of the biocatalyst was 479.12 ± 14.35 U g⁻¹.

2.2.2 Synthesis of poly(styrene-*co*-divinylbenzene) magnetized with modified magnetite

The poly (styrene-*co*-divinylbenzene) matrix (STY-DVB-M) was synthesized following the method of Bento et al. (2017) [10]. Magnetite, required for polymer synthesis, was synthesized by co-precipitation of iron ions. Iron (II) chloride (0.6 mol L⁻¹) and iron (III) chloride (1.2 mol L⁻¹) solutions were mixed and kept at 65°C under stirring. Sodium hydroxide (4 mol L⁻¹) was added slowly until pH 11. After reaching the desired pH, the ferric solution was kept at 65°C under stirring for 30 min. The flask was placed on a magnet to precipitate the magnetic particles. The resulting black material was decanted and washed successively with ultrapure water and a 1:1 ethyl acetate/water solution until the supernatant reached pH 7.0. The mixture was vacuum filtered and oven dried at 60°C for 18 h to afford magnetite particles.

Magnetite was modified with oleic acid to increase its hydrophobicity. Briefly, 3.25 g of magnetite, 40 mL of oleic acid, and 80 mL of ultrapure water were added to a beaker and kept under stirring for 15 min. The mixture was vacuum filtered, washed with ethanol to remove the residual oleic acid, and oven dried at 60 °C for 18 h.

The polymer support was synthesized by suspension polymerization, in which styrene and divinylbenzene monomers comprised the organic phase, toluol and heptane were respectively the high-affinity and low-affinity solvents, AIBN was the initiator, and the aqueous phase was an aqueous PVA solution. The reaction was conducted in a 1 L glass reactor at 400 rpm and 70°C under an inert atmosphere (nitrogen gas). After polymerization, particles were vacuum filtered and washed sequentially with ultrapure water at room temperature, ultra pure water at 50°C, acetone, and ethanol to remove the aqueous phase and residual reagents. The material was then oven dried at 60°C for 18 h and sieved to 24–80 mesh size using an electromagnetic sieve shaker (Spencer).

2.2.3 Immobilization of *C.antarctica* lipase on STY-DVB-M

STY-DVB-M was immersed in heptane at a ratio of 1:10 (w/v) and kept under stirring for 2 h on a shaker. Excess heptane was removed, and flasks received the addition of 100 µL of 5 g L⁻¹ PEG 1500 and 250 µL of lipase per gram of STY-DVB-M. The mixture was homogenized and then kept still for 18 h at 4 °C. Finally, immobilized lipase was vacuum filtered and washed with heptane until the moisture content was reduced to less than 10%.

2.2.4 Substrate preparation

The substrate for isopropyl palmitate synthesis was prepared in 500 mL glass flasks using appropriate amounts of myristic acid, isopropanol, and 10% (w/v) molecular sieves previously activated.

2.2.4.1 Esterification in shake flasks

Isopropyl myristate synthesis was carried out at 50 °C and 200 rpm in shake flasks containing 20 mL of substrate and 10% (w/v) biocatalyst. At predetermined intervals (0.15, 30, 45, 60, 90, 120, 180, and 240 min), aliquots of approximately 0.1 g were withdrawn to monitor product formation.

2.2.4.2 Continuous esterification in a fixed-bed bioreactor

Fixed-bed bioreactor experiments were carried out in a jacketed glass column (Diogolab®) with a height of 166 mm, an internal diameter of 11 mm, and an internal volume of 15.8 mL. The height/diameter relationship was defined on the basis of previous work [11]. The reactor was packed with 4.3 g of biocatalyst, and substrates were fed using a peristaltic pump (Sci-Q 400, Watson-Marlow). The reaction was conducted at 50 °C. The reaction scheme is shown in Figure 1.

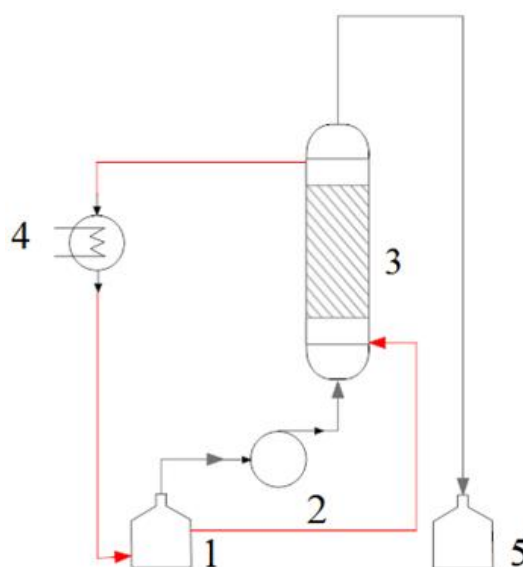


FIGURE 1 – Experimental lay out of the packed bed reactor used in the synthesis of isopropyl myristate under continuous flow, where: 1-feeding reservoir; 2-peristaltic pump; 3-glass column; 4-thermostated bath; 5 – product reservoir.

Space-time was calculated according to Equation 1:

$$\tau = \frac{V_{working}}{Q} \quad (1)$$

In which: V= Reactor working volume reactor (mL) and Q= flow rate (mL /min).

The reactor working volume was calculated as the difference between the total reactor volume and the volume occupied by the biocatalyst. The density of the biocatalyst was 1.11 g L⁻¹, calculated according to Simões et al. (2015) with modifications [12].

2.2.4.3 Strategy for water removal from the reaction medium

To remove the water formed during the esterification reaction and thus shift the equilibrium to the side of ester formation, we operated the reactor in a two-stage configuration with a water extraction column (packed with molecular sieves) between the two bioreactors.

The experimental system comprised two jacketed glass columns (166 mm in height, 11 mm in internal diameter, and 15.8 mL in internal volume), each packed with 4.3 g of biocatalyst, and a water extraction column containing 10.8 g of molecular sieves. The immobilized enzyme/molecular sieve relationship was determined by Freitas et al. (2011) [13] and corresponds to a 1:1.25 (w/w) ratio. Molecular sieves were replaced in the water extraction column every 3 days of continuous operation. Figure 2 illustrates the two-stage reactor system.

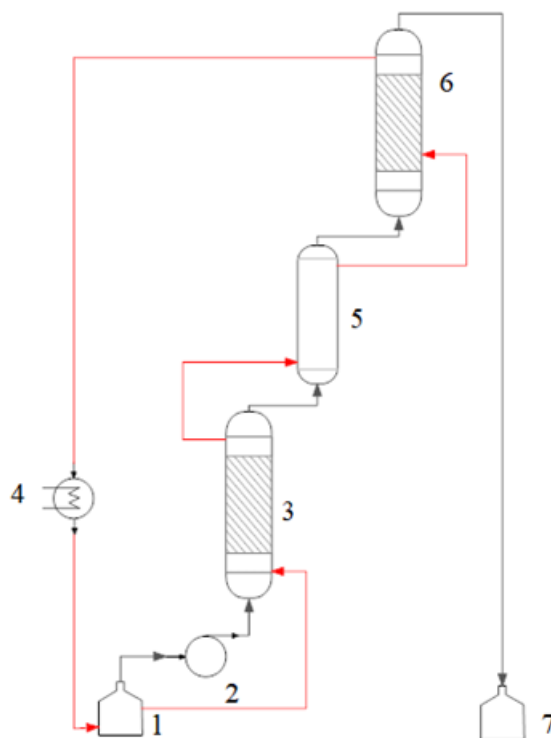


FIGURE 2- Experimental set up used for esterification of isopropanol with myristic acid in two-stage packed bed reactor connecting with water column extractor: 1-feeding reservoir; 2-peristaltic pump; 3 and 6-enzyme packed columns; 4-bath for temperature control; 5-column with molecular sieves and 7-product reservoir.

2.2.5 Determination of isopropyl myristate content

Quantification of isopropyl myristate was performed using a PerkinElmer[®] Clarus 580 gas chromatograph equipped with a flame ionization detector (FID) and a 5% diphenyl, 95% dimethyl polysiloxane capillary column coated with glass fiber. Isopropyl myristate standard was synthesized following the methods proposed by Vilas Bôas et al. (2017) [14]. FID was supplied with synthetic air at 400 mL min⁻¹ and H₂ at 40 mL min⁻¹. N₂ was used as carrier gas at a flow rate of 0.2–1.0 mL min⁻¹. Detector and injector were maintained at 250 °C.

Temperature program was started at 105 °C for 7 min with an N₂ flow rate of 0.2 mL min⁻¹, followed by ramp to 200 °C at 20 °C min⁻¹ and hold for 1 min and ramp to 280 °C at 25 °C min⁻¹ and hold for 2 min, totaling 17.95 min of analysis. The injection volume was 1 µL of a 1:1 mixture of sample and internal standard (8.0 g L⁻¹ hexanol in heptane medium).

2.2.6 Monitoring of esterification by quantification of the limiting reagent (myristic acid)

Concentration of myristic acid was monitored by titration with aqueous KOH solution (0.04 mol L⁻¹) using phenolphthalein as indicator. Aliquots (0.1 g) of the reaction medium were added to 10 mL of ethanol. Esterification yield was calculated on the basis of the stoichiometric ratio of synthesized ester and considering that myristic acid was completely converted to isopropyl myristate, according to Equation 2:

$$\text{Esterification yield (\%)} = \left(\frac{C_{\text{ester}}}{C_i} \right) * 100 \quad (2)$$

In which: C_i = Initial concentration of the limiting reactant (myristic acid, mmol·L⁻¹) and C_{ester} = Ester concentration at a given time (mmol·L⁻¹).

2.2.7 Operational stability of the biocatalyst

The operational stability of the biocatalyst was evaluated according to the method proposed by Pires-Cabral et al. (2010) [15]. The highest esterification yield was used as the reference activity (100% catalytic activity), and the residual activity was determined by the ratio of esterification yield at a given time to the highest esterification yield. The best linear fit to the data was calculated using Origin 9.0.

III. RESULTS

3.1 Influence of acid/alcohol molar ratio on isopropyl myristate synthesis in shake flasks

Aiming to obtain the optimal experimental condition for the synthesis of isopropyl myristate in solvent-free medium, we tested the following acid/alcohol molar ratios: 1:5, 1:10, and 1:15. The 1:5 acid/alcohol molar ratios is the minimum required for solubilization the fatty acid at 50 °C.

Vadgma, Odaneth, and Lali (2015) [1] reported that the optimal temperature for the activity of *C. antarctica* lipase, the same lipase used in the present work, immobilized on acrylic resin (Novozym 435) was 50–60 °C. Thus, 50 °C was the reaction temperature used in the present study. Results are shown in Figure 3.

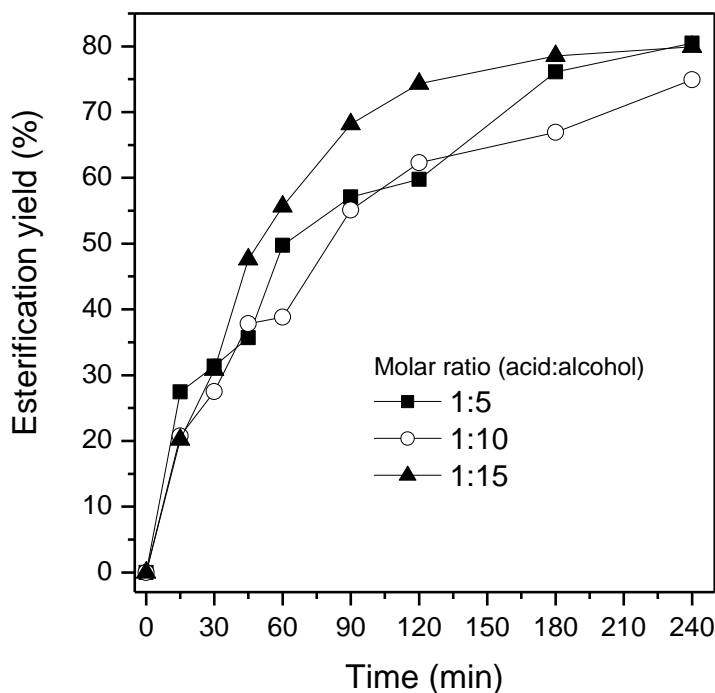


FIGURE 3–Esterification progress in the synthesis of isopropyl myristate catalyzed by CALB-STY-DVB-M at 50°C, in shake flasks, using different molar ratio acid to alcohol and 10% (m/v) of biocatalyst in solvent free medium

No significant differences were observed among the esterification yields obtained under the three conditions. Yields of approximately 80% were obtained in 240 min of reaction. The reaction yield was not influenced by the increase in the proportion of isopropyl alcohol (reagent).

3.2 Study of flow direction in the single-stage continuous fixed-bed bioreactor

The acid/alcohol molar ratio of 1:15 was chosen for this experiment, as the viscosity of the reaction medium is proportional to the concentration of fatty acids; that is, the lower the concentration of fatty acids, the lower viscosity of the reaction medium. It is known that the use of a high-viscosity reaction medium in a fixed-bed bioreactor can cause pressure drop, affecting the substrate flow rate and thereby reducing the reaction yield [16].

Continuous esterification was carried out in a fixed-bed reactor operating in upflow and down flow modes to investigate the influence of flow direction on the synthesis of isopropyl myristate. Feed can be pumped down flow, which leads to a lower bed pressure drop as a result of the action of gravitational force, or upflow, which minimizes flow channeling. In upflow mode, however, the maximum flow rate is limited, as it cannot exceed the minimum fluidization velocity; otherwise, the bed will fluidize [16-18]. A feed rate of $0.0405 \text{ mL min}^{-1}$ was used, totaling a space time of 5 h. Results are exhibited in Figure 4.

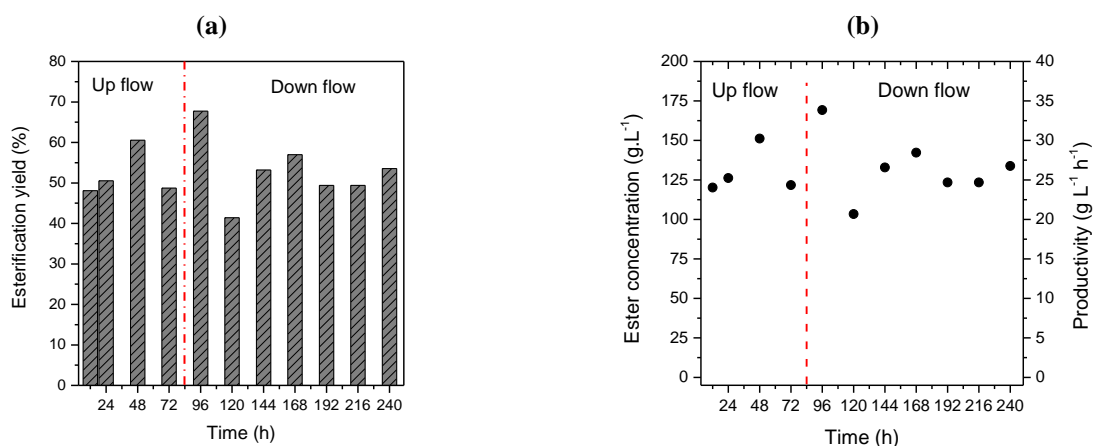


FIGURE 4 – a) Esterification yield and b) Ester concentration and volumetric productivity in the isopropyl myristate synthesis carried out under continuous flow in packed bed bioreactor catalyzed by CALB-STY-DVB-M at 50°C, using up flow and down flow rates (space-time 5h).

As shown in Fig. 4a, mean esterification yields of 55% were obtained regardless of the flow direction. However, down flow resulted in higher stability and less bubble formation inside the bed, probably because of the lower pressure drop.

Freitas et al. (2011) [13] synthesized monoglycerides from babassu oil using lipase from *Burkholderia cepacia* immobilized on SiO₂-PVA and observed significant differences in monoglyceride yields between the two reactor configurations: a 5% yield was obtained with upflow, whereas a 22% yield was obtained with downflow. According to the authors, this result was due to insufficient homogenization of the substrate when pumped upflow, which hampered catalysis. Fidalgo et al. (2016) studied the influence of flow direction on biodiesel production using Novozym 435 and obtained similar yields (approximately 90%) in both flow directions [9]. These results might be associated with the low feed rate used in each study, as was the case of the present work. The down flow mode was used in subsequent experiments.

3.3 Study of feed rate in the single-stage continuous fixed-bed bioreactor

To increase the performance of the evaluated system, we investigated the influence of feed rate on isopropyl myristate synthesis. Feed rates of 0.025 mL min⁻¹ and 0.01 mL min⁻¹ were tested, resulting in space-times of 8 and 20 h, respectively. Figure 5 shows the results obtained with an 8 h space-time, and Figure 6 presents the results obtained with a 20 h space-time

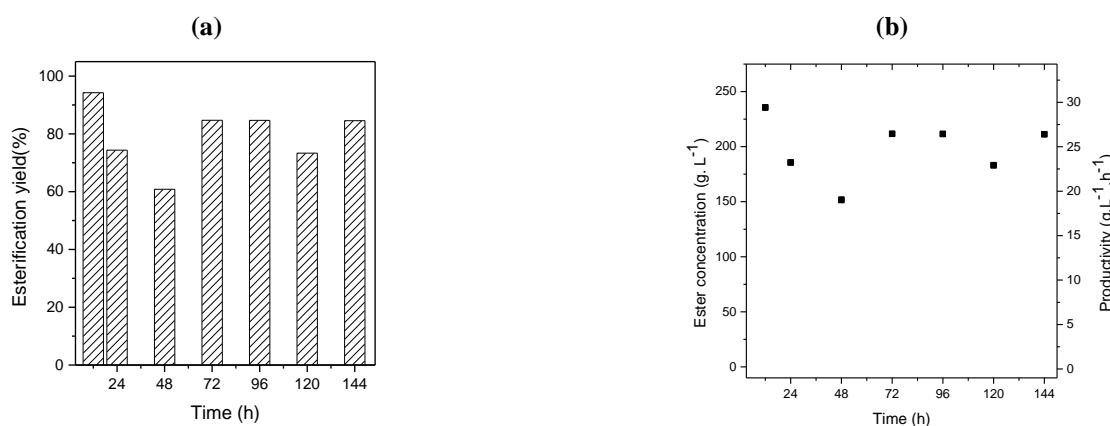


FIGURE 5 – a) Esterification yield and b) Ester concentration and volumetric productivity in the isopropyl myristate synthesis carried out under continuous flow in packed bed bioreactor catalyzed by CALB-STY-DVB-M at 50°C, using down flow rates (space-time 8h).

A space time of 8 h resulted in an 80% yield and 25 g L⁻¹ h⁻¹ productivity. Similar results were found by Vadgama et al. (2015) [1], who studied the continuous synthesis of isopropyl myristate by esterification of myristic acid with isopropanol in a fixed-bed bioreactor using Novozym 435 as biocatalyst. The operating conditions were acid/alcohol molar ratio of 1:15, 60°C, and space time of 5 h. Yields of approximately 80% were obtained.

An esterification yield of 70% and a productivity of $8 \text{ g L}^{-1} \text{ h}^{-1}$ (Fig. 6b) were obtained with a space time of 20 h. The lower yield, compared with that obtained with a space time of 5 h, can be explained by the increase in water formed during the esterification reaction, contributing to the reversion of esterification as a result of the longtime of contact between substrate and biocatalyst. An option to minimize this effect is to remove the water formed in the reaction medium.

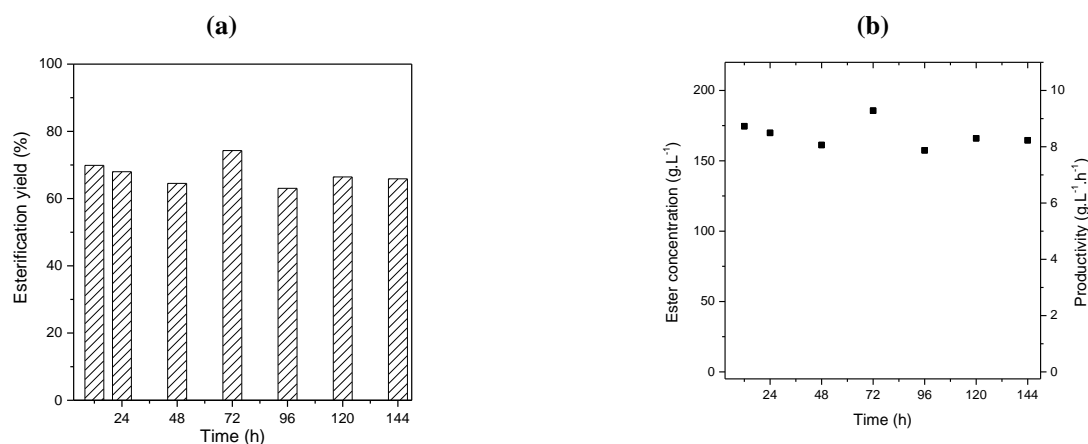


FIGURE 6— a) Esterification yield and b) Ester concentration and volumetric productivity in the isopropyl myristate synthesis carried out under continuous flow in packed bed bioreactor catalyzed by CALB-STY-DVB-M at 50°C, using down flow rates (space-time 20h).

3.4 Synthesis of isopropyl myristate in a two-stage fixed-bed bioreactor with a water extraction column

As stated earlier, the water formed during esterification affects substrate conversion; lower conversions are obtained with increased water contents [19]. Several strategies for water removal are described in the literature, such as pressure reduction; use of drying agents (molecular sieve or silica gel), hydrophilic solvents, or salt pairs; and dry air bubbling [20]. Furthermore, reduction of residual fatty acid in the reaction medium facilitates the separation and purification of the product.

We evaluated the performance of a water extraction column containing molecular sieves in the continuous system studied. A feed rate of 0.04 mL min^{-1} was used, which corresponds to a space time of 10 h. Results are shown in Figure 7.

Under these operating conditions, conversion to isopropyl myristate was almost complete. Esterification yield and productivity were 99% and $25 \text{ g L}^{-1} \text{ h}^{-1}$, respectively. These results indicate that molecular sieves were effective in removing water from the reaction medium, which shifted the equilibrium toward ester formation and consequently increased the yield. In addition, the extraction column provided greater stability to the system. No variations were observed during 6 days of operation.

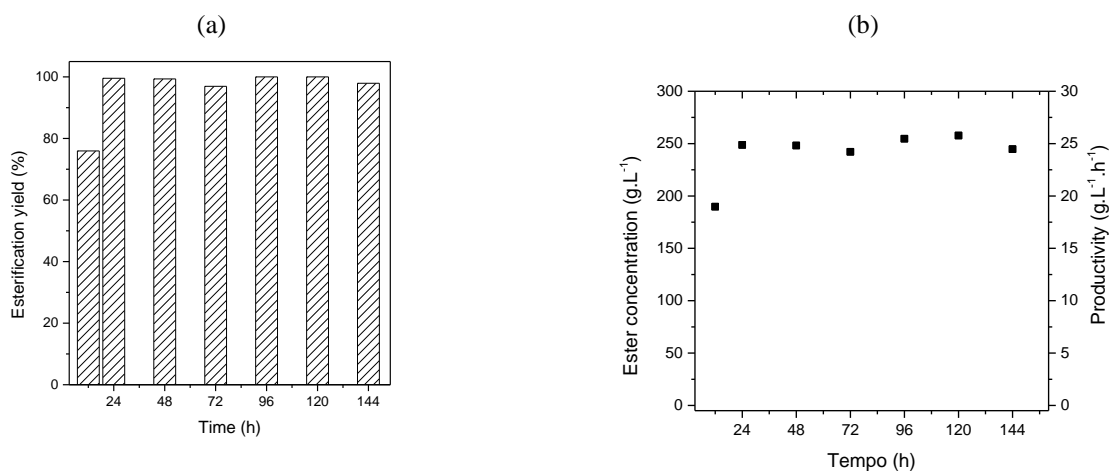


FIGURE 7 -a) Esterification yield and b) Ester concentration and volumetric productivity in the isopropyl myristate synthesis carried out under continuous flow in two stage packed bed bioreactor coupling with water extraction column catalyzed by CALB-STY-DVB-M at 50°C, using down flow rates (space-time 10h).

3.5 Operational stability of the biocatalyst

The operational stability of immobilized enzymes is a parameter of fundamental importance in reactions carried out for long periods. The operational stability of *C. antarctica* immobilized on STY-DVB-M was evaluated in the single-stage ($\tau = 8$ h) and the two-stage ($\tau = 10$ h) packed-bed reactor systems. Figure 8 shows the residual activity of the biocatalyst in both processes.

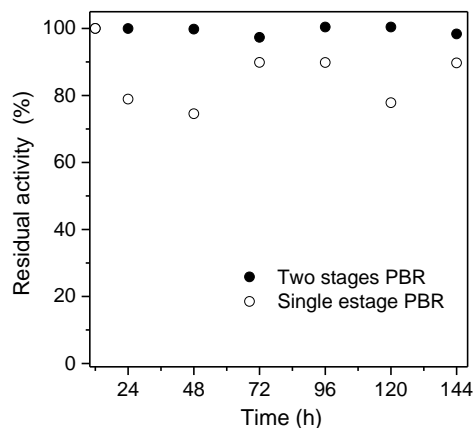


FIGURE 8 – Residual esterification activity of the lipase from *Candida antarctica* immobilized on STY-DVB-M quantified on continuous runs carried on packed bed reactors (single stage and two-stages)

The biocatalyst had a half-life of approximately 35 days ($K_d = 0.00137 \text{ h}^{-1}$) in the single-stage reactor, whereas, in the two-stage reactor, the half-life was 51.7 days ($K_d = 0.000559 \text{ h}^{-1}$). The use of the water extraction column significantly increased the half-life of the biocatalyst (by approximately 150%), which made the process more stable.

Similar results were observed by Freitas et al. (2011) [13]. The authors studied the synthesis of monoglycerides by esterification of oleic acid and glycerol with *Penicillium camemberti* lipase immobilized on SiO_2 -PVA as biocatalyst in a continuous fixed-bed reactor. An increase in the half-life of the enzyme (total of 19 days) was achieved with the use of a water extraction column. In a study by Lee et al. (2013) [21], who used lipase Novozym 435 to synthesize erythorbyl laurate, a two-fold increase in the half-life of the biocatalyst was observed with the use of a potassium resin to remove water from the reaction medium.

The results suggest that the biocatalyst can be used for several cycles of operation without significant loss of productivity, an economic and environmental advantage. This bioprocess is a competitive alternative to conventional chemical processes.

IV. CONCLUSION

No significant differences in esterification yields were observed among the three tested acid/alcohol molar ratios (1:5; 1:10, and 1:15). Therefore, an acid/alcohol ratio of 1:15 was used for the synthesis of isopropyl myristate in a continuous packed-bed reactor, as it resulted in lower substrate viscosity and, consequently, greater operability. The down flow mode was selected because it caused a low bed pressure drop as compared with the upflow mode.

In the single-stage reactor, the highest yield and productivity were obtained with a space-time of 8 h. The extraction column used in the two-stage reactor was effective in removing the water formed during esterification and in increasing the stability of the bioprocess. Almost complete conversion of myristic acid to isopropyl myristate was obtained, and the half-life of the biocatalyst increased by approximately 150%.

The results were satisfactory, and reaction and operational conditions were established for the continuous enzyme-catalyzed synthesis of isopropyl myristate in solvent-free medium conducted in a fixed-bed bioreactor. The bioprocess is an interesting option for large-scale industrial application

ACKNOWLEDGEMENTS

This study was financed in part by the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior - Brazil (CAPES) - Finance Code 001 and was supported by the São Paulo Research Foundation - Brazil (FAPESP) (grants nos. 2016/17833-3 and 2016/10636-8).

REFERENCES

- [1] Vadgama, RN, Odaneth AA, Lali, AM, Green synthesis of isopropyl myristate in novel single phase medium Part II: Packed bed reactor (PBR) studies, *Biotechnol. Rep.*, vol. 8, pp.105-109, October 2015.
- [2] Zhang Y, Zhang XY, Chai JL, Cui XC, Pan J, Song JW, Sun B, Lu JJ, The phase behavior and solubilization of isopropyl myristate in microemulsions containing hexadecyl trimethyl ammonium bromide and sodium dodecyl sulfate, *J. Mol. Liq.*, vol. 244, pp. 262-268, September 2017.
- [3] Zhao C, Quan P, Liu C, Li Q, Fang L, Effect of isopropyl myristate on the viscoelasticity and drug release of a drug-in-adhesive transdermal patch containing blonanserin, *Acta Pharm. Sin. B.*, vol. 6, pp. 623-628, May 2016.
- [4] Gonçalves CGS, Marsaioli AJ, Biocatalysis: facts and trends, *Quim. Nova*, vol. 36, n. 10, pp.1587-1590, October 2013.
- [5] Zheng J, Wei W, Lan X, Zhang Y, Wang Z, Fluorescent microplate assay method for high-throughput detection of lipase transesterification activity, *Anal. Biochem.*, vol. 549, pp 26-28, July 2018.
- [6] Cai Q, Hu C, Yang N, Wang Q, Wang J, Pan, H, Hu Y, Ruan C, Enhanced activity and stability of industrial lipases immobilized on spherical bacterial cellulose, *Int. J. Biol. Macromol.*, vol. 109, pp. 1174-1181, January 2018.
- [7] Liu D, Chen J, Shi Y, Advances on methods and easy separated support materials for enzymes immobilization, *Trend. Anal. Chem.*, vol. 102, pp. 332-342, March 2018.
- [8] Qi H, Du Y, Hu G, Zhang L, Poly(carboxybetaine methacrylate)-functionalized magnetic composite particles: A biofriendly support for lipase immobilization, *Int. J. Biol. Macromol.*, vol. 107, pp. 2660-2666, October 2018.
- [9] Fidalgo, WRR, Ceron A., Freitas L, Santos JC, de Castro HF, A fluidized bed reactor as an approach to enzymatic biodiesel production in a process with simultaneous glycerol removal, *J. Ind. Eng. Chem. - Korean Society of Industrial and Engineering Chemistry*, vol. 36, pp. 217-223, May 2016.
- [10] Bento HBS, de Castro HF, de Oliveira PC, Freitas L, Magnetized poly(STY-co-DVB) as a matrix for immobilizing microbial lipase to be used in biotransformation, *J. Magn. Magn. Mater.*, vol. 426, pp. 95-101, January 2017.
- [11] Damstrup ML, Kill S, Jensen AD, Sparsø FV, Xu X, Process development of continuous glycerolysis in an immobilized enzyme-packed reactor for industrial monoacylglycerol production, *J. Agric. Food Chem.*, vol. 55, pp. 7786-7792, August 2007.
- [12] Simões AS, Ramos L, Freitas L, Santos JC, Zanin G.M., de Castro, HF, Performance of an enzymatic packed bed reactor running on babassu oil to yield fatty ethyl esters (FAEE) in a solvent-free system, *Biofuel*, vol. 2(2), pp. 242-247, June 2015.
- [13] Freitas L, Silva GS, Santos JC, Oliveira PC, de Castro HF, Strategies to remove water formed as by-product on the monoolein synthesis by enzymatic esterification performed on packed bed reactor, *Eur. Food Res. Technol.*, vol. 233, pp. 743-750, September 2011.
- [14] Vilas Bôas RN, Biaggio FC, Giordani DS, De Castro HF, Enzymatic synthesis of isopentyl caprylate using fusel oil as feedstock, *Quim. Nova*, vol.40, n. 5, pp. 541-547, January 2017.
- [15] Pires-Cabral P, da Fonseca MMR, Ferreira-Dias S, Esterification activity and operational stability of *Candida rugosa* lipase immobilized in polyurethane foams in the production of ethyl butyrate, *Biochem. Eng. J.*, vol. 48, pp. 246-252, January 2010.
- [16] Poppe JK, Fernandez-Lafuente R., Rodrigues RC, Ayub MAZ, Enzymatic reactors for biodiesel synthesis: Present status and future prospects, *Biotechnol. Adv.*, vol. 33, pp. 511-525, February 2015.
- [17] Aguiéiras ECG, Cavalcanti-Oliveira, ED, Freire DMG, Current status and new developments of biodiesel production using fungal lipases, *Fuel*, vol. 159, pp. 52-67, June 2015.
- [18] Chisti Y, Bioreactor design, in: *Basic Biotechnology*. 3rd ed., Ratlede C, Kristiansen B. New York: Cambridge University Press, 2006, Cap.7, pp.181-200.
- [19] Foresti ML, Ferreira ML, Chitosan-immobilized lipases for the catalysis of fatty acid esterifications, *Enzyme Microb. Technol.*, vol. 40, pp. 769-777, June 2007.
- [20] Xu X, Engineering of enzymatic reactions and reactors for lipid modification and synthesis, *Eur. J. Lipid. Sci. Tech.*, vol. 105, pp. 289-304, February 2003.
- [21] Lee DE, Park KM, Choi SJ, Shim JH, Chang P, Enhancing operational stability and exhibition of enzyme activity by removing water in the immobilized lipase-catalyzed production of erythorbyl laurate, *Biotechnol. Prog.*, vol. 29 (4), pp. 882-889, May 2013.