Hydrophobic immobilization of *Burkholderia cepacia* lipase onto octyl-silica for synthesis of flavors esters

Imobilização hidrofóbica da lipase de *Burkholderia cepacia* em sílica-octil para síntese de ésteres aromatizantes

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ABSTRACT

Burkholderia cepacia lipase (BCL) was immobilized onto silica modified with octyl groups (OS) and the biocatalyst (BCL-OS) was evaluated as its performance in the synthesis in organic medium (synthesis of flavor esters as a model). The maximum support loading was 0.375 g enzyme/g support, yielding a biocatalyst with an activity of 1197 U/g support at pH 7.0 and 50 °C in the hydrolysis of olive oil. The biocatalyst BCL-OS showed to be 9-fold more stable than the free lipase at 60°C in buffer solution (absence of substrates), with an increase of half-life from 16 to 144 h. The physical-chemical characterization of silica, octyl silica, and BCL-OS biocatalyst allowed confirming the immobilization of BCL onto the modified silica. The biocatalyst had an excellent performance in the synthesis of flavor esters, yielding more than 85% esterification yield (based on acid consumption) for acetic and butyric acids as acyl donors, and ethanol, butanol and hexanol as acyl acceptors. The biocatalyst could be recycled by ten 5 h-cycles of butyl butyrate syntheses at 37°C in heptane, retaining around 80% of its initial activity. Therefore, these results indicate that the BCL immobilized onto silica modified with octyl groups is a promising biocatalyst for application in organic syntheses.

Keywords: octyl-modified silica, hydrophobic immobilization, lipase, esterification, flavor esters.

RESUMO

A lipase de Burkholderia cepacia (BCL) foi imobilizada em sílica modificada com grupos octilas (OS) e o biocatalisador (BCL-OS) foi avaliado quanto ao seu desempenho na síntese em meio orgânico (síntese de ésteres aromatizantes como modelo). O carregamento máximo do suporte foi de 0,375 g enzyme/g support, produzindo um biocatalisador com uma atividade de 1197 U/g support a pH 7,0 e 50 °C na hidrólise do azeite de oliva. O biocatalisador BCL-OS mostrou-se 9 vezes mais estável que a lipase livre a 60 °C em solução tampão (ausência de substratos), com um aumento da meia-vida de 16 para 144 h. A caracterização físico-química da sílica, sílica-octil e biocatalisador BCL-OS permitiu confirmar a imobilização da BCL na sílica modificada. O biocatalisador teve um excelente desempenho na síntese de ésteres aromatizantes, atingindo rendimentos de esterificação acima de 85% (com base no consumo de ácido) para ácidos acético e butírico como doadores de acila, e etanol, butanol e hexanal como aceptores de acila. O biocatalisador pode ser reciclado por dez ciclos de 5 h de síntese de butirato de butila à 37 °C em heptano, mantendo cerca de 80% de sua atividade inicial. Portanto, esses resultados indicam que a BCL imobilizada em sílica modificada com grupos octilas é um biocatalisador promissor para aplicação em sínteses orgânicas.

Palavras-chave: sílica modificada com grupos octis, imobilização hidrofóbica, lipase, esterificação, ésteres aromatizantes.
INTRODUCTION

The green chemistry approach is conducting the chemical industry to develop more sustainable processes and products (ALMEIDA et al., 2017). In this context, the use of biocatalysts is a good choice by an industry aiming for an environmentally friendly and green process (ANGAJALA; PAVAN; SUBASHINI, 2016). Enzymes are proteins capable of catalyzing complex chemical reactions under mild experimental conditions, in addition to their selectivity and high catalytic activity (CHOI; HAN; KIM, 2015; MANOEL et al., 2015; ZHANG et al., 2014). In this way, enzymes can become excellent catalysts to substitute the classical chemical ones (CHOI; HAN; KIM, 2015; DICOSIMO et al., 2013; KUMAR et al., 2016; LI; DONG, 2016). Lipases (glycerol ester hydrolases, EC 3.1.1.3) are one of the most versatile and important biocatalysts. They have wide applications in food, cosmetic, pharmaceutical, fine chemical, oleochemical, biodiesel, and detergent industries, among others (ANGAJALA; PAVAN; SUBASHINI, 2016; HASAN; SHAH; HAMEED, 2006; KAPOOR; GUPTA, 2012; MOTA et al., 2020; SARNO et al., 2017; ULRICH; PETER, 2009).

To use enzymes in industrial processes, in many cases, it is necessary to increase their operational stability (CARVALHO; LIMA; SOARES, 2015; DICOSIMO et al., 2013). Therefore, the search for biocatalysts with increased stability is one of the major challenges in the last decades. Among the several approaches used, the immobilization of enzymes by adsorption or covalent linking on solid supports, encapsulation into sol-gel matrices, among others, is a well-established and successful strategy to insolubilize and stabilize enzymes (BARBOSA et al., 2014; BARBOSA et al., 2016; DICOSIMO et al., 2013; KOPP et al., 2015; LISBOA et al., 2018; MANOEL et al., 2015; SANTANA et al., 2018; SARNO et al., 2017; VESCOVI et al., 2016; VESCOVI; DOS SANTOS; TARDIOLI, 2017; ŽIVKOVIĆ et al., 2015).

The choice of a methodology and a suitable support for immobilization is critical to obtain a suitable insolubilized biocatalyst aiming its application at an industrial scale. For that, it is desirable that the biocatalyst is active, stable, easily recovered from the reaction medium at the reaction end, and reusable (CARVALHO; LIMA; SOARES, 2015; NIKOLIĆ et al., 2020). The critical choice of an enzyme immobilization strategy is based on the efficiency of the enzyme in the desired process, the costs of the immobilization procedure, the toxicity of the immobilization reagents, and the desired final properties of the immobilized biocatalyst (DICOSIMO et al., 2013; KHARRAT et al., 2011; THANGARAJ et al., 2019). Regarding to the solid materials, their physical-chemical properties, as well as their cost are critical variables to be considered in the design of a suitable enzyme/support system. Porous silica can fulfill these requirements (BERNAL; MESA, 2017).

In the case of prefabricated porous silica, the introduction of functional groups on its surface can improve its properties, such as mechanical resistance, porosity, and hydrophilic/hydrophobic balance.
(BARBOSA et al., 2016; CARVALHO, Nayara B.; LIMA; SOARES, 2015; LIMA et al., 2015; VESCOVI et al., 2016). Several surface modifying agents can promote a suitable chemical, physical, and/or morphological modification of the support surface; thus, contributing to synthesize an immobilized biocatalysts with high catalytic efficiency and operational stability to fulfill the requirements of an industrial process (BARBOSA et al., 2014; BARBOSA et al., 2016; CARVALHO et al., 2013; SHELDON; VAN PELT, 2013; SOARES et al., 2006; SOUZA et al., 2013).

Most lipases have a polypeptide chain called lid covering their active site (KHAN et al., 2017). In homogeneous medium, they are in equilibrium between an open and a closed form, making they inactive in the last form. But, in the presence of interfaces (e.g., oil drops, air bubbles, etc.) the equilibrium is displaced towards to the open form, where the activity is restored (KIM et al., 1997; PALOMO et al., 2004). Because that, the adsorption of lipases onto a hydrophobic support can permanently open the lid; thus, exposing the active site and increasing the enzyme activity (hyperactivation) in the open configuration. This mechanism is called interfacial activation (MANOEL et al., 2015). Hyperactivation of several lipases immobilized onto silica modified with hydrophobic groups have been previously reported, e.g., Thermomyces lanuginosus lipase (TLL) immobilized onto silica magnetic microparticles functionalized with octyl groups (KOPP et al., 2015), porcine pancreas lipase (PPL) and TLL immobilized onto silica functionalized with octyl groups (MACHADO et al., 2019; VESCOVI; DOS SANTOS; TARDIOLI, 2017), and Pseudomonas fluorescens lipase (PFL) immobilized onto silica functionalized with octyl, octyl+glutaraldehyde, octyl+epoxy and octyl+glyoxyl groups (VESCOVI et al., 2017).

In this study, porous silica surface was modified with octyl groups aiming to increase the hydrophobicity of the material to allow the hydrophobic adsorption of Burkholderia cepacia lipase (BCL). The immobilized BCL was characterized by adsorption-desorption of N₂, thermogravimetric analysis (TG), and Fourier-transform infrared spectroscopy (FT-IR). After, the performance of the immobilized lipase was evaluated in organic medium by using esterification of carboxylic acids (acetic and butyric acids) with short- and medium-chain alcohols (methanol, ethanol, butanol, hexanol, and octanol) as a reaction model. Important flavoring additives for foods, pharmaceuticals, and cosmetics have been synthetized by lipases, such as butyrate and acetate esters (CORRADINI et al., 2016; DOS SANTOS; DA SILVA CRUZ; TARDIOLI, 2017). On contrary to the flavors chemically synthetized, these compounds produced by enzymatic route are recognized as natural, increasing their market value (ABBAS; COMEAU, 2003).
2 RESULTS E DISCUSSION

2.1. LIPASE IMMOBILIZATION

Porous silica functionalized with octyl moieties (hereinafter named octyl silica) was evaluated as support for immobilization of BCL. Figure 1 shows the schematic representation of the hydrophobic immobilization mechanism.

![Figure 1. Schematic representation of the hydrophobic adsorption of BCL onto silica functionalized with octyl moieties (octyl-silica). The 3D structure of BCL was obtained from Protein Data bank (code 1YS1).](image)

The biocatalysts were prepared by offering to the immobilization loads of 100 to 600 mg<sub>protein</sub>/g<sub>support</sub>. Maximum hydrolytic activity of the biocatalysts (1197 U/g<sub>support</sub>) was achieved offering 500 mg<sub>protein</sub>/g<sub>support</sub> (Figure 2), for which it was achieved 75% immobilization yield. For higher loads, a very small increase in the biocatalyst activity was observed, indicating a saturation of the support surface. The maximum loading, 375 mg<sub>protein</sub>/g<sub>support</sub> (corresponding to 75% of immobilization yield when 500 g<sub>protein</sub>/g<sub>support</sub> was offered), was higher than those previously reported by Lima et al. (2015) and Blanco et al. (2004), who reported maximum octyl silica loading of 168 and 200 mg/g, respectively. The larger amount of BCL adsorbed onto octyl silica may be probably due to hydrophobic adsorption of lipase-lipase multilayers over the maximum loading of the support to yield support-lipase monolayers (BLANCO et al., 2004).

Figure 3 shows the effects of different pHs (a) and temperatures (b) in the hydrolytic activity of free and immobilized BCL. It can be observed that both lipase forms, free and immobilized, showed the highest activity at neutral pH (7.0) and at 50°C. However, the BCL adsorbed onto octyl silica was capable to retain a higher activity at higher temperature (60% of the maximum activity at 80°C, while the free lipase retained only 30%). The higher stability of the immobilized BCL at high temperatures may be explained by a higher rigidity of the enzyme molecules, at least a fraction of
molecules, which have a higher resistance to deleterious conformation changes under high temperatures (FAN et al., 2017). The same profile of hydrolytic activity vs. pH was observed until pH 7.0 for free and immobilized BCL. However, at pH 9.0 approximately 90% of the maximum enzyme activity was maintaining for the immobilized BCL, while the free lipase was fully inactivated. Similar findings were reported for BCL immobilized on poly(3-hydrobutyrate-co-hydroxyvalerate) (PHBV) by Martins et al. (2016), who reported maximum activity of immobilized BCL at pH 7.0 and 50 °C.

![Graph](image-url)

**Figure 2.** Enzymatic activity (□) and yield (●) of immobilizing BCL on octyl-silica as a function of offered enzyme load (100-600 mg protein/g support). Immobilization conditions: 25°C, 4 h, pH 7.0 (10 mM sodium phosphate buffer).

![Graphs](image-url)

(a) ![Graphs](image-url)

(b)

**Figure 3.** Temperature–activity (a) and pH-activity (b) profiles for free (●) and immobilized (■) BCL. Hydrolytic activities of olive oil (10 min reaction) were measured at pH 7.0 (100 mM sodium phosphate buffer in the temperature range 30 - 80 °C) and 50°C (100 mM buffers in the pH range 3.0 – 9.0), respectively. The maximum enzyme activities were taken as 100% and the relative activities are expressed as mean ± s.d.
2.2. DETERMINATION OF THE KINETIC PARAMETERS AND THERMAL STABILITY

The kinetic parameters for free and immobilized BCL were estimated assuming that the kinetic of initial rates vs. substrate concentration follows the classical Michaelis-Menten model (data not shown). The estimated $K_m$ for free and immobilized BCL was 665 mM, showing that the enzyme affinity by the substrate is not changed with the immobilization, while $V_{max}$ was reduced from 3333 to 1668 μmol.g$^{-1}$.min$^{-1}$ after the immobilization. This reduction in the catalytic efficiency of the enzyme may be attributed mainly to intraparticle diffusional delays (ASMAT; HUSAIN; AZAM, 2017; BLANCH; CLARCK, 1996). The thermal stabilities of free and immobilized BCL were investigated by measuring residual hydrolytic activities after enzyme incubation at 60 °C for different periods of time (Figure 4).

![Inactivation profiles of free (■) and immobilized (●) BCL at 60°C. Residual hydrolytic activities of olive oil (10 min reaction) were measured at pH 7.0 (100 mM sodium phosphate buffer) at 37 °C. Enzyme activities at the initial time were taken as 100% and the residual activities are expressed as mean ± s.d.](image)

Figure 4. Inactivation profiles of free (■) and immobilized (●) BCL at 60°C. Residual hydrolytic activities of olive oil (10 min reaction) were measured at pH 7.0 (100 mM sodium phosphate buffer) at 37 °C. Enzyme activities at the initial time were taken as 100% and the residual activities are expressed as mean ± s.d.

The results showed that the immobilized BLC was 9-fold more stable than the free counterpart (half-lives of 144 and 16 h, respectively). Increase in the thermal stability of others lipases adsorbed onto hydrophobic surfaces have been previously reported (BERNAL; ILLANES; WILSON, 2014; BLANCO et al., 2004; LIMA et al., 2015; VESCOVI et al., 2017, 2016; VESCOVI; DOS SANTOS; TARDIOLI, 2017) as a typical behavior of the high affinity of lipases to hydrophobic surfaces (RODRÍGUEZ; MARTINEZ; BERNAL, 2020). Similar results were also found to *Rhizopus oryzae* lipase covalently immobilized on silica nanoparticles modified with epoxy groups (ASHJARI; MOHAMMADI; BADRI, 2015). However, the use of modified supports with octyl groups, besides to allow an enzyme stabilization, allow increasing the catalytic activity of the immobilized biocatalyst due to interfacial activation, which is difficult to achieve by using hydrophilic surfaces (VESCOVI; DOS SANTOS; TARDIOLI, 2017).
2.3. PHYSICO-CHEMICAL AND MORPHOLOGICAL CHARACTERIZATION

Table 1 shows the results of specific surface area, volume and average pore size of porous silica, octyl silica support (OS), and BCL adsorbed to OS (BCL-OS). It is possible to observe a small decrease in the silica surface area from 79.6 to 77.3 m²/g, as well as in the volume (0.67 to 0.64 cm³/g) and pore diameter (33.9 to 31.2 nm) after functionalization with octyl moieties. Large differences in the pore volume and average pore diameter are not expected because the octyl chains have small size compared to the silica pore diameter (BLANCO et al., 2004). On the other hand, the BCL-OS biocatalyst showed a decrease in all parameters (specific surface area, volume and average pore size), which is consistent with the fact of the support is loaded with the lipase (BARBOSA et al., 2016), especially when the support is highly loaded with enzyme.

Table 1. Textural data obtained from nitrogen adsorption isotherms for silica, octyl silica (OS) and BCL immobilized on octyl silica (BCL-OS).

| Sample  | BET surface area (m²/g) | BJH desorption cumulative pore volume (cm³/g) | BJH desorption average pore diameter (nm) |
|---------|-------------------------|-----------------------------------------------|------------------------------------------|
| Silica  | 79.6 ± 0.2              | 0.67                                          | 33.9                                     |
| OS      | 77.3 ± 0.9              | 0.64                                          | 31.2                                     |
| BCL-OS  | 48.7 ± 0.5              | 0.49                                          | 17.09                                    |

Thermogravimetric analysis (Table 2) showed that the unmodified silica and octyl silica showed more significant mass losses in the region II (200-600 °C), 3.2 and 4.7%, respectively. The difference in the loss of mass may be attributed to the octyl chains. After the immobilization of BCL, the loss of mass in the region II increased to 5.9, probably because the thermal decomposition of the enzyme molecules.

Table 2. Mass loss data (wt.%) obtained by TG analysis for silica, octyl silica (OS) and BCL immobilized on octyl silica (BCL-OS).

| Sample   | Region I (0-200 °C) | Region II (200-600 °C) | Region III (600-800 °C) | Total mass loss (%) |
|----------|---------------------|------------------------|-------------------------|---------------------|
| Silica   | 2.2                 | 3.2                    | 0.8                     | 6.2                 |
| OS       | 2.4                 | 4.7                    | 0.5                     | 7.8                 |
| BCL-OS   | 2.3                 | 5.9                    | 0.5                     | 8.7                 |
Similar behavior was previously reported by Lima et al. (2015), who also immobilized *Pseudomonas fluorescens* lipase on silica functionalized with octyl groups. Studies performed by Meera et al. (2011) also indicates a significant loss of mass in the region I for the functionalized support, who related the mass loss to the presence of modifying agent moieties in the support surface. The immobilized biocatalyst (BCL-OS) showed a total mass loss of approximately 9 wt.%, mostly in the region II (5.9 wt.%), very likely to the organic compounds (C, H, O and N) volatilization from the octyl moieties (4.7 wt.%) and enzyme protein (1.2 wt.%).

FTIR spectra of the octyl silica, free BCL and BCL-OS biocatalyst can be seen in Figure 5. The free lipase showed a typical spectrum of proteins with bands associated with amines in the range of 3278 cm\(^{-1}\) and amide I in the range of 1640 cm\(^{-1}\) caused by C–O stretching vibrations of peptide linkages, as reported by Portaccio et al. (2011). For the octyl silica, it was observed the presence of siloxane groups (Si-O-Si) in the range of 787 cm\(^{-1}\) and internal asymmetric Si–O–Si stretching (between 1000-1100 cm\(^{-1}\)) from the silica matrices and silanol groups (Si-OH) (BARBOSA et al., 2016; MOHIDEM; BIN MAT, 2012). For the BLC-OS, bands referring to amines, amide I and silane groups were observed, which is consistent with the lipase immobilization.

**Figure 5.** FTIR spectra for octyl silica (OS) (black line), free *Burkholderia cepacia* lipase (BCL) (blue line) and BCL adsorbed to octyl silica (BLC-OS) (red line).

### 2.4. PRODUCTION OF FLAVOR ESTERS

Esterification reactions of butyric and acetic acids (acyl donors) with different acyl acceptors (methanol, ethanol, butanol, hexanol, and octanol) were used to evaluate the performance of the BCL-OS biocatalyst. Figure 6 shows that except to methanol and octanol, esterification yields were higher than 80% for both acyl donors. The effect of the reaction time in the synthesis of flavor esters from
butyric acid catalyzed by BCL-OS biocatalyst was evaluated up to 24 h at the same conditions above. Figure 7a shows that maximum esterification yield was achieved at short reaction time, as following: 88 ± 2% after 8 h reaction using ethanol, 85 ± 2% after 5 h reaction using butanol, and 85 ± 1% after 10 h reaction using hexanol. Similar results were previously reported by Fallavena et al. (2014) to the esterification of butyric acid with ethanol and butanol (conversions above 80%), catalyzed by Rhizomucor miehei lipase immobilized onto Duolite ES 562. Matte et al. (2016) also reported similar findings in the synthesis of butyl butyrate catalyzed by Thermomyces lanuginosus lipase (TLL) immobilized covalently via epoxy groups onto Immobead 150. The authors reported a maximum conversion of 84% at 40 °C after 4 h reaction using an acid:alcohol molar ratio of 1:3. In another work, was reported the syntheses of short-chains alkyl esters by esterification of acetic and butylic acids with several alcohols, e.g., ethanol, 1-butanol, 1-hexanol, and isoamyl alcohol, catalyzed by several immobilized lipases (lipases from Candida Antarctica type B (CALB), Pseudomonas fluorescens (PFL) and Thermomyces lanuginosus (TLL). PFL immobilized onto octyl silica yielded high reaction conversions (>97%) at 37°C after 24 h reaction. Particularly, in the synthesis of butyl butyrate, reaction conversion greater than 90% was achieved by PFL immobilized on octyl silica after 2 h reaction at 37 °C (DE LIMA et al., 2018).

Using methanol as acyl acceptor, the very low esterification yield may be attributed to the inactivating and/or inhibitory effect of this alcohol on the lipase activity, a widely reported behavior (AMINI et al., 2017; WU et al., 2017; ZHAO et al., 2015). In fact, the inhibition of Candida
antarctica lipase in the esterification reaction of adipic acid in the presence of methanol for the synthesis of adipate esters was previously reported (CHAIBAKHSH et al., 2009). The low conversion using octanol may be probably due to the increase in the viscosity of the reaction medium, which hinders an efficient mass transfer rate (GULDHE et al., 2015).

Figure 7b shows the effect of the biocatalyst concentration (from 0.5% to 1.5%, w/v) on the esterification yield of butyric acid with three acyl acceptors (ethanol, butanol and hexanol). For all the alcohols evaluated, the maximum esterification yield (around 90% after 24 h reaction) was obtained using 0.5-1.0% (w/v) of biocatalyst concentration. Above that value, it was not observed esterification yield increase, probably due to mass transfer limitations into the reactor, because the stirring may be impaired by high solid loads. Padilha et al. (2012) reported the similar esterification yield (around 90% valeric acid consumption) in the synthesis of ethyl valerate catalyzed by BCL encapsulated into alginate beads at 37 °C, 150 rpm stirring, and 0.5 M of substrates (valeric acid and ethyl alcohol) in heptane. However, this yield was achieved using a very high biocatalyst concentration into the reaction (20%, w/v) and longer time reaction (120 h).

![Figure 7a](image1.png) ![Figure 7b](image2.png)

**Figure 7.** Profiles of esterification yield vs. time reaction (a) and effect of biocatalyst concentration (b) in the syntheses of flavor esters catalyzed by BCL-OS biocatalyst loaded with 0.375 g protein/g support. Reactions conditions: 37 °C, butyric acid:alcohol molar ratio of 1:1 (0.1 M acid in heptane), 250 rpm stirring for 24 h, and 20 mg/mL of molecular sieve.

Corradini et al. (2016) also reported high solid loads into the reactor for the synthesis of n-propyl acetate catalyzed by TLL immobilized by physical adsorption onto hydrophobic mesoporous supports (polyhydroxybutyrate (PHB) and poly-methacrylate (PMA)) with immobilized protein concentration of 36.5 mg protein/g support. Maximum esterification yields of acetic acid (59 and 94% based on acid consumption) were achieved using biocatalyst concentrations of 20 and 16.5% (w/v), respectively.
However, high esterification yield (84.3% based on acid consumption) was achieved after short reaction time (50 min) using high concentration of substrates (2.0 M n-propanol and acetic acid) in heptane. These comparisons show that octyl silica is a good support for immobilization of lipases, allowing it to be highly loaded with the enzyme. This property contributed to achieve high esterification yield using low solid load into the reaction system, reflecting in higher volumetric productivities.

The reusability of a biocatalyst is a prerequisite to the economic viability of an industrial bioprocess (ASMAT; HUSAIN; AZAM, 2017). Thus, the reusability of the BCL-OS biocatalyst was evaluated in the synthesis of butyl butyrate, as shown in Figure 8. The biocatalyst shows high operational stability, retaining its activity above 80% up to eleven 5h-batches, and above 50% after twenty-one 5h-batches. The high operational stability of the biocatalyst in the organic medium (synthesis of butyl butyrate) may be attributed to the high density of functional groups (octyl groups) on the support surface, contributing to a great rigidification of the tridimensional structure of the immobilized enzyme molecules. Besides, the high support hydrophobicity allows a strong interaction lipase-support; thus, preventing enzyme inactivation and/or enzyme leaching to the reaction medium, mainly when the reaction is carried out in organic medium (VESCOVI et al., 2017; VESCOVI; DOS SANTOS; TARDIOLI, 2017). The activity loss along the reuse batches (mechanical stirred) could be attributed to the shear of silica under mechanical stress. Under these conditions, fine particles may be lost during the recovery and washing steps between each reaction cycle (ASMAT; HUSAIN; AZAM, 2017).

Figure 8. Operational stability of BCL-OS loaded with 375 mg protein/g support in the synthesis of butyl butyrate at 37°C, using an acid:alcohol molar ratio of 1:1 (0.1 M acid in heptane), 1% (m/v) biocatalyst concentration, 20 mg/mL molecular sieve, and reaction time of 5 h. The maximum esterification yield (based on acid consumption) at the first 5h-batch (85 ± 2%) was defined as 100%, and all values are expressed as mean ± s.d.
Barbosa et al. (2014) evaluated the performance of BCL immobilized onto silica aerogel in the hydrolysis of olive oil at 37°C for 30 min. The authors reported that the immobilized enzyme retained above 80% of its initial activity until the 10th cycle, but the activity was decreased to 50% after the 13th cycle. On the other hand, Matte et al. (2016) reported similar findings compared to our work in the synthesis of butyl butyrate using TLL immobilized covalently onto Immobead 150 (86% activity retention after eight cycles). In another work, Corradini et al. (2016) reported lower operational stability for the TLL immobilized by physical adsorption onto PMA in the synthesis of n-propyl acetate at 29 °C for 50 min. The biocatalyst could retain only 50% of its initial activity after 6 cycles of 50 min.

3 MATERIAL AND METHODS

3.1 MATERIALS

*Burkholderia cepacia* lipase (BCL, Amano lipase, E.C.3.1.1.3) and octyltrietoxysilane (OCTES) were purchased from Sigma Aldrich Co. (St. Louis, MO, USA). Porous silica (Immobead S60S) was purchased from Chiral Vision (Leiden, The Netherlands). Extra-virgin olive oil with low acidity (Carbonell®, Brasil) for hydrolysis assays was acquired from the local market. The substrates for esterification reactions (*i.e.*, glacial acetic acid, butyric acid, methanol, ethanol, propanol, butanol, hexanol, and octanol, all >99% purity) were purchased from Sigma Aldrich Co. (St. Louis, MO, USA). Molecular sieve UOP type 3A (3 Å pore diameter and 1/16 particle size) was purchased from Fluka Analytical (St. Louis, MO, USA). All other chemicals were of analytical grade from Synth and Vetec Química Fina Ltda (São Paulo, Brazil).

3.2 SUPPORT ACTIVATION

Porous silica was chemically modified according to the methodology described by Vescovi et al. (2016). Briefly, silica was treated with HCl solution (0.1 M) under reflux for 3 h, followed by washing with distilled water until neutral pH, and drying at 50 °C for 12 h. 1 g of dry and treated silica was added into 20 mL of OCTES: toluene solution (1:10, v/v), the mixture was kept under reflux for 4 h at 110 °C. The silica functionalized with octyl groups (octyl silica, abbreviated here as OS) was washed with toluene, acetone, and distilled water, and dried at 40 °C for 24 h.

3.3 LIPASE IMMOBILIZATION

The lipase immobilization onto OS was carried out at 25 °C and pH 7.0 (10 mM sodium phosphate buffer), as following: 1 g of OS was suspended in 10 mL of enzymatic solution prepared in 10 mM phosphate buffer pH 7.0, containing different protein loads (100 to 600 mg of enzyme
powder), and kept under stirring for 4 h. After, the BCL immobilized onto octyl silica (BCL-OS) was washed with 10 mM phosphate buffer pH 7.0, and stored at 4 °C. The hydrolytic activity of the BCL-OS was measured using olive oil emulsion as substrate.

3.4. ENZYMATIC ACTIVITY ASSAY

The hydrolysis activity of free BCL and BCL-OS was measured by hydrolysis of emulsified olive oil, as described by Soares et al. (2004). One unit (U) of enzyme activity was defined as the amount of enzyme that releases 1 µmol of fatty acid per min (µmol·min⁻¹) under the assay conditions (37 °C, pH 7.0, 80 rpm stirring). The overall immobilization yield, η (%), was calculated according to Eq. (1):

$$\eta(\%) = \frac{U_s}{U_o} \times 100$$  

(1)

where $U_s$ is the total enzyme activity measured in the immobilized BCL and $U_o$ is the total enzyme activity offered to the immobilization.

3.5. BIOCHEMICAL CHARACTERIZATION, KINETIC PARAMETERS AND THERMAL STABILITY

The optimum pH of free BCL and BCL-OS was determined by measuring the hydrolysis activity for 10 min in the pH range of 3.0 to 9.0 at 37 °C, using 5.0 mL of olive oil emulsion and the following buffers in the concentration of 100 mM: citrate buffer (pH from 3.0 to 5.0); phosphate buffer (pH from 6.0 to 8.0); carbonate-bicarbonate buffer (pH 9.0). The optimum temperature of the BCL (free and immobilized) was determined by measuring the hydrolysis activity for 10 min at temperatures between 30 and 80 °C, using the olive oil emulsion at pH 7.0 as substrate. The values of $K_m$ and $V_{max}$ were estimated using the Lineaweaver-Burk linearization of the data of initial hydrolysis rate vs. substrate concentration at 50 °C, pH 7.0 (100 mM phosphate buffer) and olive oil concentration from 5 to 90% (v/v) under the best conditions of temperature, pH and enzymatic loading. Thermal stability was determined by incubating the free and immobilized BCL at the temperature of 60 °C and pH 7.0 in 100 mM phosphate buffer, and monitoring the hydrolytic activity. The residual activity was calculated as the ratio between the activity at a given incubation time and the activity at the start of the experiment.

3.6. PHYSICAL-CHEMICAL CHARACTERIZATION

Specific surface areas of silica, octyl silica and BCL-OS were calculated using BET (Brunauer-Emmett-Teller) method by measurement of nitrogen adsorption at 77 K in a NOVA 1200e Surface
Area & Pore Analyzer (Quantschrome Instruments, 11.0 version software). Pore volume and average pore diameter were calculated by BJH (Barret, Joyner and Halenda) and t-methods for mesoporous and microporous materials, respectively. Thermogravimetric (TG) curves was obtained by simultaneous measurements of TG/DTA in a DTG-60H Shimadzu thermal analyzer, under nitrogen atmosphere in a temperature ramp increasing from room temperature up to 800 °C at a heating rate of 20 °C min⁻¹. IR spectra of silica, octyl silica and BCL-OS were obtained in the wavelength range from 400 to 4000 cm⁻¹ in a CARY 630 FTIR Agilent Spectrophotometer.

3.7. FLAVOR ESTER SYNTHESIS

The synthesis of the flavors esters was carried out according to the methodology adapted from Lima et al. (2018). The synthesis were carried out at 37 °C, 250 rpm stirring, for 24 h in the presence of molecular sieve (20 mg/mL, previously activated at 250 °C for 24 h), using butyric and acetic acids as acyl donors and several alcohols (methanol, ethanol, butanol, hexanol and octanol) as acyl acceptors (ABBAS; COMEAU, 2003; DE LIMA et al., 2018; PAULA et al., 2008). The acid:alcohol molar ratio was 1:1 to an acid concentration of 0.1 M in heptane (10 mL solution in closed glass flasks) and the best enzyme concentration. The esterification reaction was monitored by acid consumption (acid-base titration with alkali solution and phenolphthalein as the indicator). The effect of the reaction time and the enzyme concentration (0.5 to 1.5%, m/v) in the esters yields was evaluated for the best operational conditions.

3.8. OPERATIONAL STABILITY TESTS

The operational stability of the BCL-OS biocatalyst was determined in esterification batches of butyric acid with butanol under optimal experimental conditions (37 °C for 5 h, acid:alcohol molar ratio of 1:1, 0.1 M acid concentration in heptane, biocatalyst concentration of 1%, m/v, and 20 mg/mL molecular sieve). At the beginning and between each reaction cycle the biocatalyst was washed with hexane and dried by vacuum suction.

4. CONCLUSIONS

*Burkholderia cepacia* lipase immobilized onto silica modified with octyl groups showed to be an excellent biocatalyst to the synthesis of flavor esters in organic solvent (heptane). Yields higher than 85% were obtained for all acids and alcohols evaluated. Besides, the biocatalyst remained highly stable in the reusability assays, maintaining its activity higher than 80% for ten 5h-cycles at 37 °C. Octyl silica could to be highly loaded (375 mg protein/g support), allowing to prepare biocatalysts highly active, thus avoiding the use of high solid loads into the reactor. The evaluation of the kinetic
parameters of free and immobilized BCL in the hydrolysis of olive oil showed that the affinity of the enzyme by the substrate did not change with the immobilization, although there was a strong indicative of intraparticle diffusional delays due to the high loading of the support. In general, BCL immobilized onto octyl silica (BCL-OS) has great potential to be evaluated in other biotechnological processes that require high stability against organic solvents.

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ABBREVIATIONS

BCL  Burkholderia cepacia lipase
OS  Silica modified with octyl groups
BCL-OS  BCL immobilized on OS
PHBV  poly(3-hydroxybutyrate-co-hydroxyvalerate)
TLL  Thermomyces lanuginosus lipase
PPL  Porcine pancreas lipase

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