Immobilization of Biotechnologically Important *Candida rugosa* Lipase onto Commercial Matrices

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Abstract— *The continual search for alternative environmentally cleaner technologies in industrial processes has led to an increase in the use of enzymatic processes globally. However, due to their physical characteristics they require immobilization in order to remain effective. The objective of this study was to investigate the immobilization of the biotechnologically important and commercially available Candida rugosa lipase (CRL) by physical interfacial adsorption onto a number of matrices to act as biocatalysts. Five different types of support were tested: i) macroporous silica (synthetic inorganic), ii) polyhydroxybutyrate (natural organic), iii) polypropylene (synthetic organic), iv) polymethacrylate (synthetic organic), and v) polystyrene-divinylbenzene (synthetic organic). Results generated during this study showed that from the group of materials tested, polystyrene-divinylbenzene gave the best results with the highest amount of immobilized protein (8.10 ± 0.31 mg/g) and a good immobilization yield (90.35% ± 1.53%). The efficiency of protein immobilization was found to be highest when carried out at pH4.5, which is close to the isoelectric point of the enzyme.*

Keywords—Biocatalysts, Candida rugosa, immobilized protein, lipase, physical adsorption, support.

I. INTRODUCTION

Over recent decades, the search for green / environmentally friendly / cleaner technologies (balanced with the need to remain economically viable) for use in almost all industrial chemical processes has seen the increase in the use of enzymatic methodologies to achieve these targets. This has resulted in the enzyme market experiencing a high growth rate; it is estimated that in the period 2018-2023, the global enzyme market will grow at an annual rate of 4.9% and rise from US $5.5 Billion in 2018 to US$7 Billion in 2023 [1].Within the global enzyme market, there are six groups of enzymes which stand out: proteases, carbohydrases, lipases, phytases, polymerases and nucleases.

The interest in these biocatalysts goes beyond their ability to simply increase the rate a chemical reaction (i.e. the role of a traditional catalyst); they offer alternatives for many chemical processes due to their unique properties of biodegradability, specificity and selectivity in reactions [1-3].

Of specific interest in this study, Lipases are extremely versatile enzymes and are used in a variety of applications: in addition to simple hydrolysis reactions they can catalyse esterification and transesterification reactions and are also frequently used in the cosmetics, food, flavouring, beverage, paper, lubricant, detergent, effluent treatment, biosensor and biodiesel industries[2-4].

The industrial application of lipases (and other enzymes) in large-scale processes is often limited by the high cost and stability issues faced under industrial conditions; they are fragile structures which are sensitive to high temperature, variations in pH and various solvents. In addition, it can be difficult to separate them from some reaction systems, which limits their recovery and re-use which in turn may also lead to the contamination of the final product [5,6]

In order to overcome some of these limitations, the use of enzymes that are immobilized is a strategy that has been adopted to protect the enzyme, however in order to remain as an efficient active biocatalyst, the selection of an appropriate support material and application protocol are key factors [7]. Support can be from various origins: organic / inorganic / natural / synthetic and may also have a variety of porosities (porous, microporous, mesoporous, macroporous). The overall success of the immobilization is dependent on the chemical, mechanical and morphological compatibility between the biocatalyst and

the surface material to which it is confined. Such compatibility factors include functional groups, hydrophobicity / hydrophilicity, internal geometry, surface area, mechanical resistance and porosity [5,8]

In the literature, lipases have been shown to be successfully immobilized through various immobilization methods including adsorption, entrapment, covalent coupling, cross-linking reversed micelles and ion pairs, with the adsorption, entrapment and covalent coupling methods being the most common [9-11].Physical adsorption involves enzymes being physically attached to the support through weak electrostatic forces such as van der Waals forces, hydrophobic interactions or hydrogen bonding. Whereas entrapment (as the name suggests) is where the enzymes are trapped within a polymeric material (sometimes a gel) which itself is attached to the support substrate. In comparison, covalent bonding is where the enzyme is covalent bonded by some of its amino acids to a coupling group on the support substrate [12,13]

Of these methods, the physical adsorption process is attractive from an operational point of view as it has a number of benefits: i) it requires mild conditions to achieve and is a relatively easy process, ii) it generally has a low cost, iii) it eliminates the addition of chemical agents, and iv) by the desorption of inactive enzymes from the support surface of the biocatalyst it allows the reuse of the material [4,5]

Furthermore, one of the fundamental characteristics of lipases is their ability to adsorb hydrophobic surfaces through the hydrophobic pocket present in the region of the active site, which acts on the modulation of its catalytic activity [14]. This situation favours the formation of hydrophobic interactions, which are typical of the physical adsorption process.

Considering these factors, this study was designed to study hydrophobic materials of different origins to determine the best support for the immobilization of *Candida rugosa* lipase by physical adsorption. The evaluation of the influence of immobilization pH on the catalytic activity of the prepared biocatalysts was also assessed.

II. MATERIALS AND METHODS

2.1 Materials

Candida rugosa microbial lipase (type VI), polyhydroxybutyrate, polymethacrylate (Diaion® HP-2MG) and polystyrene-divinylbenzene (Diaion® HP-20) were purchased from Sigma-Aldrich® (St. Louis, USA). Macroporous silica (Immobead S60S) and polypropylene (Immobead IB-S500) were purchased from Chiral Vision (Leiden, Netherlands). Low-acid olive oil purchased locally (Carbonell). All other reagents, solvents, and gum Arabic were obtained from Synth and Vetec (São Paulo, SP).

2.2 Immobilization of Lipase by Physical Adsorption

Five different types of supports were tested: macroporous silica (synthetic inorganic), polyhydroxybutyrate (natural organic), polypropylene (synthetic organic), polymethacrylate (synthetic organic) and polystyrene-divinylbenzene (synthetic organic). Each material was tested separately by the following method which itself was adapted from the method presented by Mendes *et al* [15,16].

Firstly, the selected support was soaked in 95% ethanol for 2 hours at 25°C. After this step, the support materials were vacuum filtered and further washed with ethanol to produce "pre-treated supports". These pre-treated supports (0.5 g) were then individually incubated in 9.5mL of enzyme solution (containing an initial protein loading of 13.6 mg protein per gram of support) in 10mM phosphate buffer at pH7.0 for 24 hours. Following the incubation period, the prepared "biocatalyst" (immobilized *Candida rugosa* microbial lipase on the support) was vacuum filtered, washed with distilled waterand stored at 4°C.

2.3 Determination of Protein

The concentration of immobilized protein (IP) was determined by the method developed and published by Bradford [17]. The efficiency of the immobilization procedure was performed by determining the concentration of protein in the immobilization supernatant before and after the procedure using the calculation shown in (1).

$$IP = \frac{V \times (C_0 - C_t)}{M} \quad (1)$$

Where: IP is the mass of immobilized protein per gram of support (mg/g), V is the volume of the enzyme solution (mL), C_0 is the initial protein concentration (mg/ml), C_t is the residual protein concentration (mg/ml) after a given incubation time, and M is the support mass used for immobilization.

2.4 Determination of Hydrolytic Activity

The hydrolytic activity (HA) was determined using the Soares *et al.* hydrolysis method, which involves the hydrolysis of emulsified olive oil[18]. The substrate was treated by an emulsion containing 50g olive oil and 50g of a 3% (w/w) gum Arabic solution. The reaction solution was prepared: 5 mL of substrate, 4.9 mL of sodium phosphate buffer solution (100mM, pH7.0) and 0.1g of immobilized enzyme or 0.1 mL of enzyme in soluble form or the immobilization supernatant were added to a 125 mL Erlenmeyer flask. The flasks were incubated at 37^{o}C for 5min in a thermostatic bath whilst shaking at 150rpm. After the incubation period, the reaction was quenched by the addition of 10 mL of a 1:1 v/v mixture of acetone and ethanol (92.5%). The fatty acids released were then quantified by titration with a 20mM aqueous sodium hydroxide (NaOH) solution, using phenolphthalein as an indicator [19].

The apparent hydrolytic activity (HA) of the biocatalysts (measured in Units/g or Units/ml) was calculated according to the methodology of Soares *et al.* [18], according to(2).

$$\mathrm{HA} = \left(\frac{(\mathrm{V_t} - \mathrm{V_c}) * 10^3 * \mathrm{Mol}}{\mathrm{M_e} * \mathrm{t}}\right) \tag{2}$$

Where V_t is the volume (mL) of NaOH titrant solution for the test sample; V_c is the volume (mL) of the NaOH titrant solution used in titration of the control; Mol is the molarity (mol/L) of the titrant solution; M_e is the mass (g or mL) of enzyme used (proportional to the aliquot used) whether free or immobilised; t is the reaction time (minutes).

The immobilization yield (IY) calculated as a percentage was determined using (3).

$$\mathrm{IY}\ (\%) = \left(\frac{\mathrm{HA_0} - \mathrm{HA_f}}{\mathrm{HA_0}}\right) \mathrm{x}\ 100\% \tag{3}$$

Where HA_0 and HA_f are the apparent hydrolytic activity (measured in U/g or U/mL) of the enzyme in solution before and after immobilization, respectively.

The specific hydrolytic activity (HAE) of the biocatalysts (measured in Units/g or Units/mL) was calculated according to (4).

$$\mathrm{HAE} = \frac{\mathrm{HA}}{\mathrm{IP}} \tag{4}$$

Where HAE is the apparent hydrolytic activity (U/g) of the prepared biocatalysts and IP (mg/g) is the concentration of immobilized protein.

2.5 Influence of pH on the Physical Adsorption of Lipase

The influence of pH on the adsorption process was determined in the range of pH4.0 to 8.0 with an ionic strength of 10mM [15,16]. In this study, sodium acetate solutions (to achieve pH4.0, 4.5 and 5.0) and sodium phosphate solutions (to achieve pH6.0, 7.0 and 8.0) were used in the preparation of the enzymatic solutions for immobilization. The suspensions (0.5g carrier in 9.5mL of enzyme solution) were incubated in sealed Duran flasks (250 mL and kept stirring (200 rpm) in a rotary incubator for a maximum period of 24 hours at 25^{o}C. At the end of the immobilization process, aliquots (0.5mL) were removed for the experimental determination of both the hydrolytic activity and residual protein concentration of the immobilization supernatant. The hydrolytic activity of the immobilized derivative was also determined after being vacuum filtered and washed with distilled water.

III. RESULTS AND DISCUSSIONS

The properties of the commercially available support substrates selected for the *Candida rugosa* lipase immobilization study are summarised in Table 1.

TABLE 1
PROPERTIES OF THE COMMERCIALLY AVAILABLE SUPPORT SUBSTRATES SELECTED FOR THE *Candida rugosa* LIPASE IMMOBILIZATION STUDY

Support	Commercial Name	Surface Area (m^2/g)	Pore Size (Å)
Polyhydroxybutyrate	Polyhydroxybutyrate	17.1 *	31.0 *
Polymethacrylate	Diaion HP2-MG	500.0 **	170.0 **
Polypropylene	Immobead IB-S500	647.8 ***	91.8 ***
Polystyrene-divinylbenzene	Diaion HP-20	500.0 **	260.0 **
Silica	Immobead 560S	74.8 #	193.0 #

Sources: *SILVA et al.[20]; **Sigma-Aldrich; ***CHIRAL VISION; #LIMA et al.[6]

Experimental results obtained during this study demonstrating the catalytic properties of the prepared biocatalysts are shown in Table 2.

TABLE 2
CATALYTIC PROPERTIES OF THE IMMOBILIZED *Candida rugosa* LIPASE ON THE SELECTED SUPPORTS

Support	Immobilization Yield (IY) [%]	Immobilized Protein (IP) [mg/g_s]	Apparent Hydrolytic Activity (HA) [U/g_s]	Specific Hydrolytic Activity (HAE) [U/mg_P]
Polyhydroxybutyrate	95.22 ± 5.07	4.67 ± 0.38	395.43 ± 75.41	84.69 ± 16.15
Polymethacrylate	89.16 ± 0.15	7.70 ± 0.59	50,59 ± 1.73	6.57 ± 0.23
Polypropylene	66.16 ± 1.53	6.75 ± 0.17	261.22 ± 5.20	38.69 ± 0.77
Polystyrene-divinylbenzene	90.35 ± 1.53	8.10 ± 0.31	74.20 ± 33.82	9.16 ± 4.17
Silica	73.18 ± 0.46	4.63 ± 0.47	59.33 ± 11.06	12.82 ± 2.39

Source: author data (where g_s is gram of support and mg_p is gram of protein

As the data presented in Table 2 shows, high yields of immobilization were obtained using silica (73%), polymethacrylate (89%) and polystyrene-divinylbenzene (90%), which is probably due to the large pore size that allows high lipase retention – the crucial requirement in the preparation of active biocatalysts. These supports have been widely used in the preparation of various biocatalysts through the immobilization of several lipases in the synthesis of important esters (biolubricants and biodiesel), through esterification and transesterification reactions [6,10,11].

Candida rugosa lipase is a globular protein with a reported molecular volume of 50Å × 42Å × 33Å and a molecular diameter of approximately 51Å as reported by Gao *et al* [21]. As the data presented in Table 2 shows, the mean pore size of the supports tested was greater than the molecular diameter of the lipase except for polyhydroxybutyrate. The polyhydroxybutyrate contains pores that have a diameter smaller than that of the enzyme, which coupled with the high immobilization yield (95.22% ± 5.07%), indicates that the adsorption is preferentially on the external area of the support. Polypropylene produced a lower immobilization yield (66.16% ± 1.53%), but it also produced a high value for hydrolytic activity (261.22 ± 5.20 Units/g), which when considering how close the pore size is to the diameter of the enzyme, this suggests that the immobilization also occurs in the external region. These results indicate that *Candida rugosa* lipase can be adsorbed both on the external and internal surfaces of the support.

Due to its high immobilization yield and hydrolytic activity, polyhydroxybutyrate could be considered an adequate support, however as the immobilization occurs only on the external area of the support (resulting in a gain in hydrolytic activity) the biocatalyst effectively becomes unprotected, which means that it is vulnerable to being denatured if used under harsh industrial processing conditions, which makes the polyhydroxybutyrate a unattractive option.

The results shown in Table 2 and discussed thus far, clearly indicate that polystyrene-divinylbenzene and polymethacrylate are the two most suitable supports. Both have similarly high Immobilization Yields (IY) and quantity of Immobilized Protein (IP); however, when considering the apparent hydrolytic activities (HA) and specific hydrolytic activities (HAE), then we see a difference, which indicates that polystyrene-divinylbenzene is the better option. These differences in hydrolytic activities are most likely due to the difference in pore sizes (as shown in Table 1) where the polystyrene-divinylbenzene (260.0Å) has a larger pore size than the polymethacrylate (170.0Å). Having the larger pore size may allow better accessibility of the

substrate molecules to the biocatalytic microenvironment whilst simultaneously leaving the biocatalyst protected from external environment conditions.

Thus, polystyrene-divinylbenzene was selected as the most suitable support substrate for the second phase of testing where the effect of pH on adsorption was investigated, and the results are summarised in Table 3.

TABLE 3
INFLUENCE OF PH ON THE CATALYTIC PROPERTIES OF *Candida rugosa* LIPASE IMMOBILIZATION IN POLYSTYRENE-DIVINYLBENZENE.

pH	Immobilization Yield (IY) [%]	Immobilized Protein (IP) [mg/g_s]	Apparent Hydrolytic Activity (HA) [U/g_s]	Specific Hydrolytic Activity (HAE) [U/mg_P]
4.0	90.27 ± 6.88	6.63 ± 0.05	32.53 ± 4.52	4.94 ± 0.04
4.5	89.21 ± 0.29	6.74 ± 0.03	76.84 ± 9.62	11.40 ± 0.04
5.0	89.31 ± 1.50	7.34 ± 0.08	31.85 ± 6.34	4.34 ± 0.05
6.0	85.10 ± 1.69	1.49 ± 0.31	18.59 ± 5.37	12.78 ± 2.68
7.0	84.84 ± 1.54	2.11 ± 0.16	29.52 ± 1.96	14.02 ± 1.03
8.0	87.76 ± 1.08	4.96 ± 0.05	29.69 ± 4.14	5.99 ± 0.06

Source: author data (where g_s is gram of support and mg_p is milligram of protein)

As the data presented in Table 3 shows, there appears to be no significant influence of pH variation on the yield of immobilization (IY). However, the pH does appear to have influenced both the apparent hydrolytic activity (HA) and specific hydrolytic activity (HAE) of the biocatalyst. This is most likely to be because the active site on the *Candida rugosa* lipase has an α-helix structure which is referred to as a "cap" or "flap". This "cap" is composed of amino acids with amphiphilic properties and the pH of the microenvironment can influence the movement of this "cap", which are reflected in the values of enzymatic activity as reported by María *et al* [22].

With regard to the values measured for the Immobilized Protein (IP), the maximum value obtained (7.34 ± 0.08 mg/g) was recorded at pH5. However, there is a further factor which must be taken in consideration. The isoelectric point of *Candida rugosa* lipase is around pH4.2 [23]. At the isoelectric point, maximal hydrophobic interaction between lipase and the support surface (interfacial activation mechanism) which favours the enzyme adsorption process and the partitioning of substrate molecules to the surface of the biocatalyst can be expected. Additionally, it is known that the immobilization pH is an important factor in the preparation of active biocatalysts since they influence both the surface load of the supports, and the degree of ionization of the protein molecules [24].

This isoelectric point effect can be observed when looking at the hydrolytic activities recorded in Table 3. At pH4.5, both the apparent hydrolytic activity (HA) and specific hydrolytic activity (HAE) are significantly higher than the results obtained at pH5.0. As the Immobilization yield and immobilized protein content have similar values at both pH4.5 and pH5.0, the hydrolytic activity results clearly show that the most appropriate pH condition for immobilization is pH4.5 (close to the isoelectric point of lipase).

IV. CONCLUSION

The results obtained during this study indicate that polystyrene-divinylbenzene is a very good material for a support structure in the immobilization of *Candida rugosa* lipase, especially when carried out at pH4.5, which is close to its isoelectric point (pH 4.2). In addition, the physical properties of polystyrene-divinylbenzene allowed the *Candida rugosa* lipase to be immobilized preferentially within the support where the biocatalyst is protected from variations in a reaction environment, reducing the possibility of denaturation. Additional future work we focus on the stability of the prepared biocatalyst when applied in biotransformation reactions.

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