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Covalent attachment of lipases on glyoxyl-agarose beads: Application in fruit flavor and biodiesel synthesis

Adriano A. Mendes[a,b,*], Heizir F. de Castro[c], Raquel L.C. Giordano[b]

[a] Institute of Chemistry, Federal University of Alfenas, 37130-000 Alfenas, MG, Brazil
[b] Department of Chemical Engineering, Federal University of São Carlos, 13565-905 São Carlos, SP, Brazil
[c] Department of Chemical Engineering, Engineering School of Lorena, University of São Paulo, 12602-810, Lorena, SP, Brazil

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ABSTRACT

The aim of this work was to prepare biocatalysts to catalyze the synthesis of butyl butyrate by esterification reaction, and the synthesis of biodiesel by transesterification of palm and babassu oils with ethanol. Lipase preparations Lipolase® (TLL1) and Lipex® 100L (TLL2) from *Thermomyces lanuginosus* and Lipase AK from *Pseudomonas fluorescens* (PFL) were immobilized on glyoxyl-agarose beads prepared by activation with glycidol (Gly) and epichlorohydrin (Epi). The influence of immobilization time, lipase source and activating agents on the catalytic activity of the biocatalysts were evaluated in both aqueous and organic media. TLL1 immobilized on glyoxyl-agarose by 24 h of incubation resulted biocatalysts with high hydrolytic activity (varying from 1347.3 to 1470.0 IU/g of support) and thermal-stability, around 300-fold more stable than crude TLL1 extract. The maximum load of immobilized TLL1 was around 20 mg of protein/g of support. The biocatalyst prepared exhibited high activity and operational stability on the butyl butyrate synthesis by esterification after five successive cycles of 24 h each (conversion around 85–90%). Immobilized TLL1 and PFL were active in the synthesis of biodiesel by transesterification reaction. Maximum transesterification yield (≥98.5% after 48 h of reaction at 45 °C) was provided by using palm oil as feedstock.

1. Introduction

Lipases (triacylglycerol hydrolases E.C. 3.1.1.3) are enzymes that *in vivo* catalyze the hydrolysis of triacylglycerols to glycerol and free fatty acids. These enzymes, *in vitro*, also catalyze esterification, transesterification and interesterification reactions in non-aqueous systems [1–3]. A typical feature of lipases is the so-called "interfacial activation", although some lipases have been identified that do not undergo increased activity in the presence of hydrophobic interfaces. A polypeptide chain called "lid" or "flap" covers its active site, making it inaccessible to solvent and substrates in many instances (closed conformation). The lid undergoes conformational changes during the process of interfacial activation, allowing substrate molecules access to the active site (open conformation) [4–6].

The limitations of the industrial use of lipases have been mainly due to their high cost, which may be overcome by proper immobilization techniques on solid supports. The immobilization facilitates the recovery and further reuse of the biocatalyst, avoids enzyme aggregation and autolysis and increases flexibility of reactor design. Furthermore, additional stabilization of the immobilized enzyme three-dimensional structure may be achieved if an increase in the rigidification of the macromolecule structure is promoted [7–10]. Lipases have been immobilized by different protocols as physical adsorption on hydrophobic and ionic exchange supports, covalent attachment on highly activated supports and encapsulation in inorganic and organic supports [11–21]. Although there are several methods to immobilize enzymes, the immobilization/stabilization by multipoint covalent attachment on activated supports presents some practical advantages when compared with other immobilization methods: the immobilization process can be controlled and different supports can be tested without difficulty. Furthermore, the enzyme molecules become more rigid, and thus more resistant to conformational changes induced by heat and organic solvents than the corresponding unmodified ones [7–9,12,15–20,22–24]. A great increase in the stability of several enzymes after their multipoint covalent attachment on glyoxyl-supports has been widely reported [7–9,12,15,20,23]. The stability factor for each enzyme depends on the enzyme structure, on the

* Corresponding author at: Institute of Chemistry, Federal University of Alfenas, 37130-000 Alfenas, MG, Brazil. Tel.: +55 35 3299 1399.
E-mail addresses: adriano.mendes@unifal-mg.edu.br, adriano_amendes@yahoo.com.br (A.A. Mendes).

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Table 1
Catalytic properties of crude lipase extracts used in the present work.

Lipase	Source organism	Designation	Supplier	Protein (mg/g)	Activity[c] (IU/g)	Specific activity (IU/mg protein)	$t_{1/2}$ at 70 °C (min)
TLL1[a]	*T. lanuginosus*	Lipolase®	Novozymes	17.9	3422.5	191.2	4.8
TLL2[a]	*T. lanuginosus*	Lipex® 100L	Novozymes	23.7	4161.7	175.6	5.4
PFL[b]	*P. fluorescens*	AK	Amano	20.5	4459.0	217.5	2.5

[a] Liquid lipase preparation.
[b] Solid powder lipase preparation.
[c] Activity measured on the olive oil emulsion hydrolysis (pH 8.0, 37 °C).

number of aldehyde groups generated in the support and on the immobilization conditions [7–10,15–18,20,22–24].

In this work, three lipase preparations from *Pseudomonas fluorescens* (PFL) and *Thermomyces lanuginosus* (Lipolase® – TLL1 and Lipex® 100L – TLL2) were immobilized by covalent attachment on glyoxyl-agarose beads. The effect of the incubation time, activating agent (glycidol or epiclorohydrin) and lipase source on the catalytic activity and thermal-stability of the biocatalysts was investigated. The immobilization parameters were assayed in hydrolysis of emulsified olive oil. The biocatalysts were also used to catalyze butyl butyrate (pineapple flavor) synthesis by direct esterification in heptane medium. Operational stability tests were performed for 5 successive cycles of 24 h each in butyl butyrate synthesis. Fatty acid ethyl ester (biodiesel) synthesis by transesterification of babassu and palm oils with ethanol in solvent-free systems was also performed. Recently, some studies have focused on the transesterification of triglycerides with short-chain alcohols such as methanol or ethanol to produce biodiesel catalyzed by immobilized lipases prepared by multipoint covalent attachment on glyoxyl-supports [15–17]. However, the application of palm and babassu oils as feedstocks in biodiesel production catalyzed by biocatalysts prepared by the above mentioned strategy has not been reported yet.

The immobilization of the lipases on glyoxyl-supports occurs *via* nucleophilic attack of amine groups from Lys residues on the enzyme surfaces (pK of 10.5) to the aldehyde groups of the supports to form Schiff's bases (C=N double bonds) [7–9,12,16–18,22–24]. After immobilization, the biocatalysts prepared were then incubated in sodium borohydride solution for the reduction of the Schiff's bases, which transform them in stable covalent bonds, as well as change the reactive aldehyde groups to the inert hydroxyl groups, an important step in the immobilization process [7,12,16,24]. The representative scheme of preparation and immobilization of lipases on glyoxyl-supports, *e.g.* agarose beads, has been previously reported [16].

Lipases from *Pseudomonas fluorescens* and *Thermomyces lanuginosus* are able to form bimolecular aggregates at low enzyme concentrations [20,25]. Thus, the covalent attachment of the lipases on glyoxyl-agarose beads was carried out in the presence of Triton X-100 (0.15%, v/v) to obtain fully dispersed immobilized lipase molecules oriented toward the reaction medium [16,19,20].

2. Materials and methods

2.1. Materials

Lipase preparations Lipolase® (TLL1) and Lipex® 100L (TLL2) from *Thermomyces lanuginosus* were kindly donated by Novozymes (Araucária, Brazil) and Lipase AK from *Pseudomonas fluorescens* (PFL) was purchased from Amano Enzyme Inc. (Nagoya, Japan), and used as received without further purification. The characteristics of these lipase preparations are presented in Table 1. Agarose beads 6B-CL (Sepharose™ 6B-CL) was acquired from Amershan Biosciences (Uppsala, Sweden). Epichlorohydrin (Epi), glycidol (Gly), butyric acid, Triton X-100, Bradford reagent and bovine serum albumin were purchased from Sigma–Aldrich Co. (St. Louis, USA). Sodium borohydride, sodium periodate and anhydrous butanol were obtained from Vetec (Sao Paulo, Brazil). Gum arabic was acquired from Synth (São Paulo, Brazil). Olive oil (low acidity) from Carbonell (Spain) was purchased at a local market. Anhydrous ethanol (minimum 99.5%, m/m) was supplied by Chromoline (SP, Brazil). Refined bleached palm oil was a kind gift from Agropalma (Belém, Brazil) and babassu oil kindly supplied by Pulcra (Jacareí, Brazil). The fatty acid composition of palm and babassu oils has been previously reported [26].

2.2. Preparation of glyoxyl-agarose beads

Agarose beads were activated with glycidol and epichlorohydrin to produce glyoxyl-support. For glycidol activation, 10 g beads were added to a solution composed of 3 mL distilled water, 5 mL 1.7 M NaOH solution containing 0.15 g sodium borohydride ($NaBH_4$). Following this, 3.6 mL of glycidol was slowly added and the mixture kept at 0 °C for 15 h [27]. For epichlorohydrin activation, 10 g beads were suspended in 100 mL 2 M NaOH solution containing 0.6 g $NaBH_4$. Then, 10 mL of epichlorohydrin were slowly added and the suspension was also kept at 0 °C for 15 h [28]. Glyceryl-agarose (prepared by activation with glycidol and epichlorohydrin) was suspended in 60 mL Milli-Q water and then added 30 mL 100 mM sodium periodate solution to produce glyoxyl groups [27]. The suspensions were kept under slight stirring for 2 h at room temperature. Glyoxyl-agarose beads were thoroughly rinsed with Milli-Q water and vacuum dried. The density of aldehyde groups for activated agarose beads by glycidol was 95 μmol/g wet support [29]. For the activation with epichlorohydrin, the densidy of aldehyde and epoxy groups was 102 and 6 μmol/g wet support, respectively [12].

2.3. Immobilization procedure

Immobilization of lipase preparations on glyoxyl-agarose was carried out by adding 1.0 g of support to 9 mL of a solution at pH 10.05 (buffer sodium bicarbonate 100 mM) containing 0.15% (v/v) of Triton X-100 and 5 mg protein. The suspensions were kept under mild stirring in an orbital shaker during at different time of incubation varying from 4 to 72 h at room temperature. The immobilization was followed by measuring the hydrolytic activity and protein concentration in the supernatant solution. The immobilization of TLL1 on glyoxyl-agarose was further tested by offering different loadings of protein (5.0, 10.0, 30.0, 60.0 and 80 mg/g of support) to determine the support saturation enzymatic load. After the enzyme immobilization step, 1.0 mg/mL sodium borohydride was added to the immobilization suspension and kept under agitation during 30 min at 25 °C. After this, the biocatalysts were filtered (Whatman filter paper 41) and thoroughly rinsed with 200 mM buffer sodium phosphate pH 7.0 and finally washed thoroughly with distilled and Milli-Q water.