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Evaluation of immobilized lipases on poly-hydroxybutyrate beads to catalyze biodiesel synthesis

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ABSTRACT

Five microbial lipase preparations from several sources were immobilized by hydrophobic adsorption on small or large poly-hydroxybutyrate (PHB) beads and the effect of the support particle size on the biocatalyst activity was assessed in the hydrolysis of olive oil, esterification of butyric acid with butanol and transesterification of babassu oil (*Orbignya* sp.) with ethanol. The catalytic activity of the immobilized lipases in both olive oil hydrolysis and biodiesel synthesis was influenced by the particle size of PHB and lipase source. In the esterification reaction such influence was not observed. *Geobacillus thermocatenulatus* lipase (BTL2) was considered to be inadequate to catalyze biodiesel synthesis, but displayed high esterification activity. Butyl butyrate synthesis catalyzed by BTL2 immobilized on small PHB beads gave the highest yield (≈90 mmol L^{-1}). In biodiesel synthesis, the catalytic activity of the immobilized lipases was significantly increased in comparison to the free lipases. Full conversion of babassu oil into ethyl esters was achieved at 72 h in the presence of *Pseudozyma antarctica* type B (CALB), *Thermomyces lanuginosus* lipase (Lipex® 100L) immobilized on either small or large PHB beads and *Pseudomonas fluorescens* (PFL) immobilized on large PHB beads. The latter preparation presented the highest productivity (40.9 mg of ethyl esters mg^{-1} immobilized protein h^{-1}).

1. Introduction

Lipases (triacylglycerol ester hydrolases, EC 3.1.1.3) are enzymes that in nature catalyze the cleavage of ester bonds in tri-, di-, and monoacylglycerols to glycerol and free fatty acids [1]. In organic medium, lipases also catalyze esterifications, transesterifications and interesterifications [1–4]. These enzymes are applied in several industrial processes including synthesis of biopolymers, production of pharmaceuticals, agrochemicals, cosmetics, flavors, treatment of waste rich in lipids and biodiesel synthesis by transesterification of triglycerides with short-chain alcohols [1–17].

With respect to the biodiesel synthesis, and despite the fact that an enzymatic process is still not commercially developed, a number of articles have shown that enzyme holds promise as catalyst [6,7,18]. These studies consist mainly in optimizing the reaction conditions (vegetable oil, alcohol, molar ratio, solvent, temperature, lipase source and immobilizing support) in order to establish the characteristics for industrial applications [6–14].

Lipases have been immobilized in different organic/inorganic supports by physical adsorption, encapsulation, covalent attachment or cross-linking [8–34]. For application in an organic medium, a strong enzyme-support interaction is not required. Under these conditions, the enzyme is insoluble in the apolar medium and physical adsorption may be a suitable method of immobilization. This method is easy, cheap and allows a simple recycling of the support at the end of the enzyme's life. However, the chance of desorption of the enzyme is the main disadvantage of this immobilization procedure [19].

The immobilization by physical adsorption on hydrophobic supports is explained through an important property of the lipases, namely interfacial activation in the presence of hydrophobic interfaces. Lipases have an oligopeptide chain "lid" that covers their active site making them inaccessible to substrates. In the absence of an interface, the active site is secluded from the reaction medium showing "closed conformation". However, in the presence of a hydrophobic interface, important conformational rearrangements take place resulting in the "open conformation" [35–37]. In this case, lipases are strongly adsorbed to hydrophobic interfaces through the hydrophobic face of the "lid" that cover their

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active site, an hydrophobic surrounding of the active center of the lipase. Hence, lipases recognize these surfaces as similar to those of their natural substrates (drops of oil), yielding "immobilized open structures" [30–34].

Commercially available immobilized lipases are, in general, immobilized by physical adsorption on organic matrices such as *Pseudozyma antarctica* type B immobilized on Lewatit VPOC 1600 that consists of poly(methyl methacrylate-*co*-divinylbenzene) (Novozym® 435) and *Mucor miehei* immobilized on macroporous anion exchange resin (Lipozyme® RM IM) from Novozymes [38–40]. These biocatalysts have high costs, mainly associated with the support price [20]. Therefore, the use of cheaper supports in the lipase immobilization has been proposed as an alternative to the expensive supports for further applications in large scale processes. Several inexpensive hydrophobic supports have been already used in the lipase immobilization by physical adsorption such as egg shell [21], phyllosilicates [10,22], bentonite [23], polypropylene [12,13,24–27], and porous styrene-divinylbenzene [28,29].A promising hydrophobic support that can be also used is the poly-hydroxybutyrate (PHB) beads, a polymer derivative of poly-hydroxyalkanoates (PHAs) that is produced as an energy storage material by many bacteria. Physical and mechanical properties of this polymer are similar to those of synthetic thermoplastics such as polypropylene [41]. The glass transition temperature is in the range from 0 to 30 °C while the melting temperature is near 180 °C. The densities of crystalline and amorphous PHB are 1.26 and 1.18 g cm^{-3}, respectively, and present Young's modulus of 3.5 GPa and the tensile strength of 40 MPa. PHB is highly soluble in halogenated solvents such as chloroform and dichloromethane [41,42]. As PHAs have been demonstrated to be a family of biopolymers with good biodegradability and biocompatibility [43], PHB has been investigated for use in surgical suture [44], nerve repairer [45] or soft tissue repairer [46] and matrices as cell growth supporting materials [47]. Recently, it has become of industrial interest to evaluate these polyesters for a wide range of applications [47]. However, their use as a support for lipase immobilization still has been scarcely reported in the literature.

The aim of this work was to immobilize five microbial lipase preparations from several sources such as two enzymatic preparations from *Thermomyces lanuginosus* commercially available as Lipolase® (TLL1) and Lipex® 100L (TLL2), *P. antarctica* type B (CALB, also known as lipase B from *Candida antarctica*), *Geobacillus thermocatenulatus* (named BTL2) and *Pseudomonas fluorescens* (PFL) by physical adsorption on small or large PHB beads to catalyze the transesterification of babassu oil with ethanol in a solvent-free medium. Immobilization parameters were estimated in the hydrolysis of olive oil and butyl butyrate synthesis (pineapple flavor). The ability of the immobilized BTL2 on SPHB to catalyze aliphatic esters from carboxylic acids (butyric, lauric and oleic) with short-chain alcohols (ethanol and butanol) was also investigated.

The main desirable characteristic of lipases for biodiesel production from triacylglycerols is the tolerance towards short-chain alcohols such as methanol and ethanol, high catalytic activity and broad specificity [6,8–12,48,49]. *P. antarctica* type B (CALB) has a very small lid and does not suffer from an increase in activity by interfacial activation [50]. It is constituted of 317 amino acids with a molecular weight of 33 kDa [39,51], dimension of 30 Å × 40 Å × 50 Å and isoelectric point of 6.0 [39,52]. CALB has been the most studied lipase for biodiesel production in various reaction systems. It also catalyzes acyl transfer reactions of various oils and acyl acceptors (alcohols or esters) showing high resistance to organic solvents, high thermal stability and broad substrate specificity [48,49]. Lipolase® (TLL1) and Lipex® 100L (TLL2) are alkalophilic lipase preparations from *T. lanuginosus* [53] and generally regarded as region-specific for *sn*-1,3 positions [54]. However, their specificity can be influenced by many issues such as media, substrates, and even acyl migration from the *sn*-2 position to the *sn*-1 or *sn*-3 positions in partial glyceride [55,56]. Both of them are single chain protein consisting of 269 amino acids with a molecular weight of 31.7 kDa, isoelectric point of 4.4 and a size of 35 Å × 45 Å × 50 Å [54,57]. *P. fluorescens* lipase (PFL) is a non-specific enzyme with a molecular weight of 33 kDa and isoelectric point at pH 4.0 [58,59]. This lipase also displays high catalytic activity at alkaline region [60]. Both PFL and TLL present a true lid which isolates the active center of the lipases from the medium (closed form). It has been found that these lipase preparations are able to aggregate into bimolecular structures even at moderate enzyme concentrations [50,59]. These two lipase preparations also have been reported to be efficient biocatalysts for the transesterification reaction with high alcohol:oil molar ratio in solvent-free systems [8–12]. These properties make these lipase preparations good biocatalysts for biodiesel synthesis by transesterification of babassu oil in solvent-free systems. *G. thermocatenulatus* (BTL2) is a thermoalkalophilic and non-specific lipase consisting of 389 amino acids with molecular weight of 43 kDa and isoelectric point of 7.4 [61–63]. BTL2 is the first crystallized lipase with double lid, which implies in a complex catalytic mechanism [63]. It has a dimension based on its crystal structure of 73.07 Å × 129.08 Å × 127.49 Å [63,64]. Although BTL2 is highly active in aqueous systems [61,62,65], its use in organic systems to produce alkyl esters by transesterification of triglycerides with short-chain alcohols (biodiesel) and direct esterification of carboxylic acids with short-chain alcohols has not been reported in the literature yet.

2. Materials and methods

2.1. Materials

Lipase preparations from *P. antarctica* type B (CALB), *T. lanuginosus* named Lipolase® (TLL1), Lipex® 100L (TLL2) from Novozymes (Araucária, Brazil) and *P. fluorescens* (PFL) from Amano Enzyme (Nagoya, Japan) were used without further treatment. Lipase from *G. thermocatenulatus* cloned in *E. coli* (BTL2) was produced as previously described [62]. The catalytic properties of the lipase preparations are presented in Table 1. SPHB (average particle diameters of 75–90 μm) and LPHB (average particle diameters of 750–1180 μm) were acquired from PHB Industrial (São Paulo, Brazil). Anhydrous ethanol was purchased from Cromoline (São Paulo, Brazil). Butanol and carboxylic acids (butyric, lauric and oleic) were purchased from Merck (Darmstadt, Germany). Babassu oil was courtesy of Cognis (São Paulo, Brazil) having the following composition in fatty acids (w/v): 3.50% caprylic, 4.50% capric, 44.7% lauric, 17.5% myristic, 9.70% palmitic, 3.1% stearic, 15.2 oleic, and 1.80% linoleic, with 709.4 g mol^{-1} average molecular weight. All the other reagents were of analytical grade.

2.2. Determination of hydrolytic activity

Hydrolytic activities (HA) of soluble and immobilized lipases were assayed by the hydrolysis of olive oil emulsion, according to the methodology described by Soares et al. [66], with slight modifications. The substrate was prepared by mixing 50 g of olive oil with 150 g of gum arabic solution (3 wt.%). The reaction mixture containing 5 mL of the emulsion, 5 mL of 100 mmol L^{-1} phosphate buffer (pH 8.0) and soluble (0.2 mL, 0.5 mg mL^{-1}) or immobilized (0.1 g) lipase was incubated for 5 min at 37 °C. The reaction was stopped by the addition of 10 mL of commercial ethanol. The liberated fatty acids were titrated with 20 mmol L^{-1} sodium hydroxide solution in the presence of phenolphthalein as indicator. One international unit (IU) of activity was defined as the amount of enzyme required