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Immobilization of porcine pancreatic lipase on poly-hydroxybutyrate particles for the production of ethyl esters from macaw palm oils and pineapple flavor

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ABSTRACT

Poly-hydroxybutyrate particles (PHB) were used as support to immobilize porcine pancreatic lipase (PPL). The biocatalysts prepared were tested in the synthesis of pineapple flavor by esterification of butanol and butyric acid in heptane medium, and in the synthesis of ethyl esters by transesterification of macaw palm pulp (MPPO) and macaw palm kernel (MPKO) oils with ethanol in solvent-free systems. The effect of protein loading on the biocatalyst activity was assessed in olive oil hydrolysis. Maximum hydrolytic activity of 292.8 ± 8.60 IU/g was observed. Langmuir isotherm model was applicable to the adsorption of PPL on PHB particles. Maximum immobilized protein amount was 24.3 ± 1.70 mg/g. The optimal pH and temperature in hydrolysis reaction for the immobilized PPL were at pH 8.5 and 50 °C, while for the crude PPL extract were at pH 8.0 and 45 °C. Immobilized PPL exhibited full hydrolytic activity after 2 h of incubation in non-polar solvents. In esterification reaction, optimal conversion was around 93% after 2 h of reaction. After six esterification cycles, the biocatalyst retained 63% of its initial activity. The biocatalyst prepared attained transesterification yield of 50% after 48 h of reaction for MPKO and 35% after 96 h of reaction for MPPO.

1. Introduction

Over the last years there has been an increasing interest in the production of esters, a large family of organic compounds with broad applications in medicine, biology, chemistry and industry. Fatty acid alkyl esters (FAAE) from triglycerides, so-called biodiesel, and short-chain esters (flavor and fragrances) have been considered very interesting from the industrial point of view. FAAE of vegetable oils, animal fats or waste cooking oils and greases, is an alternative fuel to the conventional diesel from petroleum [1]. Since FAAE from renewable sources, it does not contribute to new carbon dioxide emission – one of the factors responsible for the greenhouse effect. The other main characteristic of this fuel is the almost total absence of sulphur and the low production of soot particulate after combustion [1–3]. FAAE are industrially obtained by transesterification of triglycerides with short-chain alcohols, mainly methanol and ethanol [2,3]. Short-chain esters, another important class of compounds of industrial interest, are volatile compounds widely used in flavor and fragrance applications in food, beverages, pharmaceuticals and personal care industries [4–7]. Among them, butyl butyrate has been widely used in food industry due to its pleasant pineapple flavor [6,7]. The production of these esters in industrial scale has been preferentially performed by chemical catalysts such as alkali or acids resulting in high conversion levels in short reaction time [2,3,7]. However, there are major drawbacks of such chemical processes, as several problems during the steps of removal of catalyst from the product and the wastewater treatment, and excessive consumption of energy [2,7]. On the other hand, the reactions catalyzed by lipases can overcome these problems which are performed at lower temperature to save energy, and it exhibits high selectivity, leading to products with high purity and fewer side products [2,4,7].

Lipases (triacylglycerol ester acylhydrolases, EC 3.1.1.3) are enzymes that catalyze the hydrolysis of triglycerides to glycerol and free fatty acids. In non-aqueous medium, lipases also catalyze esterification, transesterification and interesterification reactions [2–11]. These enzymes are applied in several industrial processes including the synthesis and degradation of engineering thermoplastics, production of pharmaceuticals, agrochemicals, cosmetics,

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flavors and fragrances, emulsifiers, structured lipids, biodiesel synthesis and fatty acids concentrated [8–11]. However, their application is often limited by their sensitivity to temperature, and also by the difficulties in their recovery and recycling. The immobilization of enzymes, including lipases, on solid supports is among of the most effective methods to improve their stability, separation and reusability [12–14].

Lipases have been immobilized by using different protocols such as adsorption on ionic exchange resins and hydrophobic matrices, covalent attachment on highly activated supports, encapsulation in organic and inorganic matrices and cross-linking [12–18]. The immobilization of lipases by physical adsorption on hydrophobic supports has been suggested as a simple way to obtain the open and stabilized form of the biocatalysts [19–23]. This immobilization strategy is a reversible process which allows easy desorption of the enzyme after its inactivation by using dissociation agents (e.g., detergents, guanidine, etc.) enabling the reuse of the support [20–23]. Moreover, this immobilization procedure has been used in the purification of lipases preparations from several sources by selective adsorption of the enzyme on hydrophobic supports at low ionic strength [19–25].

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 (named "lid" or "flap") 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". In this case, lipases are strongly adsorbed on hydrophobic interfaces through the hydrophobic face of the "lid" that cover their active site, a hydrophobic area surrounding of the active center of the enzyme. Hence, lipases recognize those hydrophobic surfaces as similar to those of their natural substrates (oil droplets) [19–25].

The immobilization of lipases by physical adsorption on hydrophobic supports has been widely reported [18–32]. In recent years, considerable efforts have been put into the studies of using hydrophobic natural and synthetic organic supports for lipase immobilization [5,6,18–32]. A promising hydrophobic support that can be used in lipase immobilization by adsorption is the poly-hydroxybutyrate (PHB). Its application as support for the immobilization of lipases from several sources has been recently reported for the production of highly active biocatalysts in both aqueous and non-aqueous media [29].

In the present investigation, we report the preparation of active biocatalysts by immobilizing PPL by physical adsorption on PHB particles for further application in the synthesis of esters of industrial interest such as pineapple flavor by esterification of butanol and butyric acid in heptane medium, and in the synthesis of ethyl esters by transesterification of macaw palm oils with ethanol in solvent-free systems. The first aim of the study was to characterize the surface area and porous diameter of the support by B.E.T. analysis. Following this, the effect of the protein loading on the catalytic activity of the biocatalysts was assayed in the hydrolysis of olive oil emulsion. The influence of pH, temperature and organic solvents on the hydrolytic activity of PPL was also studied. The effect of the immobilized PPL amount and reactants concentration on the butyl butyrate synthesis (pineapple flavor) was initially studied. After, it was verified the influence of enzyme concentration, temperature and molecular sieve concentration on ester conversion to obtain the optimal experimental conditions using factorial design and response surface methodology analysis. Finally, the operational stability of the biocatalyst was performed in consecutive cycles of butyl butyrate synthesis in heptane medium. Vegetable oils from macaw palm, so-called macauba (*Acrocomia aculeata*), a perennial, fruit-producing palm tree native of tropical forests and typically Brazilian [33], were used as feedstocks in ethyl ester synthesis by transesterification reactions in solvent-free systems. Among oleaginous plants, macaw palm is the second most productive (1500–5000 kg oil/ha), inferior only to palm oil (*Elaeis guineensis*). Besides the high productivity achieved after four years of growth, *A. aculeata* can produce for over 100 years, and that gives this species a great potential for biodiesel production [34]. Macaw palm oil is extracted from the pulp (MPPO) and fruit's seeds – kernel (MPKO). Here, we selected both macaw palm oils because their application in ethyl esters synthesis by transesterification reaction still is few reported by the literature.

To our knowledge, this is the first report of the use of PHB particles as support to immobilize porcine pancreatic lipase for further application in organic medium to catalyze ester syntheses of industrial interest such as butyl butyrate (pineapple flavor) and ethyl esters. In this work, PPL was selected because has been considered very attractive for industrial applications due to its accessibility, high stability in organic medium and broad specificity for biotransformation of non- and natural substrates. The structural features, biochemical properties and potential applications of PPL in reactions of industrial interest have been recently reported by Mendes et al. [11].

2. Materials and methods

2.1. Materials

Enzymatic extract from porcine pancreatic lipase type II (PPL) supplied by Sigma–Aldrich Co. (St. Louis, USA) was used without further treatment. The enzymatic extract is a solid preparation with a specific activity of 33.3 IU/mg of protein and 155.0 mg protein/g of solid. PHB particles (average particle diameters of 75–90 μm) were acquired from PHB Industrial (São Paulo, Brazil). Molecular sieve UOP type 3 Å (rod, size 1/16 in.) was purchased from Fluka Analytical (USA). Anhydrous ethanol (minimum 99.8%) and butanol were purchased from Synth (São Paulo, Brazil). Butyric acid was acquired from Sigma–Aldrich Co. (St. Louis, USA). Organic solvents such as N,N-dimethylformamide (DMF), methanol, commercial ethanol (minimum 95.0%), acetone, diethyl ether, hexane and heptane were purchased from Synth (São Paulo, Brazil). Olive oil (low acidity) from Carbonell (Spain) was purchased at a local market. Macaw palm pulp oil (MPPO) was acquired from Paradigma Óleos Vegetais Ltd. (Carmo do Parnaíba, MG, Brazil) having the following composition in fatty acids (% m/m): 17.6% palmitic, 4.0% palmitoleic, 2.0% stearic, 58.6% oleic, 16.0% linoleic, and 1.0% linolenic, with 865.1 g/mol average molecular weight. Macaw palm kernel oil (MPKO) was purchased from Associação dos Pequenos Produtores Rurais de Riacho D'Antas e Adjacências-Fazenda Riacho D'Antas (Montes Claros, MG, Brazil) having the following composition in fatty acids (% m/m): 5.4% caprylic, 3.9% capric, 36.1% lauric, 10.2% myristic, 8.7% palmitic, 3.6% stearic, 27.7% oleic and 3.4% linoleic, with 729.4 g/mol average molecular weight.

2.2. Surface area determination

The surface area measurements were performed by adsorption using nitrogen as adsorbate. The samples were previously degassed to below 50 mmHg at room temperature and the analyses were performed at 77 K using liquid nitrogen. The equilibrium interval was 5 s. The surface area was calculated using the B.E.T. method. Pore diameter based on the BJH calculation was evaluated by the B.E.T. apparatus software (Quantachrome Instruments, NovaWin2 version 2.2).