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Operational stabilities of different chemical derivatives of Novozym 435 in an alcoholysis reaction

María Villalba, Carlos M. Verdasco-Martín, Jose C.S. dos Santos, Roberto Fernandez-Lafuente, Cristina Otero*

Department of Biocatalysis, Institute of Catalysis and Petroleochemistry, CSIC, C/Marie Curie 2 L10, Madrid 28049, Spain

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ABSTRACT

Industrial use of Novozym 435 in synthesis of structured lipids and biodiesel via alcoholysis is limited by mass transfer effects of the glycerides through immobilized enzymes and its low operational stability under operation conditions. To better understand this, differently modified Novozym 435 preparations, differing in their surface nature and in their interactions with reactants, have been compared in the alcoholysis of *Camelina sativa* oil. The three modifications performed have been carried out under conditions where all exposed groups of the enzyme have been modified. These modifications were: 2,4,6-trinitrobenzensulfonic acid (Novo-TNBS), ethylendiamine (Novo-EDA) and polyethylenimine (Novo-PEI). Changes in their operational performance are analyzed in terms of changes detected by scan electron microscopy in the support morphology.

The hydrophobic nature of the TNBS accelerates the reaction rate; *t*-ButOH co-solvent swells the macroporous acrylic particles of Lewatit VP OC 1600 in all biocatalysts, except in the case of Novo-PEI. This co-solvent only increases the maximal conversions obtained at 24 h using the modified biocatalysts. *t*-ButOH reduces enzyme inactivation by alcohol and water. In a co-solvent system, these four biocatalysts remain fully active after 14 consecutive reaction cycles of 24 h, but only Novo-TNBS yields maximal conversion before cycle 5. Some deposits on biocatalyst particles could be appreciated during reuses, and TNBS derivatization diminishes the accumulation of product deposits on the catalyst surface. Most particles of commercial Novozym® 435 are broken after operation for 14 reaction cycles. The broken particles are fully active, but they cause problems of blockage in filtration operations and column reactors. The three derivatizations studied make the matrix particles more resistant to rupture.

1. Introduction

The alcoholysis of vegetable oils is a synthetic route to obtain products of high interest for different industrial sectors, such as biodiesel [1–4] and nutraceuticals. Biodiesel is added to conventional fuels to decrease the impact of transport on the environment. Lipids and phospholipids with nutritional and therapeutic properties can be selectively obtained via alcoholysis [5–7]. This reaction has been used for the selective removal of undesired fatty acids from fats and oils [8].

Catalysts used to mediate alcoholysis reactions can be chemical (acid or basic) or enzymatic. Nowadays, clean technologies replace the traditional chemical catalysts by biocatalysts. Lipases are the type of enzymes involved in the synthesis of biodiesel and structured glycerides. Some advantages of lipolytic catalysts are: (i) they convert both, combined and free fatty acids. Hence, soaps are not formed with the fraction of free fatty present in cheap oil feedstocks [9], being separation and purification of the reaction products easier and cheaper; (ii) they work under mild conditions of pressure and temperature, (iii) they have lower energy requirements, and (iv) are biodegradable. However, the industrial application of biocatalysts is limited, because they are labile and expensive. Hence, they must be stabilized, for example via immobilization in a support.

In solvent free media, the reaction between a short alcohol and a vegetable oil is characterized by the immiscibility of these two reagents. Short chain alcohols form a separated liquid phase that inactivates the enzyme [10–13]. Moreover, glycerol and water are accumulated during the process as a hydrophilic layer surrounding and coating the immobilized catalyst [14].

* Corresponding author.
E-mail address: cotero@icp.csic.es (C. Otero).

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The negative effect of polar substrates and products on the biocatalyst can be mitigated: (i) by washing the biocatalyst between reaction cycles with a solvent like *t*-butanol, permits to recover most of its lost activity [15], (ii) adding *t*-butanol to the reaction mixture, since it is a good solvent of glycerol and short alcohols [14], and is not converted in the scale of time that primary alcohols are completely transformed [15], and (iii) by modification of the enzyme structure and/or microenvironment in the support particles, by different chemical or physical procedures [16].

Candida antarctica B (CAL B) is a 33 kDa MW protein with the active site in a channel with large hydrophobic area surrounding the entrance. This lipase has not a real lid covering the active center [17]. This permits the immobilization of CALB on hydrophobic supports via interfacial activation. However, the properties of the enzyme molecules adsorbed to these supports (e.g., the specific activity of the immobilized biocatalyst) depend on the properties of the support surface [18].

Novozym® 435 is a commercial immobilized preparation of CAL B in a macroporous acrylic polymer resin (Lewatit VP OC 1600), where the lipase is adsorbed through hydrophobic interactions with the surface of the support pores [19]. This immobilization mechanism means that the population of immobilized enzymes is homogenous and all the enzyme molecules are immobilized involving the adsorption of the open form of the lipase via interfacial activation. [20]. Novozym® 435 is widely and successfully used in either, synthesis [21] and hydrolysis [22] reactions of ester linkages in anhydrous and aqueous media, respectively. However, the industrial applicability of Novozym® 435 is limited, due to the support disintegration by strong shear forces and protein leaching by emulsifiers and emollient products (alkyl esters and free fatty acids) [23].

Recently, the favorable effect of a silicon coating of this biocatalyst has been described [24], as a result of protection effect of the polymer covering the catalyst particles, which reduces the particles breakage under working conditions.

In this work Novozym® 435 was modified to obtain different modifications of the enzyme surface. A more hydrophobic enzyme surface was obtained using with 2,4,6-trinitrobenzensulfonic acid (TNBS) [25]. The biocatalyst modification with ethylene diamine (EDA) after carbodiimide activation was carried out to change anionic groups of the enzyme by cationic groups [25,26]. This cationic nature of the enzyme surface means that the enzyme surface capacity to adsorb anions increased after EDA modification. Finally, Novozym® 435 was treated with an ionic polymer (polyethylenimine, PEI) to generate a very hydrophilic and cationic shell around the enzyme molecules via physical interactions [27–29]. All the modifications were performed using conditions where 100% of the exposed groups were altered, that way we should have a homogeneous population of enzyme molecules. These modifications have probed to alter the enzyme specificity and selectivity, increasing or decreasing the enzyme activity versus certain substrates, while having the opposite result with another one [16,30].

The effects of these modifications on the lipase properties strongly depend on the immobilization protocol, reaction and substrate utilized [16,27,31]. In the case of CALB immobilized on octyl-agarose, a immobilization following a similar mechanism than the one employed to produce Novozym 435, these modifications even improved its hydrolytic activity versus certain substrates [25]. Now, in this new work we have analyzed the effects of these modifications on the Novozym® 435 operational stability in anhydrous medium, using them as catalysts in the reaction of biodiesel synthesis. Special emphasis has been payed to the effect of the modification preserving the physical integrity of the biocatalysts particles, that have been reported to become dissolved under the reaction conditions.

2. Materials

Camelina oil was donated by Camelina Company (Madrid, Spain). The macroporous resin immobilized lipase Novozym® 435 (lipase B from *C. antarctica*, CALB) was a generous gift from Novozymes A/S (Spain). Hexadecane and *t*-butanol were HPLC grade from Sigma-Aldrich (Madrid, Spain). Ethylenediamine (EDA), *N*-(3-dimetylaminopropyl)-*N*-ethylcarbodiimide (DEC), 2,4,6-trinitrobenzensulfonic acid (TNBS), polyethylenimine (PEI), Mn 10,000, Mw 25,000, were from Sigma-Aldrich (Madrid, Spain). Methanolic-HCl (0.5 N) and a fatty acid standard Supelco 37 Component FAME Mix were purchased from Supelco (Bellefonte, PA, USA). *n*-Hexane, chloroform, methanol and ethanol were HPLC grade from Scharlau (Barcelona, Spain).

3. Methods

3.1. Free fatty acid content

The free fatty acid content of camelina oil was determined as follows [32]:

1 g of sample was dissolved in 20 mL of chloroform. Next, 25 mL ethanol and several drops of phenolphthalein were added to this solution. The mixture was homogenized and titrated with a sodium hydroxide 0.02 M solution, until the appearance of pink color in the solution of interest.

The free fatty acid (FFA) content, as weight percent, was calculated as the equivalent oleic acid by Eq. (1).

$$(\%)FFA = \frac{(V_s - V_b) \times M_{NaOH} \times 282.5}{100 \times m_s} \times 100 \quad (1)$$

where "$V_{S''}$ and "$V_{B''}$ are the milliliters of sodium hydroxide solution used for titration of the sample and the blank, respectively. "$M_{NaOH''}$ is the molarity of the sodium hydroxide solution, 282.5 is the molecular mass of oleic acid, and "$m_{s''}$ is the mass in grams of the sample whose content of free fatty acids is to be determined.

3.2. Chemical and physical modification of Novozym® 435

3.2.1. Solid phase amination

Ten grams of Novozym® 435 was suspended in 100 mL of 1 M EDA at pH 4.75 under continuous stirring [33]. Modification started with the addition of solid DEC (to a final concentration of 10 mM). After 2 h of gentle stirring at 25 °C, the aminated derivate had more than 95% of the exposed carboxylic groups modified [34,35]. This was confirmed by comparing the developed color after titration with TNBS, that did not increase using 100 mM DEC or 2 M EDA. These preparations were washed with distilled water and incubated in 1 M hydroxyl amine at pH 8 for 12 h to recover the Tyr residues that could have been modified by DEC [36]. Finally, the immobilized enzyme preparation was washed with distilled water and *t*-butanol and stored at 4 °C.

3.2.2. Modification of the primary amino groups of Novozym® 435 with TNBS

12 g of Novozym® 435 were added to 100 mL of a solution of 0.1% (w/v) TNBS in sodium phosphate at pH 8.0 and the mixture was incubated for 60 min at room temperature. Then, the modified enzyme preparation was washed with distilled water and *t*-butanol [25].

3.2.3. Physical modification of Novozym® 435 with ionic polymer, PEI

A mass of 10 g of Novozym® 435 was added to 100 mL of PEI at pH 7 at the desired concentration of the polymer [27,28]. The biocatalysts were filtered and washed several times with distilled water and *t*-butanol.