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Effect of chemical modification of Novozym 435 on its performance in the alcoholysis of camelina oil

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ABSTRACT

Alcoholysis of oils mediated by immobilized lipases are limited by mass transfer effects on substrates. In this work, Novozym® 435 lipase was subjected to seven different chemical derivatizations. The effects of changes in the enzyme surface and changes of the support particles size, on substrates mass transfer restrictions were studied on the alcoholysis of Camelina oil in the presence or not of *t*-butanol as co-solvent.

Significant changes of the support particle size were detected after their chemical modification. The particle size of Lewatit VP OC 1600 support of Novozym® 435 diminished in solvent-free systems. Alcoholysis rates in *t*-butanol media were enhanced caused by two favorable effects of this solvent: substrates dissolution and support swelling. This latter effect was not sufficient to promote protein desorption during processing. The hydrophobic environment created by 2,4,6-trinitrobenzensulfonic acid (TNBS) derivatization favoured the oil conversion. The TNBS derivative was also more stable than Novozym® 435 in methanolysis with solvent.

Scanning electron microscopy revealed that after 14 reaction cycles of 24 h, a large proportion of biocatalyst particles were broken; however, matrix rupture did not cause biocatalysts inactivation. All modifications studied seemed to protect the support particles from breaking. Accumulated product particles on all biocatalysts surfaces did not impose significant mass transfer restrictions to substrates, but prevented protein desorption in urea solution.

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1. Introduction

The alcoholysis of vegetable oils with short alcohol moieties is an important route for production of biodiesel and structured lipids with nutritional and therapeutic properties [1–4]. Most of the syntheses of structured lipids are carried out under kinetic control. By contrast, complete alcoholysis of fats and oils produces biodiesel, where thermodynamic control is essential [5–8].

In solvent-free media, the alcoholysis reaction is characterized by the immiscibility of these two reagents (oil and short alcohols) [9]. Short chain alcohols inactivate the enzyme and their concentration in the reaction mixture must be very low [10]. The step-wise addition of ethanol increases the activity and operational stability of the biocatalyst [11]. Moreover, the high water content of waste and cheap oils has a negative effect on the alcoholysis reaction [12]. Glycerol and water are formed during the process; they progressively form a hydrophilic layer and inactivate the immobilized catalyst along the reaction cycles [12].

Several approaches have been pursued to mitigate the negative effect of polar substrates and products on the biocatalyst: washing the biocatalyst between reaction cycles [13,14], an immersion pretreatment of the biocatalyst with *t*-butanol [13], or a co-solvent addition to the reaction mixture [12]. The use of a very hydrophobic support to immobilize the enzyme may also reduce the adsorption of polar substrates [15,16].

Alcoholysis reactions can be mediated by a chemical (acid or basic) or enzymatic catalysts. However, only enzymes have the advantage of high regio- and chemo selectivities [17]. Unlike basic catalysts, biocatalysts convert fatty acids present as glycerides as well as their free forms. In biotransformations of cheap oil feedstocks, soaps are not formed [18]. This fact reduces the expenses of separation and purification of the reaction products [19].

Lipases are the enzymes normally employed to catalyze the alcoholysis of oils, either soluble or immobilized in supports of

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different nature (organic, inorganic) [20]. Novozym® 435 is an immobilized commercial lipase from *Candida antarctica* B (CAL B) immobilized in a macroporous acrylic polymer resin. CAL B is a member of the α/β-hydrolase fold family with a Ser-Asp-His catalytic triad. It is a 33 kDa MW protein with a channel leading to the active site and a large hydrophobic area surrounding the entrance of this channel. It is one of the few lipases without any real lid covering the active center [21]. In Novozym® 435 preparation, the lipase is adsorbed though hydrophobic interactions with the support [22,23]. In spite of the wide application of this catalyst in successful synthesis and hydrolysis of ester linkages [24,25], problems associated with the enzyme support disintegration by strong shear forces and protein desorption by emulsifiers and emollient products (alkyl esters and free fatty acids), limit the industrial applicability of Novozym® 435 [26,27]. To avoid these problems, recently composite particles of the commercial Novozym® 435 and silicone have been described as a good alternative [26].

Immobilized lipase microenvironment can be changed by using solid phase chemical modification [28]. The full amination of the enzyme with ethylendiamine, after carbodiimide activation of the carboxylic groups, produces a profound change in the ionic interactions on the enzyme surface, so that many ionic bridges are changed to ionic repulsions [29]. Moreover, the enzyme surface capacity to adsorb anions is increased. Modification with glutaraldehyde, affecting mainly to the primary amino groups of the enzyme, may produce a double effect [30]. First, it is possible to get intra or intermolecular chemical crosslinking, that should improve enzyme stability [31]. Second, it produces a slight hydrophobization of the enzyme surface. A similar hydrophobization effect of the primary amino groups may be expected after TNBS modification [32]. Both the catalytic activity and the amount of enzyme adsorbed to supports, depend on the hydrophobicity of support surfaces [33]. The polymers used in this work produced a certain degree of hydrophilization of the enzyme surface; namely, aldehyde dextran which is a polyol [34] and polyethylenimine which is a poly-cation [29]. These two polymers decrease the void volume of the support pores. Generally, the effects of modifications on the lipase properties are strongly dependent on the immobilization protocol, reaction and substrate utilized [35].

The objective of this work was to investigate the diffusional limitations of substrates though immobilized biocatalysts on the alcoholysis of vegetable oils. The results comprise the construction of seven different modified derivatives of a commercial preparation of Novozym® 435, and their study in alcoholysis of *Camelina sativa* oil with and without *t*-butanol solvent, in stirred tank reactors. Several different chemical modifications were made to the commercial biocatalyst. The obtained derivatives were either more hydrophilic, more hydrophobic and/or had different charge distributions on the protein surface.

Changes on the protein (activity, desorption, aggregation) and on the support were studied after several consecutive reaction cycles.

2. Materials

Camelina oil was a kind donation of the Camelina Company (Madrid, Spain). The macroporous resin immobilized lipase Novozym® 435 (lipase B from *Candida 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), glutaraldehyde (25% aqueous solution), 2,4,6-trinitrobenzensulfonic acid (TNBS), polyethylenimine (PEI), Mn 10,000, MW 25,000, dextran sulphate (DS) 9000, 20,000 Av. wt, Triton X-100 and sodium dodecyl sulphate (SDS) were from Sigma-Aldrich (Madrid, Spain). Methanolic-HCl (0.5 N) and a fatty acid standard Supelco 37Component 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 [36]:

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 0.02 M sodium hydroxide, until the appearance of a constant pink color in the mixture.

The free fatty acid (FFA) content, as weight%, was calculated as oleic acid equivalents using Eq. (1).

$$(\%)\,FFA = \frac{(V_s - V_b) \times M_{NaOH} \times 282,5}{100 \times m_s} \times 100 \qquad (1)$$

where "V_S" and "V_B" are the mL 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 were suspended in 100 mL of 1 M EDA at pH 4.75 under continuous stirring [37]. The 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 derivative had more than 95% of the exposed carboxylic groups modified [38,39]. 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 [40]. Finally, the immobilized enzyme preparations were 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 0.1% TNBS (w/v) 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 [41].

3.2.3. Glutaraldehyde modification of Novozym® 435

Novozym® 435 or the aminated derivate were incubated with 0.1% (v/v) glutaraldehyde solution in 25 mM sodium phosphate buffer at pH 7 and 25 °C for 1 h under mild stirring. This range of operating conditions permitted to fully modify the primary amino groups of the enzyme with just one glutaraldehyde molecule [31]. The suspension was then filtered and washed with water to remove the excess of glutaraldehyde. 5 g of both were added to 200 mL of 0.1 M sodium borate containing 1 mg mL^{-1} of $NaBH_4$ at pH 8.5 and 4 °C; the reaction mixture was continuously stirred for 30 min. The reduced biocatalysts were filtered and washed several times with distilled water and *t*-butanol.

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