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Biosynthesis of ethyl butyrate by immobilized recombinant *Rhizopus oryzae* lipase expressed in *Pichia pastoris*

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ABSTRACT

A recombinant *Rhizopus oryzae* lipase expressed in *Pichia pastoris* has been immobilized in three different kinds of supports: EP100, Eupergit®CM and Octadecyl-Sepabeads. These immobilized derivatives have been used to catalyze the synthesis of ethyl butyrate. The effect of different parameters on initial reaction rate and yield has been studied for each biocatalyst. The variables selected for the study were: temperature, water content, shaking speed, enzyme loading, type of solvent and substrates molar ratio. The results showed the EP100 derivative as the best choice in terms of initial rate and yield but, when the residual activity as well as the reutilization capacity was studied, Octadecyl-Sepabeads exhibits the highest stability, so it was finally chosen as the best biocatalyst.

1. Introduction

Flavors are widely used in cosmetic and pharmaceutical industries. They also represent a quarter of the world market for food additives [1]. The production of these compounds could be carried out by their direct extraction from fruits or by chemical synthesis. The first process entails a high cost because of the expensive treatment of fruit, the downstream process and the shortage of some natural materials. However, the compounds obtained by this process could be labeled as "natural".

On the other hand, the chemical synthesis entails high temperatures and inorganic catalyst. Moreover, the obtained compound could not be labeled as "natural", reducing this fact the final prize of the product [2,3].

The use of enzymes in flavor synthesis comes up as a great solution because the obtained compounds could be labeled as "natural" and it has been also reported that they show better odor and color [1,2]. Moreover, this method could reduce the cost of the process compared to the direct extraction from fruits.

Lipases (glycerol ester hydrolases, EC. 3.1.1.3) are a group of hydrolytic enzymes very appreciated in industry whose function is to catalyze the hydrolysis of esters. However, in non-aqueous solvents these enzymes are able to catalyze synthesis, transesterification and interesterification reactions [4–9]. Short chain aliphatic esters represent a high percentage of all flavor compounds and they have been successfully synthesized by these enzymes from different sources [2,10–15].

Rhizopus oryzae lipase has high regioespecificity – it acts only at the 1- and 3-locations – because it belongs to a group of lipases that are active against esters of primary alcohols [16–20]. This enzyme has been cloned and produced in *Pichia pastoris* in our research group and it has also been characterized and immobilized on different kinds of supports [21–24]. It was found that the protein extract, obtained from the cultures of recombinant *P. pastoris*, contained two active isoforms of lipase which showed higher specific activity than native lipase [23]. Although the recombinant *R. oryzae* lipase expressed in *P. pastoris* (rROL) showed low stability in solution, a slight improvement of it was obtained when it was immobilized on several supports. It was also determined the optimum ratio of protein:support to carry out the immobilization, in order to obtain the most active biocatalyst for its use in biocatalysis [24].

The aim of the present work is to apply the recombinant *R. oryzae* lipase expressed in *P. pastoris* in the biosynthesis of ethyl butyrate which has a pine-apple aroma. The enzyme has been used immobilized on three different kinds of supports: by adsorption on EP100 and Octadecyl-Sepabeads, and by covalent binding in Eupergit®CM. The effect on the initial reaction rate and yield of different parameters such as temperature, water content, shaking speed, enzyme loading, type of solvent and substrates molar ratio, has been studied for each biocatalyst. Moreover, the stability of the derivatives

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has been analyzed by their residual activity and their reutilization capacity.

2. Materials and methods

2.1. Lipase

rROL is obtained by a mixed substrates fed-batch cultivation of a recombinant *P. pastoris* strain using methanol as inducer [22]. The culture broth was centrifuged and microfiltered to remove the biomass. The supernatant was concentrated by ultrafiltration with a Centrasette® Pall Filtron (Pall Corporation, NY, USA) system equipped with an Omega membrane of 10 kDa cut-off, and subsequently dialyzed against 10 mM Tris–HCl buffer pH 7.5 and thereafter lyophilized [25].

2.2. Chemicals

Accurel EP100 polypropylene powder was a generous gift from Akzo Nobel (Stockholm, Sweden). Eupergit®CM and Octadecyl-Sepabeads were purchased from Sigma (St. Louis, USA) and Resindion (Binasco, Italy), respectively. Butyric acid, ethanol, hexane, heptane, isooctane and 4-methyl 2-pentanol were analytical grade.

2.3. Lipase activity assay

Lipase activity assay was followed spectrophotometrically in a Cary Varian 300 (Varian Inc., Palo Alto, USA) spectrophotometer at 30 °C in 200 mM Tris–HCl + 5 mM $CaCl_2$ buffer at pH 7.25 using the Roche lipase colorimetric kit (Roche kit 11821792, Mannheim, Germany) which contains as substrate 1,2-di-O-lauryl-rac-glycero-3-(glutaric acid 6-methylresorufin ester), as previously described [26]. The measurement was done at 580 nm and every analysis was carried out in duplicate, except the analysis of suspension samples which were carried out in quadruplicate.

2.4. Total protein determination

Protein concentration was determined by the method of Bradford using bovine serum albumin as standard [27]. All samples were analyzed on triplicate.

2.5. Immobilization process

Immobilization of rROL on EP100 and Eupergit®CM was carried out as previously described [24]. The obtained derivatives had 0.49 UA/mg support for rROL-EP100 and 0.43 UA/mg support for rROL-Eupergit®CM.

The immobilization of rROL on octadecyl Sepabeads (rROL-Sepabeads) was performed at Instituto de Catálisis y Petroleoquímica (CSIC, Spain) as described [28]. Its activity was 8.38 UA/mg.

2.6. Synthesis reactions

The synthesis reactions of ethyl butyrate in organic media were carried out in 10 mL screw-capped vials using ethanol and butyric acid as substrates. Temperature and shaking were maintained constant at the desired conditions in an incubator (Optic Ivymen System, Barcelona, Spain).

The reaction volume was 10 mL with the solvent and the concentration of substrates selected in each case. The solvents as well as substrates were previously dried for 24 h with molecular sieve UOP type 3 (Sigma Aldrich, St. Louis, USA). The initial solution was added to the biocatalyst which was previously weighted in the reaction vial. If the immobilized derivative had to be used dried, it was maintained 24 h in a dryer with silica gel before starting the reaction. The reaction was followed analyzing the butyric acid as well as the ethyl butyrate concentration for 24 h by gas chromatography in a 7890A Agilent Technologies gas chromatograph. The reaction rates were calculated by fitting data results using the Sigma Plot 11.0 software (Systat software, IL, USA).

The chromatograph was equipped with a flame ionization detector and an INNOWax (Agilent Technologies, Santa Clara, CA) (Crosslinked Polyethylene Glycol) capillary column (30 m × 0.35 mm × 0.25 μm). Injection and detector temperatures were set at 250 °C and 370 °C, respectively. The oven temperature programme was as follows: 60 °C for 6 min, temperature increase to 180 at 30 °C per min and a final plateau at 180 °C for 2 min. Helium was used as a carrier gas at a column head pressure of 15 psi. The retention time of butyric acid under these conditions is 12.6 min and 4.2 min for ethyl butyrate. Every sample was filtered previously with a 0.45 μm filter (Millipore, Bedford, USA) and mixed with equal volume of internal standard (0.4 M of 4-methyl 2-pentanol in hexane).

2.7. Residual activity assay

The biocatalysts were weighted (20 mg) in 10 mL screw-capped vials and dried for 24 h in a drier with silica gel. They were subsequently incubated in a thermostatic shaker (Optic Ivymen System, Barcelona, Spain) at 30 °C and 250 rpm in the selected organic solvent for 1 h and 24 h. After that, the immobilized derivatives were recovered by vacuum filtration and re-suspended in 1 mL of water. Their residual activities were analyzed as previously described in Section 2.3.

2.8. Reutilization of biocatalyst in ethyl butyrate synthesis

Biocatalysts were used for ethyl butyrate synthesis as described in Section 2.6, at the best conditions founded for each one. The initial activity loaded was selected in order to reach the maximum percentage yield in the minimum reaction time (100 UA rROL-EP100, 150 UA rROL-Eupergit®CM and 1500 UA rROL-Sepabeads). Biocatalysts were recovered by vacuum filtration and rinsed with 20 mL of solvent. Then the immobilized derivatives were weighted for their use in a new reaction using fresh reactants and they were used dried for 24 h or directly after the recovery.

2.9. Reactivation of rROL-EP100

After the incubation for 1 h or 24 h in hexane of rROL-EP100, as it is described in Section 2.7, the biocatalyst was recovered by vacuum filtration and incubated in water, 30 g/L of glucose or 30 g/L lactose aqueous solution in a MoviROL (P-selecta, Abrera, Spain) for 24 h at 4 °C. Then, the biocatalyst was recovered by vacuum filtration and its activity was tested as previously described in Section 2.3.

2.10. Analysis of protein desorption

The biocatalysts were weighted (40 mg) in 10 mL screw-capped vials and dried for 24 h in a drier with silica gel. Hexane (400 μL) was added and they were incubated for 24 h at 30 °C and 250 rpm in a thermostatic shaker (Optic Ivymen System, Barcelona, Spain). The hexane was then evaporated and the desorbed protein was dissolved in 400 μL of distilled water. The support was then removed by filtration with a 0.45 μm filter (Millipore, Bedford, USA). In order to detect the presence of desorbed proteins in the samples, they were analyzed by Bradford method (Section 2.4) and SDS-PAGE (Section 2.11). All experiments were carried out in duplicate.