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Interfacial activation and bioimprinting of *Candida rugosa* lipase immobilized on polypropylene: effect on the enzymatic activity in solvent-free ethyl oleate synthesis

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Abstract

Lipase from *Candida rugosa* adsorbed on polypropylene powder (CR/PP) was subjected to activation pre-treatments in order to enhance its activity in solvent-free ethyl oleate synthesis. The lipase activation achieved upon adsorption onto a hydrophobic support like PP was further enhanced through oil–water interfacial activation and bioimprinting of the immobilized catalyst. Several aliphatic hydrocarbons/buffer pH 7 mixtures were used in the pre-activation of CR/PP with specific activity increments of up to 29%. Molecular bioimprinting was also performed, with specific activity enhancement of near 70% with respect to non-treated CR/PP. The effect of several fatty acids used as templates and the water present in the reaction medium was studied. The oil–water activation and bioimprinting treatments that led to the best activities were assayed at the immobilization step. Instead of pre-treating CR/PP adsorbed in buffer medium, interfacial activation with octane/buffer and bioimprinting with a mix of fatty acids were carried out *in the immobilization vial*. The best results were found for CR/PP immobilized in 5/95 octane/buffer (v/v, %) medium. In that way, a biocatalyst with enhanced specific activity is obtained right from the immobilization vial with no need of further activation steps prior to reaction.

Keywords: Adsorbed lipase; Interfacial activation; Bioimprinting

1. Introduction

To perform lipase-mediated synthesis in solvent-free media has a number of advantages. In absence of solvent, substrate concentration can be maximized, solvent cost and solvent separation are avoided, and downstream processing is reduced. Different strategies have been adopted in order to improve enzyme activity/stability in non-aqueous media: immobilization on supports of varied nature, surface modification with amphiphiles, enzyme crystal cross-linking, introduction of new bonds by protein engineering, change of solvent nature, incorporation into reverse micelles, use of surfactant coated enzymes and molecular bioimprinting [1].

Lipases are known to contain an amino acid lid covering their active site, which opens in presence of an oil–water interface. This phenomenon, called interfacial activation, has been found for *Candida rugosa* lipase (CRL) upon its adsorption onto hydrophobic supports [2], and especially onto polypropylene powder [3]. The immobilized lipase is fixed in an "open conformation" and enhanced enzymatic activity is achieved. In this work, further lipase activation is obtained through activating pre-treatments involving liquid–liquid interfaces. Maruyama et al. have reported that the closing of the lipase lid is caused by water, and the opening is caused by an oil–water interface in oil–water two-phase system. Both, closing and opening, cannot take place in organic solvents, where the lids of the lipase molecules will be closed because of the absence of an interface. If the lids can be kept open in advance, lipase is expected to have a high activity in organic solvents [4]. In this contribution, several hydrocarbon–buffer

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mixtures have been used as pre-treatment for lipase from *C. rugosa* immobilized on polypropylene powder (CR/PP) which was later used in ethyl oleate synthesis.

Improvement of enzymatic activity and stability in non-aqueous media has also been achieved by bioimprinting [5,6]. Bioimprinting consists of loading the enzyme's active site with a substrate analogue in an aqueous solution. A complex similar to an enzyme–substrate complex is formed and small conformational changes are supposed to occur—the induced fit. Then the ligand is washed away, but the enzyme is unable to adopt its former conformation due to its rigid structure which results from strong electrostatic interactions in the media possessing low dielectric constants [5]. González-Navarro and Braco found that the benefits of molecular bioimprinting strategy seem valid, not only for organic solutions but also for solvent-free mixtures of substrates [6].

In this contribution, both interfacial activation through oil–water interfaces and bioimprinting are tested in the pre-treatment of a biocatalyst already activated upon adsorption onto a hydrophobic interface. In particular for CRL adsorbed on polypropylene, in the author's knowledge, *this is the first time* that additional activation is obtained through liquid–liquid interface-pre-treatments and/or simple bioimprinting procedures. The water presence in reaction media (something apparently prohibited for bioimprinted molecules) has also been addressed.

2. Experimental

2.1. Materials

Low-molecular weight polypropylene powder (30,000 g/mol; BET area: 23 m^2/g) was obtained by polymerization using metallocenes. *C. rugosa* AY lipase (EC 3.1.1.3) (64,000 g/mol) was kindly donated by Amano Enzyme Inc. Oleic acid (99%) was purchased from J.T. Baker. Commercial fatty acid mixture (C_{14}: 01.6%, C_{14}: 11.9%, C_{16}: 05.7%, C_{16}: 111.7%, C_{18}: 179.1%—wt.%) was purchased from Quimicar-Olavarría, Argentina). Absolute ethanol (99%) and sulphuric ether (99%) were both purchased from Dorwil. Buffer solution of pH 7 (di-sodium hydrogenophosphate) and potassium hydroxide were both from Merck. Laboratory Ceblaco provided octane used in pre-activation experiments. Isooctane (2,2,4-trimethylpentane) was purchased from U.V.E., *n*-tedradecane (99%) from Sigma and polyethylenglycol (PEG; M_w = 35,000 g/mol) from Fluka.

2.2. Immobilization procedure

Four hundred milligrams of *C. rugosa* lipase was added to 50 ml of buffer solution of pH 7, and subjected to strong stirring during 30 min in order to solubilize lipase. A filtering step was performed to retain carbohydrates and other insoluble compounds. One gram of ethanol pre-treated PP was added to lipase solution and immobilization began. Samples from supernatant solution were periodically withdrawn, filtrated with special filters for small particle's powder, and diluted up to 5 ml with buffer for UV/vis analysis of lipase content. At the end of the immobilization period, the catalyst (CR/PP) was washed with distilled water, separated from solution and dried to constant weight.

2.3. Characterization of adsorption/desorption of water for free and immobilized C. rugosa lipase

In the open literature, there are several reports on water adsorption isotherms performed on free and immobilized lipases. In these works, organic solvents are used to equilibrate the water activities selected (a_w, defined as the water vapor pressure in equilibrium with the solution divided by the vapor pressure of pure water evaluated at the same temperature [7]), and construct an adsorption isotherm [8–10]. In this work, we propose a different way to study the behavior of free and immobilized lipase: successive adsorption/desorption of water, for increasing values of a_w of the gas phase. A Cahn Electrobalance 1000 with a device containing the salt solutions at the desired a_w was used to give the desired relative humidities or water activities of the gas phase. Successive water adsorption/desorption cycles were carried out to analyze the changes in free and immobilized lipase (10 mg of sample in all the cases) upon contact with water gas at different a_w (0.33, 0.44, 0.58, 0.75, 0.84, 0.94). Blank (without sample) and samples were equilibrated until no change in weight was found (each point required one full-day work).

2.4. Interfacial activation of CR/PP with aliphatic hydrocarbon/buffer pH 7 mixtures

2.4.1. Pre-treatment procedure

Fifty milligrams of immobilized lipase (CR/PP) was typically contacted with 5 ml of octane/buffer of pH 7 mixture for 30 min. The two-phase mixture was magnetically stirred at 45 °C (a temperature equal to reaction temperature) and 1000 rpm. The oil–water interfacially activated lipase was obtained by filtration of the mixture and dried at 45 °C for 1 h. When pure octane was used as pre-treatment medium (two experiments performed), the periods of contact were 10 and 60 min.

2.4.2. Nature of the pre-treatment mixtures

Increasing amounts of octane were used for the activation mixtures. The volume of the mixtures was always of 5 ml, with percentages of octane ranging from 2.5 to 100%. Total volume was completed with buffer of pH 7 solution. In the octane/buffer relationship that produced the catalyst with highest esterification activity, *n*-tetradecane and isooctane were tested. In the best condition, the addition of 25 mg of polyethylenglycol to pre-treatment mixture was also assayed (0.5:1, w/w, PEG:CR/PP). Pre-treatment of CR/PP with oleic acid and oleic acid/buffer pH 7 in the amounts used for reac-