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Hydrolysis and oxidation of racemic esters into prochiral ketones catalyzed by a consortium of immobilized enzymes

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ABSTRACT

One-pot multi-step conversions are desirable to achieve more efficient and sustainable chemical processes. In this context, the immobilization of multi-enzyme systems allows the reusability of several stabilized biocatalysts working in cascade and orthogonal reactions to access more complex synthetic schemes. Herein, we have shown the one-pot tandem hydrolysis and oxidation of racemic esters (1-phenylethyl acetate) to yield quantitative conversion of prochiral ketones (acetophenone) catalyzed by a consortium of immobilized enzymes. Eukaryotic lipase and catalase, and microbial thermophilic alcohol dehydrogenase and NADH oxidase are covalently, irreversibly and individually immobilized onto four different carriers, achieving high immobilization yields (>95%) for all the enzymes, and residual activities >50% for both thermophilic alcohol dehydrogenase and NADH oxidase, 18% for the catalase and 10% for the lipase. This heterogeneous system efficiently recycles NAD^+ with a maximum turnover frequency (TOF) of 294 h^{-1} and can be reused for up to 10 operational cycles, retaining more than 80% of its initial activity.

1. Introduction

Selective cleavage of protecting groups and the transformation of racemic mixtures into prochiral synthons to be further asymmetrically transformed into chiral molecules are crucial steps in synthetic chemistry to manufacture pharmaceuticals, agrochemicals and other fine chemicals [1]. Interestingly, the unselective hydrolysis of ester groups followed by the unselective oxidation of the resulting alcohols yields 100% of the corresponding prochiral ketone starting from a racemic ester. These prochiral ketones are important synthons to access more interesting chiral molecules such as secondary alcohols, secondary amines or β-nitro alcohols. Recently, Wang et al. have developed a chemo-enzymatic cascade reaction to cleavage protecting ester groups leaving a primary alcohol available for a sequential gold-catalysis to yield prochiral substituted tetrahydrofurans that can be easily converted into pure diasteroisomers by selective hydrogenation of the double bond [2]. A fully biocatalytic scheme emerges as an attractive alternative to combine the hydrolysis of ester groups and the oxidation of alcohols by using a lipase and an alcohol dehydrogenase (ADH), respectively. The use of these two biocatalysts instead chemical catalysts will ensure the environmental sustainability of the process because the enzymes can efficiently work in aqueous media under mild conditions reducing both wastes and energetic demand [3]. However, if we want to quantitatively convert a racemic ester into a prochiral ketone, both lipase and ADH must be chemo and regioselective but non-enantioselective in order to hydrolyze and oxidize both enantiomers of the ester and the resulting alcohol respectively. Unfortunately, this catalytic sequence is unexploited in spite of the rich existing toolbox for both lipases and ADHs. This fact is due to that the biocatalysis community has successfully worked in searching and engineering highly enantioselective

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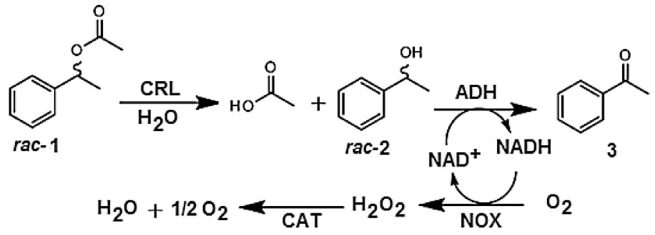

Scheme 1. One-pot tandem hydrolysis and oxidation of *rac*-1-phenylethyl acetate (*rac*-**1**) catalyzed by a consortium of 4 immobilized enzymes. CRL: Lipase from *Candida rugosa*. ADH: Alcohol dehydrogenase 2 from *Thermus thermophilus* HB2. NOX: NADH oxidase from *Thermus thermophilus HB2*. CAT: Catalase from bovine liver.

enzymes to manufacture optically pure molecules [4,5] forgetting those processes that require achiral enzymes to achieve high product yields. Hence, this tandem reaction encourages us to find non-enantioselective lipases and ADHs suitable to be integrated into a one-pot process. Nevertheless, tandem reactions involving sequential action of lipases and ADHs are rare in biocatalysis and only few artificial synthetic cascades have been developed to efficiently access 6-aminohexanoic acid [6] and poly-ε-caprolactone [7] combining lipases and ADHs among other enzymes.

Another important hurdle to implement enzymes into the industrial world is their solubility and low stability. Herein, protein immobilization addresses those two issues stabilizing the enzymes and allowing them working in consecutive operational cycles. Lastly, our group has successfully immobilized different enzyme consortiums, increasing their global stability and allowing their recyclability [8]. An optimal immobilization of the multi-enzyme system must guarantee the perfect orchestration of both activity and stability for each enzyme participating in the chemical cascade. When the immobilization protocol fails for one of the enzymes, the reusability of the immobilized systems becomes difficult as occurs for a recently reported 4-enzymes system immobilized on layered inorganic hydroxides able to synthesize phosphorylated sugars [9]. Therefore, we must immobilize each enzyme on the suitable surface and through the optimal immobilization chemistry to coordinate both activity and stability of each enzyme in order to ensure both maximum productivity and product yield in a large number of reaction cycles.

Herein, we report a consortium of immobilized enzymes which enables the quantitative transformation of racemic mixtures of esters into prochiral ketones by sequentially coupling hydrolysis and oxidation steps. This heterogeneous multi-enzyme system incorporates an NADH oxidase and a catalase to *in situ* recycle the redox cofactor (NAD^+) required for an efficient oxidation and to *in situ* eliminate the H_2O_2 produced during the NAD^+ recycling. Finally, we have evaluated different conditions to optimize the performance of such consortium of immobilized enzymes and demonstrated its re-usability.

2. Materials and methods

2.1. Materials

Racemic 1-phenylethyl acetate (*rac*-**1**), racemic 1-phenylethanol (*rac*-**2**), acetophenone (**3**) and *R* or *S*-1-phenylethanol (99%), flavin-adenine-dinucleotide sodium salt (FAD^+), *p*-nitrophenyl butyrate (*p*NPB) and *p*-nitrophenol were acquired from Sigma-Aldrich (St. Louis, IL). Nicotinamide-adenine-dinucleotide sodium salt (NAD^+) was purchased from GERBU Biotechnik GmbH (Wieblingen, Germany). 6BCL agarose beads activated with glyoxyl groups (Ag-G) was prepared as described elsewhere [10]. All other reagents were analytical grade or superior.

2.2. Enzyme production and purification

Alcohol dehydrogenase (Tt27-ADH2), and NADH oxidase (NOX) from *T. thermophilus* overexpressed in *Escherichia coli* were produced and purified according to a previous work [11,12]. Lipase 2 (BTL) from *Geobacillus thermocatenulatus* overexpressed in *Escherichia coli* was produced and purified according to a previously work [13]. Lipases from *Candida rugosa* (CRL) TVII, *Candida antarctica* B (CALB) and catalase from bovine liver (CAT) were purchased from Sigma-Aldrich (St. Louis, IL).

2.3. Immobilization of enzymes on Ag-G

Enzymatic solution of the target enzyme was adjusted at pH 10 with bicarbonate buffer 100 mM. Then, it was added to Ag-G in a ratio of 10:1 (v:w) and maintained under gentle agitation at 4 °C. Immobilization course was followed spectrophotometrically by measuring the activity in the suspension and in the supernatant. Once no activity was detected in the supernatant, the suspension was filtered and the solid biocatalyst was resuspended in 10 vol of bicarbonate buffered solution at pH 10 with 1 mg/mL of sodium borohydride and maintained under gentle agitation at 4 °C for 30 min. Finally, the solid immobilized biocatalyst was abundantly washed with 25 mM phosphate buffer at pH 7, filtered and stored at 4 °C. Particularly, the immobilization of CRL was conducted in the presence of 40% of PEG_{1800} to avoid its inactivation at pH 10 [14] and 0.05% of Triton X-100® to avoid the attachment of lipase dimmers [15,16].

2.4. Spectrophotometric determination of enzyme activities

2.4.1. Lipase activity

Lipase activity was determined by the hydrolysis of *p*-NPB as substrate. Enzyme activity was measured in 0.5 mM *p*-NPB dissolved in 25 mM sodium phosphate buffer and 1% acetonitrile at pH 7.0 and 25 °C. 200 μL of such reaction mixture were placed in one well of a 96-well microplate. To start the reaction, 5 μL of properly diluted enzyme solution or suspension was added and maintained under agitation at 25 °C in a Varioskan™ Flash Multimode Reader (Thermo Scientific). The released *p*-nitrophenol was monitored at 348 nm during 5 min of reaction. Calibration curve of *p*-nitrophenol in the same conditions was carried out. One unit of lipase activity was defined as the amount of enzyme required for release one μmol of *p*-nitrophenol per minute.

2.4.2. Alcohol dehydrogenase activity

ADH activity was measured by the hydrolysis of *rac*-**2**. Reaction mixture was composed of 10 mM of *rac*-**2** and 1 mM NAD^+ dissolved in different buffers at different pHs (pH 7 and 8 in 25 mM sodium phosphate, pH 9 and 10 in 25 mM sodium bicarbonate, as indicated). The enzymatic reaction was triggered by adding 5 μL of proper diluted enzyme or suspension to a 200 μL reaction mixture placed in one well of a 96-well microplate and maintained under agitation at 25 °C in a Varioskan™ Flash Multimode Reader (Thermo Scientific). Activity was calculated by the reduction of NAD^+ into NADH by monitoring the increment in absorbance at 340 nm. One unit of ADH activity was defined as the amount of enzyme required for the production one μmol of NADH per minute.

2.4.3. NADH oxidase activity

NADH activity was measured by the reduction of NAD^+ into NADH. Reaction mixture consisted in 1 mM NAD^+ and 0.15 mM FAD^+ in aqueous 25 mM sodium phosphate buffered solution at pH 7. Reaction was triggered by adding 5 μL of properly diluted enzyme or suspension to a 200 μL of reaction mixture placed in one well of a 96-well microplate and maintained under agitation at

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