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High-yield production of enantiopure 2-hydroxy-2-(2′-chlorophenyl) acetic acid by long-term operation of a continuous packed bed reactor

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ABSTRACT

We recently described the development of a versatile and efficient esterase (rPPE01$_{W187H}$) for the preparation of enantiopure 2-hydroxy acids. Herein, a primary amino-functionalized resin (ESR-1) was selected for immobilizing rPPE01$_{W187H}$, and the resulting immobilized enzyme, rPPE01$_{W187H}$@ESR-1, exhibited notably enhanced stability. No obvious inactivation was observed for rPPE01$_{W187H}$@ESR-1 following its incubation at 30 °C for 1440 h, whereas the half-life of the free enzyme was only 50.2 h under the same conditions. A continuous process was subsequently developed using rPPE01$_{W187H}$@ESR-1 in a packed bed reactor, which allowed for the immobilized enzyme to be conveniently recycled. The space-time yield for biocatalytic resolution of 2-acetoxy-2-(2′-chlorophenyl)acetate in this packed bed reactor reached as high as 3.34 kg L^{-1} d^{-1} following the optimization of the critical process parameters, including the initial pH, H:D ratio and flow direction. Further investigation of the operational stability indicated that the enzymatic process could be continuously operated for at least 42 d with approximately 50% conversion and nearly perfect optical purity, giving an outstanding total turnover number of 1.13×10^7 for rPPE01$_{W187H}$. The continuous, long-term and high-performance manufacturing process developed in this study therefore highlights the potential feasibility of using rPPE01$_{W187H}$ for the large-scale production of optically pure chiral 2-hydroxy acids.

1. Introduction

Optically pure 2-hydroxy acids, in particular 2-hydroxyphenylacetic acid (HO-PA) and its derivatives, are widely used as building blocks in the chemical and pharmaceutical industries [1]. For example, (*R*)-2-hydroxy-2-(2′-chlorophenyl) acetate [(*R*)-HO-CPA] is a key intermediate for the synthesis of Clopidogrel which is used for the treatment of heart attacks and strokes caused by blood clots. Many approaches have been developed for the synthesis of these compounds, including resolution of the corresponding racemic compounds via chemical agents, nitrilase, or carboxylesterase [2–7]. In our previous work, extensive studies were conducted toward establishing a versatile and effective bioprocess for the preparation of optically pure 2-hydroxy acids via the enzymatic resolution of *O*-acetylated hydroxy acid (Fig. 1). An esterase-producing *Pseudomonas putida* was primarily isolated from soil samples based on its selective deacetylation activity toward the substrate mentioned above [8]. Further improvements in both the expression level and specific activity provided the desirable engineered biocatalyst rPPE01$_{W187H}$ [9,10]. Following on from this work, the focus of our research shifted toward improving the performance and operational life of this catalyst through bioprocess and bioreactor engineering. The deactivation of the enzyme under the reaction conditions was alleviated to a certain extent by simply switching the substrate counter-ion from Na^+ to K^+ [9]. However, the application potential of this bioprocess was still hampered by the lack of long-term operation stability and high process efficiency.

The immobilization of a catalyst is considered as *a conditio sine qua non* for developing economically viable bioprocesses [11,12]. The main advantages of biocatalyst immobilization include enhanced stability and the facile separation of the catalyst from the product stream, which makes it easier for the catalyst to be reused in repeated or continuous processes. Improved robustness and recyclability characteristics can also result in significant increases in the productivity of a catalyst (i.e., the weight gain ratio of product to catalyst), which can ultimately lead to a reduction

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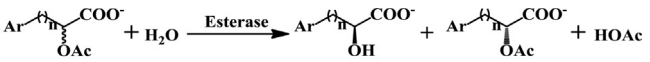

Ar = C_6H_5, *o*-Cl-C_6H_4, *m*-Cl-C_6H_4, *p*-Cl-C_6H_4, $C_{10}H_7$; n= 0, 1, 2

Fig. 1. Enantioselective hydrolysis of acetoxy acids catalyzed by rPPE01$_{W187H}$.

in the costs associated with catalyst consumption. The immobilization of free enzymes onto solid supports via physical (e.g., hydrophobic or van der Waals), ionic or covalent binding interactions represents a popular technique for the immobilization of an enzyme [13–15]. Among these different techniques, the formation of covalent binding interactions between an enzyme and a carrier system provides stronger bonds than physical or ionic binding and therefore generally confers enhanced stability to the enzyme. For example, Eupergit® C is one of the best-known carriers for covalent immobilization and numerous enzymes, such as penicillin amidase, phosphodiesterase and lipase, have been immobilized on to this support, with the resulting immobilized enzymes exhibiting remarkable increases in their stability compared with the free enzymes [16].

The type of reactor and mode of operation used for a given enzymatic process also require special consideration. Various bioreactors, such as packed bed reactor, fluidized bed reactor, and magnetically stabilized fluidized bed [17–20], have been developed for the enzymatic reactions with immobilized enzymes. and how to choose an optimum reactor for the given enzymatic process is a rather complex issue, which involves much practical considerations, such as the need for temperature and pH control and the desired through-put [20,21]. Packed bed reactors can be used to avoid enzyme attrition resulting from the collision of the enzyme particles with the impeller systems frequently used in batch reactors for immobilized particulate catalysts [22–26]. The use of a continuous mode of operation with a packed bed reactor system avoids the need for additional separation steps (e.g., filtration or centrifugation) to allow for the recycling of the catalyst during batch operations in a stirred tank reactor, as well avoiding physical enzyme losses during these operational processes. Furthermore, the use of continuous processes can allow for a decrease in the labor input of skilled workers over long-term processes. An increasing number of biocatalytic processes aiming at pharmaceutical intermediates are being performed in a continuous manner using a packed bed reactor with immobilized enzymes [14,22,27]. However, there is still a possibility that the bed pressure affected the stability of enzyme and resin, especially when the process pressure is increased beyond the lab scale production.

Herein, we have focused on improving the performance of the previously obtained rPPE01$_{W187H}$ enzyme, with the aim of developing an acceptable manufacturing route for the enzymatic resolution of racemic 2-acetoxy-2-(2′-chlorophenyl) acetate (AcO-CPA). A continuous bioprocess was developed in a packed bed reactor system using the immobilized esterase rPPE01$_{W187H}$, which was found to be much more stable than the free enzyme. The enzyme immobilization conditions, catalytic properties of the immobilized enzyme and the conditions required for the continuous operation of the packed bed reactor have been investigated in detail.

2. Materials and methods

2.1. Materials

(*R*,*S*)-2-Hydroxy-2-(2′-chlorophenyl) acetate (HO-CPA) was purchased from Guangde Chemical Co., Ltd. (Anhui, China). Glutaraldehyde (25%, v/v) was purchased from Lingfeng Chemical Reagent Co., Ltd. (Shanghai, China). All of the other chemicals and reagents used in the current study were commercially sourced as the analytical grades. The acetylation of (*R*,*S*)-HO-CPA was conducted as described previously [10]. The resins used for the immobilization of the enzyme were kindly donated by Tianjin Nankai Hecheng Science and Technology Co., Ltd. (Tianjin, China) and the informations about the resins can be obtained from the website: http://www.tjhecheng.com/en/Products.aspx?id=132. The rPPE01$_{W187H}$ enzyme was prepared as described previously [9].

2.2. Analytical methods

The conversion of (*R*,*S*)-AcO-CPA and *ee* values of the substrate and product were determined using a HPLC system (LC-10AT, Shimadzu Co., Japan) equipped with a Chiralcel OJ-H column (250 × 4.6 mm, Daicel Co., Tokyo, Japan) using hexane/isopropanol/trifluoroacetic acid (92:8:0.1, v/v/v) as the mobile phase at a flow rate of 1.0 mL min^{-1}. The separated peaks were monitored using a UV detector (SPD-10A, Shimadzu Co., Japan) at 228 nm. The activity of the enzyme was defined as previously described [9].

2.3. Enzyme immobilization procedure

The rPPE01$_{W187H}$ was immobilized onto a series of epoxy-functionalized resins (i.e., ES-1, ES-101, ES-103) according to the following procedure: the resins (0.5 g) were washed three times with 3 mL of phosphate buffer (2 M, pH 7.8) and then added to a 5 mL solution of the enzyme (2 M phosphate buffer, pH 7.8) containing 2.5 mg protein. The resulting mixtures were then shaken at 180 rpm for 20 h at 30 °C. The immobilized enzymes were collected by centrifugation and washed with phosphate buffer until no protein could be detected in the supernatant.

Activation with glutaraldehyde was necessary for resins bearing primary amino groups as their functional group (i.e., ESR-1, ESR-2, ESR-3). Samples of these resins (0.5 g) and glutaraldehyde solution (25 wt%) of 1.6 mL were added to 6.4 mL of phosphate buffer (0.2 M, pH 7.8), and the mixtures were then shaken at 180 rpm for 2 h at 30 °C. The resins were collected by centrifugation and then rinsed five times with distilled water to remove the free glutaraldehyde. The activated resins were then added to a 5 mL solution of the enzyme (0.2 M phosphate buffer, pH 7.8) containing 2.5 mg of protein, and the resulting mixtures were shaken at 180 rpm for 20 h at 30 °C. The immobilized enzymes were then collected by centrifugation and washed with phosphate buffer until no protein could be detected in the supernatant.

2.4. Activity assay of the immobilized enzyme

Small portions of the immobilized enzymes (30 mg) were added to 1 mL of phosphate buffer (100 mM, pH 6.5) containing 200 mM (*R*,*S*)-AcO-CPA, and the resulting mixtures were shaken at 30 °C for 6 min using a Heating Thermo Shaker Mixer MHR 23 (Digital HLC, Germany). The samples were then acidified by the addition of 20 μL H_2SO_4 solution (2 M) and extracted twice with ethyl acetate. The extracts were dried over anhydrous sodium sulfate and subjected to HPLC analysis. One unit of enzyme activity was defined as the amount of enzyme releasing 1.0 μmol of HO-CPA per minute under the assay conditions.

2.5. Reutilization of immobilized enzyme

Batch reactions were performed in a stirred tank reactor to evaluate the reusability of the immobilized enzymes. (*R*,*S*)-AcO-CPA (3 mmol) and K_2CO_3 (1.5 mmol) were dissolved in 10 mL of phosphate buffer (50 mM, pH 6.5) and the reaction was initiated

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