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Short Communication

Catalytic resolution of DL-tryptophan amides using the resting cells of *Flavobacterium aquatile* ZJB-09211 in a two-phase system

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ABSTRACT

The catalytic activity of *Flavobacterium aquatile* ZJB-09211 towards the kinetic resolution of DL-tryptophan amides was significantly enhanced by ethyl acetate. A maximum enzyme activity of 5118.62 U/g was obtained under the optimized conditions consisting of a mixture of ethyl acetate and Tris–HCl buffer (30:70). In a scale-up reaction, the tryptophan amide concentration was improved to 200 mM, with 49.85% (*e.e.* >99.95%) of the substrate being converted to L-tryptophan. The addition of an organic solvent to the process therefore provided an effective approach for improving the activity of the amidase that could be applied to other amidase-catalyzed bioprocesses.

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1. Introduction

The biocatalytic production of non-racemic chiral fine chemicals with isolated enzymes or whole cells has grown in significance because of the high levels of product selectivity provided by these techniques [1–4]. For example, amidases (EC 3.5.1.4) catalyze the hydrolysis reactions of a variety of different amides with the production of free carboxylate and ammonia. Amidases have also become promising synthetic tool for the synthesis of economically important chiral carboxyl acids and their derivatives, where they provide excellent levels of enantioselectivity [2]. Of the known amidases, the amino acid amidase can stereoselectively and efficiently hydrolyze L- and D-amino acid amides to their corresponding chiral amino acids and ammonia [5,6]. Amino acid amides themselves can be readily synthesized from aldehydes, hydrogen cyanide, and ammonium [7]. The amidase catalyzed asymmetric hydrolysis of amino acid amides therefore presents a promising method for the synthesis of enantiomerically pure amino acids.

A variety of different strategies has been developed to enhance volumetric productivity or reduce the costs of a particular enzymatic process, such as immobilization, reactor improvements and medium engineering [8–10]. Of these strategies, medium engineering is a widely acknowledged practical tool for improving the properties of an enzyme. The use of a second phase consisting of an organic solvent not only increases substrate loading at the same time as keeping the concentration of toxic compounds in the aqueous phase at a low level, but also effectively improves the catalytic activity of the enzyme and sometimes even the enantioselectivity [11,12]. In recent years, many reports have appeared in the literature showing that the addition of an organic solvent to the reaction medium can reduce substrate and product inhibition by reducing their concentration in the aqueous environment where the enzymatic reaction takes place [1,13].

Tryptophan (Trp) and its analogs have attracted increasing levels of attention because of their physiological functions, as well as their applications in chiral synthesis [14]. It is well-known that L-Trp and D-Trp are optically active and possess different properties and functions. Our laboratory has succeeded in isolating a new *S*-stereospecific amidase-producing strain of *Flavobacterium aquatile* ZJB-09211, which exhibited great potential for the industrial production of L-Trp by asymmetric hydrolysis of the racemic tryptophan amide. Unfortunately, however, the solubility of tryptophan amide is very poor in the water (20 mM), which restricted any improvements to the concentration of substrate and the utilization of the amidase in the catalytic process. To further improve the throughput of this biocatalytic process, herein we describe the effects of different organic solvents on the hydrolytic resolution of tryptophan amide using free cells of *F. aquatile* ZJB-09211. Enhancements in the concentration of substrate and catalytic activity of the amidase have therefore been reported for the first time in this paper by the addition of water-immiscible organic solvents.

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2. Experimental

2.1. Chemicals and reagents

Tryptophan amides were purchased from Hanhong Chemical Co., Ltd. (Shanghai, China). L-Trp, D-Trp, racemic tryptophan and other chemicals were of analytical reagent grade and purchased from J&K Scientific Ltd. (Shanghai, China).

2.2. Microorganisms and amidase production

F. aquatile ZJB-09211 was isolated through a high-throughput screening system. The cell culture of *F. aquatile* ZJB-09211 was performed in a 250 mL flasks with 50 mL of sterile medium containing (g/L): sucrose 4.3, gelatin 5.0, beer extract 8.0, ε-caprolactam 1.0, $NaNO_3$ 0.17, K_2HPO_4 1.0, KH_2PO_4 1.0, and NaCl 1.0. The flasks were incubated at 30 °C and 150 rpm for 48 h. The resulting culture broth was centrifuged at 12,000 rpm for 15 min at 4 °C. The cells were then collected for further study.

2.3. Biotransformation

The hydrolysis reaction of the tryptophan amides to L-Trp was carried out in 50 mL Erlenmeyer flasks with a screw cap at 30 °C on a rotary shaker at 150 rpm. The tryptophan amides and wet cells were added to 10 mL of a two phase reaction system in the Erlenmeyer flasks (50 mL). The reaction was conducted over a 10 min period and subsequently terminated by the addition of 20 μL of 6 M HCl. The reaction mixture was then centrifuged at 10,000 rpm and used for Trp analysis.

One unit of enzyme activity was defined as the amount of wet cells required to form 1 μmol L-trp per minute under the above conditions.

2.4. Scale-up resolution of DL-tryptophan amides

A 5-L scale reaction was performed in an 8-L stirring-tank reactor containing 100 g of wet free cells, 200 mM of DL-tryptophan amides, 1500 mL of ethyl acetate and 3500 mL of Tris–HCl buffer (50 mM, pH 7.5). The resulting mixture was stirred at 150 rpm at a constant temperature of 45 °C. Following a reaction time of 450 min, the reaction broth was centrifuged to remove the cells. The supernatant was treated by activated carbon absorption (0.3%, w/v) for 30 min and then filtered. The filtrate was collected and basified to pH 12 and the D-tryptophan amide was extracted with 3000 mL ethyl acetate for 3 times. The L-trp was crystallized from the raffinate and the extract was evaporated under reduced pressure to afford D-tryptophan amide. The product, L-Trp, was characterized by ^1H NMR, LC–MS, FT-IR spectroscopy and ^{13}C NMR. Optical purity of the product was determined by chiral high-performance liquid chromatography.

2.5. HPLC analysis

The levels of the tryptophan amides and Trp were determined by high-performance liquid chromatography (HPLC) (U3000, Dionex, USA) with a C_{18} column (250 × 4.6 mm, Dionex). The enantioseparation of L-Trp was determined by HPLC with a CHIROBIOTIC column (250 × 4.6 mm, Astec, USA).

3. Results and discussion

3.1. Selection of organic solvents and concentration of materials to be added to the reaction mixture

There have been several reports in the literature demonstrating that the addition of organic solvents can enhance the enantioselectivity and activity of biocatalytic process, especially in hydrolase (i.e. lipase, esterase and acylase) catalyzed reactions [1,15,16]. In this work, the effects of six different organic solvents on the catalytic activity and enantioselectivity of the amidase were initially examined at a concentration of 20% (v/v). As shown in Fig. 1A, the addition of ethyl acetate, cyclohexane and methyl tert-butyl ether (MTBE) significantly enhanced the enzyme activity. In particular, the enzyme activity of the amidase was enhanced to 688.83 U/g in the presence of ethyl acetate, which was 4.7 times greater than that of the neat aqueous buffer (146.61 U/g). The conversion of the substrate was also enhanced by the addition of ethyl acetate or MTBE to 49.66 or 48.83%, respectively, from a value of 45.26% for the neat aqueous buffer. The amidase from *F. aquatile* ZJB-09211 exhibited excellent levels of enantioselectivity in all of the reaction systems that we tested, providing an enantiomeric excess value (*e.e.*) greater than 99.95% in all of the test experiment.

Organic solvents at a low concentration typically reduce the mass-transfer resistance of resting cells, whereas highly concentrated organic solvents show inhibitory effects. As depicted in Fig. 1B, the amidase activity (732.89 U/g) and the conversion of substrate (49.99%) were high even when the concentration of ethyl acetate was increased up to 30% (v/v). In contrast, however, the levels of amidase activity and substrate conversion were significantly reduced when the concentration of ethyl acetate exceeded 30% (v/v). Interestingly, the amidase from *F. aquatile* ZJB-09211 exhibited excellent levels of enantioselectivity across all of the concentrations tested. The results clearly indicated that a concentration of 30% (v/v) ethyl acetate produced the most beneficial effects on the free cell activity and the product yield.

3.2. Effect of the buffer pH value on the enzyme-catalyzed resolution in a two-phase system

In this paper, we studied the effect of the pH value using two different buffers, including a phosphate buffer (6.0–8.0) (Fig. 2A) and a Tris–HCl buffer (pH value 7.0–9.0) (Fig. 2B), instead of using distilled water as the aqueous phase. The results indicated that the use of Tris–HCl buffer was significantly better than phosphate buffer as the aqueous phase across all of the pH values tested. Furthermore, the optimal pH value for the activity of the enzyme was found to 7.5 irrespective of the buffer used. The maximum enzyme activity and level of conversion of the reaction following 1 h were 3732.52 U/g and 49.95%, respectively (Fig. 2B). During the study, we also found that the selectivity of the amidase was independent of the buffer and pH value used, and that the enantiomeric excess value was more than 99.95% in all cases.

3.3. Optimization of the concentration of tryptophan amides on the enzyme-catalyzed biotransformation in the two-phase reaction system

Higher concentrations of the tryptophan amides accelerated the rate of the reaction from the perspective of the chemical equilibrium, although the low solubility of the tryptophan amide could lead to the inhibition of the enzymatic reaction process. The addition of an organic solvent can greatly improve the solubility of the tryptophan amide in the reaction system and potentially avoid these issues. To identify an optimal tryptophan amide concentration, the effects of the tryptophan amide concentration (20–200 mM) on the reaction were investigated in detail (Fig. S1). Increasing the concentration of the tryptophan amides from a low concentration (≤80 mM) led to significant increases in the activity of the amidase enzyme to a maximum (1398.74 U/g) at 80 mM. Further increases in the concentration of the tryptophan amides (>80 mM) led to a reduction on the enzyme activity of the amidase. Interestingly, however, even at a concentration of 200 mM, the enzyme activity (949.53 U/g) was still higher than the activity observed in the pure aqueous buffer.

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