Asymmetric Synthesis of (S)-α-Methylbenzylamine by Recombinant Escherichia coli Co-Expressing Omega-Transaminase and Acetolactate Synthase

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To produce (S)-α-methylbenzylamine (MBA) from acetophenone, recombinant Escherichia coli co-expressing ω-transaminase and acetolactate synthase was used as a whole-cell biocatalyst. The solvent-bridge reaction system increased the yield of the whole-cell reaction by 2.5-fold, and the inhibitory (S)-α-MBA produced in the ω-transaminase reaction solution (pH 8.0) moved into the extraction solution (pH 3.0) via an organic solvent.

Key words: acetolactate synthase; asymmetric synthesis; chiral amines; ω-transaminase

Transaminase (TA) has been studied extensively due to its potential to produce amino acids and chiral amines in biosystems.¹) TAs play important roles in amino acid metabolism and are ubiquitous in microorganisms and eukaryotic cells. The TA reaction has some features superior to other enzymes such as hydrodase and dehydrogenase, including broad substrate specificity, high enantioselectivity, and high turnover number, as well as no requirement for external cofactor regeneration.²) TAs belonging to subgroup III are called ω-TAs, and include ω-amino acid:pyruvate TA, ornithine TA, 4-aminobutyrate TA, and others.³) Unlike α-TA, ω-TA can transfer an amino group from a non-α position amino acid (such as 4-aminobutyrate) or an amine compound with no carboxylic group (such as α-MBA) to an amino acceptor (such as 2-ketoglutarate or pyruvate).³)

There are many biotechnological applications of ω-TA, and successful examples in which enantiomerically pure (R)-form compounds were synthesized via ω-TA-mediated kinetic resolution.⁴,⁵) Asymmetric synthesis of chiral amines with ω-TA (the reverse reaction of kinetic resolution) has many potential advantages over the forward reaction, including a two-fold higher theoretical yield and the ability to yield an enantiomerically pure (S)-form. Despite these properties, efficient asymmetric synthesis from ketone compounds with ω-TA has not been studied as extensively as kinetic resolution.

Asymmetric synthesis reactions are more difficult to carry out than kinetic resolution reactions due to unfavorable thermodynamic equilibrium and much more severe product inhibition. A typical example is the amination of acetophenone with alanine to produce (S)-α-MBA, where the equilibrium constant of amination is 8.81 × 10⁻⁴, and (S)-α-MBA and pyruvate are much more reactive transamination substrates than acetophene or l-alanine.⁷) Hence at least one of the products must be removed during the reaction to shift the equilibrium and reduce product inhibition.⁸) We have reported that the synthetic yield of a whole-cell reaction using Vibrio fluvialis JS17, in which pyruvate was removed by cellular metabolism, was much higher than that of the cell-free reaction.⁷)

In this study, we constructed a recombinant E. coli co-expressing ω-TA and acetolactate synthase (ALS). ALS catalyses the formation of one molecule of acetolactate from two molecules of pyruvate, and acetolactate is spontaneously decomposed to acetoin.⁹) We hypothesized that the ALS would aid in removing the inhibitory pyruvate by-product and hence shift the ω-TA reaction equilibrium further towards the asymmetric reaction (Fig. 1A). The structural gene of otaA was amplified from genomic DNA of V. fluvialis JS17 by PCR using TA-f (5'-AAAAACATATGAAACAAA-CCGCAAAGCTTG-3') and TA-r (5'-TTTTTTGGAT-CCTCAGGCAACCTCGGCAAAGAC-3') primers, and ligated into pET15b (Novagen, Darmstadt, Germany).³) The structural gene of alsS was amplified from genomic DNA of Bacillus subtilis 168 by PCR using ALS-f (5'-AAAAAAGGAATCCATGGACAAAAAGCAACAAAAAG-3') and ALS-r (5'-CCCAACTCGAGC-TAGAGCTTCTGTTTATGAG-3') primers, and ligated into pET24ma.¹⁰)

To obtain the recombinant strain (ω-TA/ALS cell) harboring the ω-TA and ALS genes, plasmids pET24ma-

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Abbreviations: ALS, acetolactate synthase; Kp, partition coefficient; MBA, methylbenzylamine; TA, transaminase
alsS were transformed into recombinant E. coli BL21/pET15b-alsS (ω-TA cell). Enzyme induction and preparation followed methods described elsewhere. Three strains of recombinant E. coli BL21 were tested (Table 1). The ω-TA activity of recombinant E. coli harboring the alsS gene is apparently capable of enhancing recombinant protein productivity, since ALS channels excess carbon to acetoin, a product that is much less toxic than acetate.

Table 1. The ω-TA and ALS Activities of Recombinant E. coli

<table>
<thead>
<tr>
<th>Strains</th>
<th>ω-TA activity (U/mg of DCW)</th>
<th>ALS activity (U/mg of DCW)</th>
</tr>
</thead>
<tbody>
<tr>
<td>E. coli BL21</td>
<td>2.5</td>
<td>0.08</td>
</tr>
<tr>
<td>ω-TA cells</td>
<td>0.08</td>
<td>0.08</td>
</tr>
<tr>
<td>ω-TA/ALS cells</td>
<td>5.5</td>
<td>0.62</td>
</tr>
</tbody>
</table>

One unit of ω-TA was defined as the amount that catalyzes the formation of 1 μmol of acetophenone in 1 min from 50 mM (S)-α-MBA and 50 mM pyruvate at 37 °C. One unit of ALS was defined as the amount that depletes 1 μmol of pyruvate in 1 min from 100 mM pyruvate at 37 °C in 100 mM phosphate buffer (pH 7.0).

The ω-TA/ALS whole-cell reaction was about 20-fold higher than that of the crude-extract enzyme reaction (0.17 mM; Fig. 1B). The initial rate of the ω-TA/ALS whole-cell reaction (31 μM/min) was higher than that of the ω-TA whole-cell reaction (17 μM/min), and the final pyruvate concentrations in respective reaction solutions were <0.05 and 0.2 mM. However, the synthetic yield (3.2 mM) of the ω-TA/ALS whole-cell reaction increased only 1.2-fold over that of the ω-TA whole-cell reaction (2.75 mM) (Fig. 1B).

We examined the stability of ω-TA within the whole cells in buffer solution, reaction solution, and reaction solution with 1 mM PLP, all at 37 °C. The ω-TA/ALS whole-cell reaction was calculated by fitting the initial rate data to the first-order inactivation equation. In the presence of 10 mM acetophenone and 100 mM alanine, the half-life of the enzyme (2.3 h) dramatically decreased as compared to that of the enzyme (10.1 h) in 100 mM phosphate buffer (pH 7.0). Although PLP is tightly bound to its apoenzyme, it helps to maintain the stability of the enzyme.

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The organic solvent-bridge reaction system consisted of 4 ml reaction solution (80 U/ml whole cells, 200 mM alanine, and 100 mM phosphate buffer, (pH 8.0)), 1 ml isooctane, and 4 ml extraction solution (200 mM acetate buffer, (pH 3.0)). Subsequently, we added 90 μmol of acetophenone (overall concentration, 10 mM) to the isooctane at 37 °C. The initial concentrations of acetophenone in the reaction, isooctane, and extraction solutions were 4.3, 55.5, and 4.3 mM respectively, as calculated from the partition coefficient of acetophenone (12.9). After a 15-h reaction, 15.5 mM (S)-α-MBA was present in the extract solution, and 0.1 mM amine was present in the reaction phase. Overall, we obtained 62.4 μmol of (S)-α-MBA from 90 μmol of acetophenone, representing a synthetic yield of 69%. The synthetic yield of (S)-α-MBA increased about 2.5-fold over that in the aqueous whole-cell reaction (Fig. 1B). Asymmetric synthesis was carried out with α-TA cells (80 U/ml) in the solvent-bridge reaction system under the conditions described above, except that α-TA/ALS cells were replaced with α-TA cells. After a 15-h reaction, 8.5 mM (S)-α-MBA was present in the extract solution, and 0.15 mM amine was present in the reaction phase. Overall, we obtained 34.6 μmol of (S)-α-MBA from 90 μmol of acetophenone, representing a synthetic yield of 38%. The synthetic yield of the reaction with α-TA/ALS cells was about 1.8-fold higher than that of the reaction with α-TA cells in the solvent-bridge reaction system. When we used cyclohexanone as an organic solvent in the solvent-bridge system with α-TA/ALS cells, the reaction produced 40.4 μmol of (S)-α-MBA.

In conclusion, we used a novel recombinant E. coli co-expressing α-TA and ALS as a whole-cells biocatalyst and the recombinant enzymes carried out (S)-α-MBA production (>99%) from acetophenone and l-alanine and inhibitory pyruvate removal respectively. We employed a solvent-bridge reaction to overcome product inhibition by (S)-α-MBA. This system led to a 2.5-fold increase in the (S)-α-MBA synthetic yield. Currently, for the large-scale production of chiral amines, the feasibility of the solvent-bridge reaction system is under study with development of a novel reactor and further optimization of reaction condition.

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References


