

An Aminotransferase from *Enhydrobacter aerosaccus* to Obtain Optically Pure β -Phenylalanine

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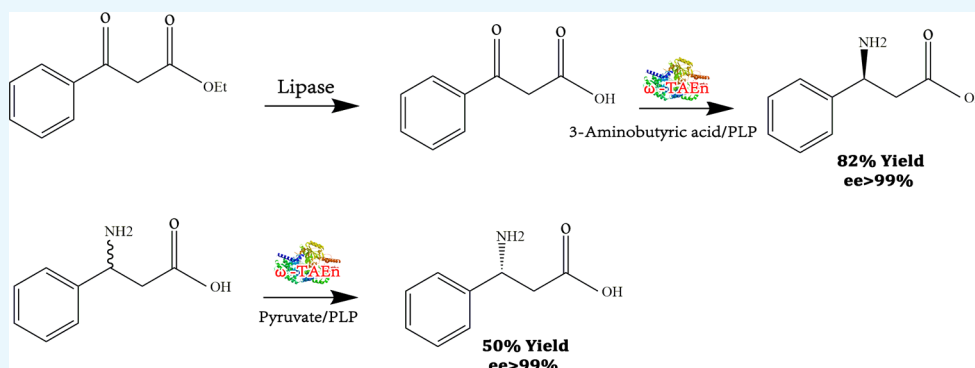
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ABSTRACT: An aminotransferase ω -TAEn was identified from *Enhydrobacter aerosaccus*. The ω -TAEn was successfully expressed in *Escherichia coli* and the obtained enzyme showed activity toward β -phenylalanine (β -phe) at optimal conditions. For optically pure (R)- β -phe, 50% yield was observed by kinetic resolution of racemic amino with pyruvate as the amino acceptor. To obtain (S)- β -phe, the lipase/ ω -TAEn catalytic system was adopted. The ω -TAEn showed strict stereoselectivity to the amino donor. The formation of (S)- β -phe was observed using 3-aminobutyric acid as the amino donor, and (S)- β -phe was obtained by asymmetric synthesis with a yield of 82%.

1. INTRODUCTION

β -Amino acids are very important chiral compounds for the synthesis of some physiologically active compounds such as peptides, peptidomimetics.^{1,2} Meanwhile, β -amino acids are interesting nonprotein amino acids since the β -amino acid peptide bonds show high stability toward protease hydrolysis, making it available for biochemical research.^{3,4} β -Phenylalanine (β -phe) is an important chiral building block for the synthesis of various pharmaceuticals, such as the antidiabetic compound sitagliptin and the anticancer drug taxol, which form the chiral center of these chiral drugs.^{5,6} It is worth mentioning that the enantiomeric purity of the compound has important effects on pharmacological activity.

Until now, methods for obtaining enantiomerically pure β -amino acids are divided into chemical synthesis, physical separation, and enzymatic catalysis. Metal catalysis and organocatalysis have been used for chemical synthesis, while expensive equipment is needed in physical separation.⁷ By contrast, enzymatic catalysis attracts more attention due to the simplicity in inserting chirality and selectivity with regard to regio- and stereochemistry in molecules.⁸ Different enzymes and various strategies have been employed to get β -amino acid.^{9–15} Among these methods, ω -transaminase (ω -TA) is one of the most prominent approaches due to high stereoselectivity and self-regenerating cofactors.^{16,17} Further-

more, the ω -TA can be applied to kinetic resolution of racemic amino acids or asymmetric synthesis of optically pure amines.^{18,19} Although some excellent ω -TAs have been reported, only a few have showed the activity to aromatic β -amino acids. Some novel ω -TAs have been reported most recently; however, the asymmetric synthesis of β -phe by these enzymes showed a low conversion.^{20–22} In this study, a new ω -TA was explored and named ω -TAEn, which was cloned, expressed, and characterized. Meanwhile, (S)- β -phe and (R)- β -phe were respectively obtained by using the ω -TAEn.

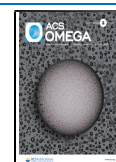
2. RESULTS AND DISCUSSION

2.1. Selection of the ω -TA Gene. A candidate protein sequence (UniPort entry UPI000A2F0B05) from *Enhydrobacter aerosaccus* (ω -TAEn), which encodes 498 amino acids, was used for the transamination to (S)- β -phe. ω -TAEn has a high degree of homology with previously reported ω -TAs from

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Mesorhizobium sp. LUK (52.45% identity), *Variovorax paradoxus* (52.4% identity), and *Burkholderia graminis* (54.02% identity) that can catalyze the ammonia transfer reaction of aromatic amino acid.^{20,23,24} To reconfirm the feature of ω -TAEn, the protein sequence was aligned with ω -transaminase from *Mesorhizobium* sp. strain LUK. K332 is where the cofactor PLP is reversibly bound to the enzyme through a Schiff-base linkage, and the chemical binding site of pyridoxal 5'-phosphate binding pocket (G191, T192, N195, E272, D305, M308, and G331) is also conserved in the new protein sequence.²¹ Since the sequence of ω -TAEn is only 50% identical compared to other sequences, in addition to the conserved sequence of ω -TA, differences in other amino acid positions may cause its unique catalytic properties. To express the ω -TAEn, the artificial gene was optimized with an N-terminal His-tag sequence in consideration of codon usage pattern in *Escherichia coli* cells.

2.2. Expression and Purification of the ω -TA. The ω -TAEn, mainly presented as a soluble protein, was expressed in *E. coli* BL21(DE3) and isolated from the crude cell extracts. Then, the ω -TAEn was confirmed by SDS-PAGE (see the Supporting Information). SDS-PAGE showed a single band between 46 and 58 kDa.

2.3. Effect of Temperature and pH. The effect of temperature and pH on ω -TAEn activity was examined, and the results are shown in Figure 1. The maximum activity was observed between 50 and 55 °C. The activity of ω -TAEn decreased sharply when the temperature was above 55 °C, and the relative activity at 65 °C dropped to 17% of that at 50 °C. The effect of pH on ω -TAEn was investigated at 50 °C, and the maximum reaction rate was observed at pH 8.0. This means that ω -TAEn preferred weak alkaline conditions, which is consistent with most ω -TAs that have been reported.^{24,25} This might be due to easier formation of the imine structure between the PLP and the enzyme at an alkaline environment. ω -TAEn showed different catalytic efficiencies at pH 8.0 in different reaction buffers, which was also observed on ω -TA from *B. graminis*. Unlike reported cases, ω -TAEn was more sensitive to pH as no catalytic activity was detected at pH 7.5 in Tris-HCl buffer.

2.4. Effect of Organic Solvent and Metal Ion. Considering the addition of the solvent in the industrial catalysis, the effect of organic solvent on the enzyme activity was examined (Figure 2). Methyl alcohol inhibited the enzyme activity more obviously, and 20% methanol caused over 80% activity loss. DMSO was gentler on the ω -TAEn. Hence, DMSO is a more suitable solvent for the production of chiral amines due to the poor solubility of the prochirality substrate in water.

The effect of six different metal ions on the enzyme was also investigated. The ω -TAEn shows a higher catalytic activity when it coexists with Ca^{2+} or Mg^{2+} ions. The metal ions Cu^{2+} and Zn^{2+} obviously inhibited the relative activities. The relative activity with addition of Zn^{2+} was less than a fifth of the control. The results indicated that heavy metal ions could be the inhibitors of the ω -TAEn. Previously, Gao et al. reported a new (*R*)-selective amine transaminase from *Fusarium oxysporum* that can be promoted by Zn^{2+} and Fe^{3+} while being inhibited by Mg^{2+} ,²⁶ which seems like the same metal ion has an opposite effect on different selective enzymes.

2.5. Enzymatic Thermal Stability. The effect of temperature on stability of the ω -TAEn was investigated (Figure 3). ω -TAEn was stable after 10 min of incubation at 55 °C, while

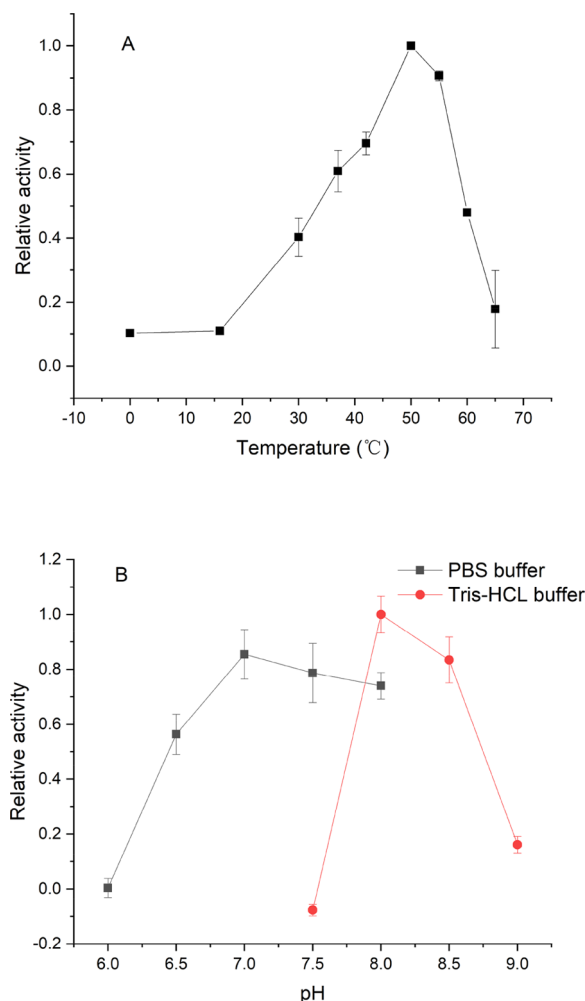


Figure 1. Effect of (A) temperature and (B) pH on the enzyme activity. The reaction was carried out in 100 mM Tris-HCl buffer containing 0.1 mM PLP, 0.025 mg/mL ω -TAEn, 10% (v/v) organic solvent, 20 mM *rac*- β -phe, and 20 mM pyruvate.

it only displayed 33% of the maximum activity at 65 °C. On the whole, ω -TAEn preferred a mild reaction condition. The interesting thing is that, when different reactants are added to the incubation process, ω -TAEn shows different thermal stabilities. At 55 °C, ω -TAEn shows reasonable stability while being incubated with PLP and pyruvic acid, and very weak catalysis with PLP and β -phe, otherwise, almost inactivated it after incubation. It seems that the PLP and pyruvic acid protect the enzyme from heat damage. Cerioli et al. confirmed that the additives can affect the enzyme storage stability.²⁷ This phenomenon is presumed to be due to the protection of active sites by PLP and pyruvic acid.

2.6. Kinetic Parameters of ω -TAEn. To determine the kinetic parameters of ω -TAEn, the initial reaction rate was detected (Figure 4). K_m and K_{cat} values of ω -TAEn to β -phe in the presence of 10 mM pyruvate were 0.70 mM and 24.15 min^{-1} , respectively. The relative smaller K_m value of ω -TAEn than those of many other ω -TAs suggests that this enzyme has a more promising potential for industrial applications.^{28,29} When the substrate reached 4 mM, the inhibition effect was observed. This was consistent with the ω -transaminase from *Mesorhizobium* sp. strain LUK, while the substrate inhibition

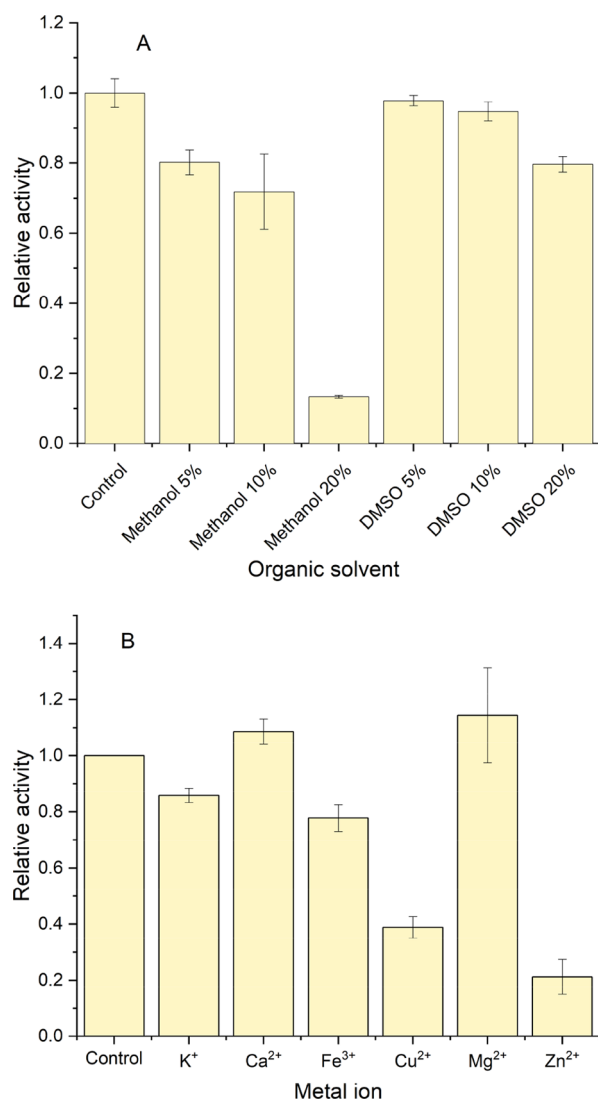


Figure 2. Effect of (A) organic solvent and (B) metal ion on enzyme activity. (A) The reaction was carried out in 100 mM Tris–HCl buffer containing 0.1 mM PLP, 0.025 mg/mL ω -TAEn, 20 mM *rac*- β -phe, and 20 mM pyruvate at 50 °C for 50 min. (B) The enzymatic reactions of ω -TA were performed in the presence of a metal ion (1 mM) for 50 min.

constant of 3.2 mM was observed in the presence of 10 mM pyruvate.²⁰

2.7. Chiral Resolution of *rac*- β -Phe. To establish the method for obtaining optically pure β -phe, ω -TAEn was used to get (*R*)- β -phe by chiral resolution of *rac*- β -phe. *rac*- β -Phe (10 mM) was completely resolved into (*R*)- β -phe (ee > 99) by ω -TAEn with 20 mM pyruvic acid as the amino acceptor during 4 h of incubation (Figure 5). The new ω -TAEn can withstand a higher catalytic temperature, which makes it more suitable to the industrial processes.

2.8. Asymmetric Synthesis of (*S*)- β -Phe. For asymmetric synthesis of (*S*)- β -phe, DMSO was added to increase the solubility of the substrate. The main obstacle to asymmetric synthesis of (*S*)- β -phe is its substrate β -phenylpyruvic acid, which is unstable due to the decarboxylation to produce acetophenone. Considering the stability of the substrate, the asymmetric synthesis process was carried out at 37 °C. Summarizing the previous research studies, L-alanine is commonly used as the amino donor for asymmetric syn-

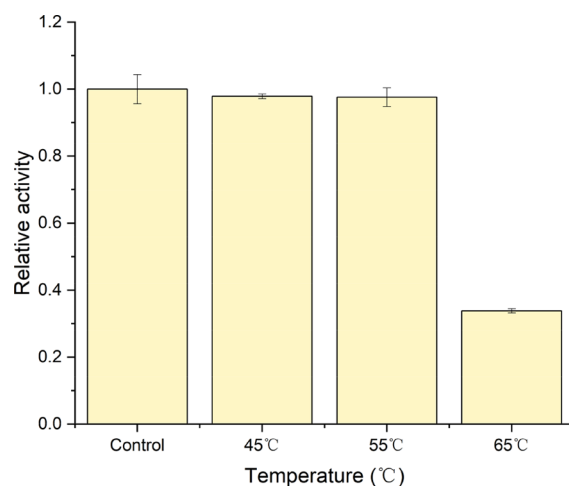


Figure 3. Enzymatic thermal stability. The enzyme was incubated at a specific temperature for 10 min in Tris buffer (100 mM, pH 8) before the reaction.

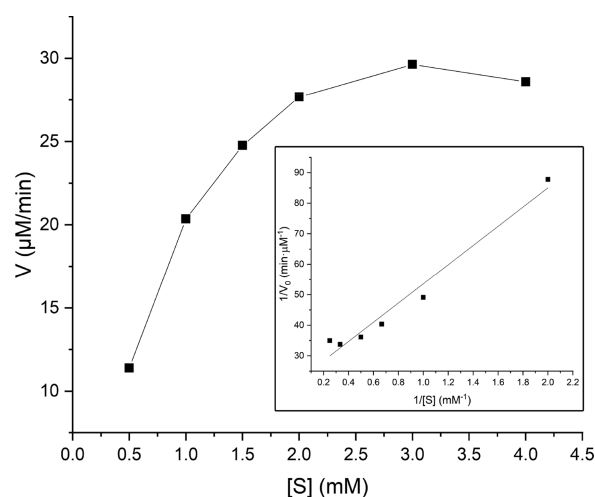


Figure 4. Michaelis–Menten plot of the reaction of ω -TAEn on *rac*- β -phe. The enzyme reaction for Michaelis–Menten plot was carried out in a reaction volume of 1 mL containing 10 mM pyruvate, *rac*- β -phe (0–4 mM), enzyme (0.025 mg/mL), and 100 mM Tris–HCl buffer (pH 8.0), and the mixture was incubated at 37 °C for 5 min.

thesis.^{20,30} The production of β -phe was not detected when using L-alanine as the amino donor, which could be due to the low activity. ω -TAEn can catalyze the formation of 2.7 mM (0.45 g/L) optically pure β -phe with 3-aminobutyric acid as the amino donor. It is difficult to have a precise quantification of the conversion, but we had measured acetophenone, the by-product, up to 4.08 mM (0.49 g/L) after 10 h.

Kim et al. combined lipase and ω -TA using ethyl benzoylacetate as an indirect substrate to reduce the loss due to decarboxylation, but the ω -TA showed a low conversion.²⁰ Then, we attempted to take this strategy. The lipase from *Candida rugosa* was used to hydrolyze ethyl phenylpyruvate. We tried to find out if the temperature can promote the reaction process because the ω -TA showed the highest activity at 50 °C. The results shows that, after 12 h of incubation, 2.74 mM (0.54 g/L) (*S*)- β -phe was obtained at 37 °C, at least 5-folds than that at 55 °C. The high temperature caused the increases in decomposition rate of the β -phe, although it lowered the hydrolysis rate of lipase. The maximum output of

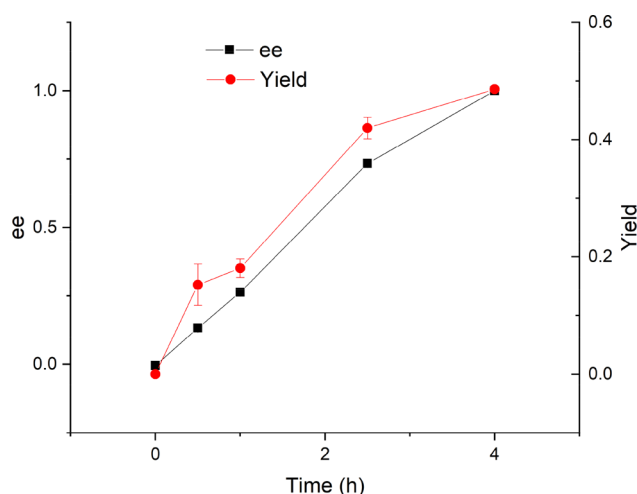


Figure 5. Reaction profile for ω -TAEn catalyzed by chiral resolution of rac - β -phe. The enzyme reaction was carried out in a reaction volume of 1 mL of Tris–HCl buffer (100 mM, pH 8.0) containing pyruvate (20 mM), rac - β -phe (10 mM), enzyme (0.045 mg/mL), and DMSO (10%, v/v), and the mixture was incubated at 50 °C.

(S)- β -phe was obtained up to 0.61 g/L at 12 h with the combination of lipase (1 mg/mL) and ω -TAEn (0.09 mg/mL) (Figure 6). The conversion rate was 40%.

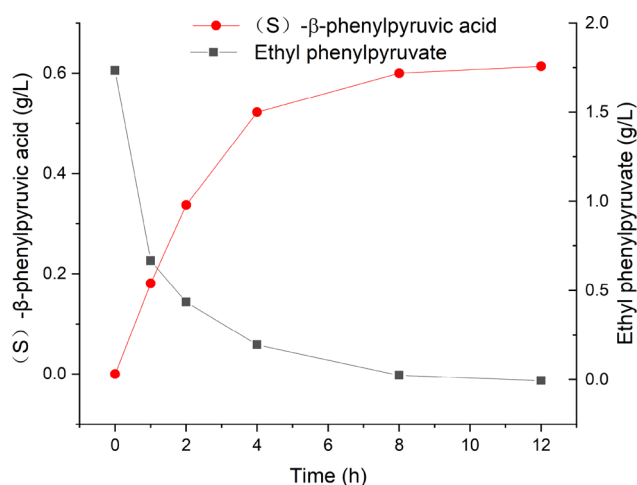


Figure 6. Asymmetric synthesis of (S)- β -phe by ω -TAEn. The reaction was carried out in Tris–HCl buffer (100 mM, pH 8) containing ethyl phenylpyruvate (10 mM), PLP (0.1 mM), DMSO (5%, v/v), DL-3-aminobutyric acid (80 mM), lipase (1 mg/mL), and ω -TA (0.045 mg/mL) at 37 °C.

Based on previous research studies, it seems that the amino donor makes a huge contribution to the conversion rate.²⁹ To get higher yields, different kinds of amino compounds were tried as an alternative to 3-aminobutyric acid. According to a previous research study, nine commonly used compounds

containing five rac -amino acids (DL-3-aminobutyric acid, DL-serine, DL-valine, DL-norleucine, and DL-glutamic acid) and four achirality amines (isopropamide, glycine, γ -aminobutyric acid, and benzylamine) were carried out with ethyl phenylpyruvate (10 mM).³¹ The (S)- β -phe was not detected while utilizing L-3-aminobutyric acid or other compounds as the amino donor, which reaffirms that idea that the ω -TAEn has high stereoselectivity to the substrate. A similar result was shown on ω -TA from *Mesorhizobium* sp. strain LUK. Previously, Mathew et al. increased the output by increasing the concentration of the amino donor ((S)- α -methylbenzylamine), but 100 mM of the amino donor inhibited the generation of products.²⁸ Then, the concentration of the amino donor was adjusted from 30 to 600 mM with 10 mM ethyl phenylpyruvate (see the Supporting Information). As expected, the increase in amino donor concentration had a direct impact on the production of (S)- β -phe. Different from the reports, the enzyme's inhibition toward the amino donor was not observed when utilizing 3-aminobutyric acid. The yield was up to 82% (Table 1), which was three times higher than the earlier report (20% yield from the 10 mM substrate).²⁰

3. CONCLUSIONS

In this study, a candidate protein sequence from *E. aerosaccus* was obtained through gene mining, the corresponding gene sequence was optimized and expressed in *E. coli*, and the acquired protein was biochemically characterized. The enzyme assays revealed the aminotransferase activity to β -amino acids, and the novel ω -TA showed high stereoselectivity and activity toward (S)- β -phe. Consistent with previous reports, the enzyme prefers alkaline environments, and the optimum temperature is about 50 °C. In this optimal condition, 10 mM rac - β -phe was successfully dissected asymmetrically to produce enantiomeric pure (R)- β -phe in the presence of an excess amount of amino acceptor within 4 h. In the resolution reaction, the generated β -phenylpyruvic acid decomposes itself under the reaction conditions, which promotes the normal progress of the reaction to obtain a high conversion rate. The asymmetric synthesis of enantiomeric pure (S)- β -phe was also performed utilizing the enzyme, and 0.91 g/L (S)- β -phe was synthesized from ethyl phenylpyruvate (10 mM) with 82% conversion rate by the way in which the ω -TA was linked to the lipase. Two reasons lead to the higher product yields: one is that the higher amino donor concentration promotes the forward direction of the reaction, and the other is due to the reduction of β -phenylpyruvic acid loss. In summary, the novel ω -TA can act as a valuable enzyme for the kinetic resolution of racemic β -phe and the asymmetric synthesis of (S)- β -phe.

4. EXPERIMENTAL SECTION

4.1. Materials. Isopropyl β -D-thiogalactopyranoside (IPTG), pyridoxal 5'-phosphate (PLP), dimethyl sulfoxide (DMSO), methanol, (S)-phenylalanine, rac -3-amino-3-phenylpropionic acid, pyruvate, 3-aminobutyric acid, and L-alanine

Table 1. Comparison of Different Transaminases in the Coupling Reaction with Lipase to Synthesize Asymmetrical β -Phe

enzyme	source	yield	enantioselectivity	ref
ω -TAIC	<i>Ilumatobacter coccineus</i>	44.20%	>99%	22
Ms- β TA	<i>Mesorhizobium</i> sp. LUK	20%		20
ω -TAPo	<i>Polaromonas</i> sp. JS666	52%	>99%	29
ω -TAEn	<i>E. aerosaccus</i>	82%	>99%	this study

were obtained from commercial corporations (Sigma-Aldrich, Aladdin, Adamas, etc.). *E. coli* DH5 α and BL21(DE3) were used as the hosts of cloning and heterologous expression. Plasmid pETDuet-1 was used as the expression vector.

4.2. Selection of the ω -TA Gene. The ω -TAPo from *Polaromonas* sp. JS666, which has been reported for showing activity toward aromatic β -amino acid, was used as the template to find new ω -TAs.²⁹ According to the BLASTP search in NCBI (<https://www.ncbi.nlm.nih.gov/>), a new protein sequence (WP_085935911.1) from *E. aerossaccus* (Strain ATCC 27094), which showed 86% similarity and 56% identity to the ω -TAPo, was selected as the candidate protein.

4.3. Construction of Plasmid and Expression of ω -TAs. The optimized gene sequence was synthesized by the Beijing Genomics Institute with pUCS7 (pUCS7- ω -TAEn) between the *Bam*HI and *Hind*III restriction sites. The purpose gene was connected to pETDuet-1 with the corresponding sites to form the gene expression vector. The plasmids were transformed into *E. coli* BL21, and the cultivated strain was screened by colony PCR using primers pETUP1 (ATGCGTCCGGCGTAGA) and T7-Terminator (ATGCGTCCGGCGTAGA).

The resulting strains were cultured in the LB culture medium. When the OD₆₀₀ reached about 0.6–0.8, IPTG (0.6 mM) was added. After 8 h of induced expression at 30 °C, the cells were harvested at 5000 rpm for 5 min at 4 °C. Cell fragmentation was achieved by constant cell disruption systems (Constant Systems) at 30 kpsi. The supernatant was purified on a Ni-NTA agarose resin obtained from Beyotime Biotechnology.

4.4. Enzyme Assays. The reaction system for detecting the specific activity of the ω -TA was composed of 1 mL of Tris–HCl buffer (0.1 M, pH 8.0) at 30 °C containing PLP (0.1 mM), *rac*- β -phe (10 mM), pyruvate (20 mM), and enzyme (0.025 mg/mL). The enzyme assay was stopped after 50 min by a water bath at 95 °C. The evaluation of enzymatic reactions was achieved by testing the generated alanine or consumed β -phe. All these experiments were done in triplicate.

4.5. Effect of Temperature, pH, Organic Solvent, and Metal Ion. To explore the impact of temperature, pH, and organic solvents on the ω -TA, an enzyme assay was carried out at various temperatures from 0 °C (ice bath) to 60 °C, in different pH buffers (phosphate buffer, pH 6–8; Tris–HCl buffer, pH 7.5–9), and at different organic solvent environments (methyl alcohol and dimethyl sulfoxide). To analyze the effect of metal ions on ω -TA, different kinds of metal ions (1 mM) were added to the reaction system. Relative activity of 100% is defined as the highest rate of alanine formation under each condition to be optimized.

4.6. Enzymatic Thermal Stability. To investigate the thermal stability of ω -TA, the enzyme was incubated at a specific temperature (45, 55, and 65 °C) for 10 min. Then, 10 mM *rac*- β -phe was added to start the reaction. The remaining activity was determined 50 min later.

4.7. Kinetic Resolution of Racemic Amines. For kinetic resolution of racemic amines, the enzyme assay was carried out in 1 mL of Tris–HCl buffer containing 10 mM pyruvate as the amino acceptor and 0.1 mM PLP, and the initial rates were detected at various substrate concentrations from 0.5 to 4 mM.

4.8. Chiral Synthesis of (S)- β -Phe. To investigate the ability to synthesize asymmetrical β -phe, β -phenylpyruvic acid, the direct substrate of ω -TA, was obtained from ethyl

phenylpyruvate (see the Supporting Information). Asymmetric synthesis of (S)- β -phe was also carried out through a combination of lipase and ω -TA. Lipase (1 mg/mL) and ω -TA (0.045 mg/mL) were added in 1 mL of Tris–HCl buffer containing ethyl phenylpyruvate (10 mM), PLP (0.1 mM), amino donor, and 5% DMSO as the cosolvent. The enzyme assay was carried out at 50 and 37 °C for 12 h, and generation of β -phe was detected.

4.9. Analytical Conditions. The β -phe was detected by HPLC using a Crownpak CR-I column (3 mm \times 150 mm, Daicel Co., Japan) at 210 nm with isocratic elution of perchloric acid solution (pH 1)/methanol (20/80, v/v) at 0.1 mL/min. The alanine was detected using an OA-5000 column (4.6 mm \times 150 mm, SUMICHIRAL Co., Japan) at 245 nm with an elution of copper sulfate solution (2 mM)/acetonitrile (90/10, v/v) at 1 mL/min. All HPLC detections were carried out at room temperature.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at <https://pubs.acs.org/doi/10.1021/acsomega.9b03416>.

Nucleotide sequence of ω -TAEn, details of β -phenylpyruvic acid preparation, graph of the SDS-PAGE of ω -TAEn, graph of temperature effect on the tandem reaction between lipase and ω -TAEn, graph of asymmetric synthesis of (S)- β -phe with different 3-aminobutyric acid concentrations, HPLC diagram of the *rac*- β -phe sample, and amino acid alignment among the ω -TAEn and other related proteins (PDF)

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Notes

The authors declare no competing financial interest.

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