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Efficient access to L-phenylglycine using a newly identified amino acid dehydrogenase from *Bacillus clausii*†

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An amino acid dehydrogenase from *Bacillus clausii* (*Bc*AADH) was identified and overexpressed in *Escherichia coli* BL21(DE3) for the preparation of L-phenylglycine from benzoylformic acid. Recombinant *Bc*AADH was purified to homogeneity and characterized. *Bc*AADH could catalyse reductive amination and oxidative deamination at optimum pHs of 9.5 and 10.5. Furthermore, *Bc*AADH has a broad substrate spectrum, displaying activities toward various aromatic and aliphatic keto acids. When coexpressed with glucose dehydrogenase from *Bacillus megaterium*, the potential application of *Bc*AADH in the preparation of L-phenylglycine was investigated at a high substrate loading and low biocatalyst addition. As much as 400 mM benzoylformic acid could be fully reduced into L-phenylglycine within 6 h at >99.9% *ee*. With merely 0.5 g DCW L^{-1}, 200 mM benzoylformic acid was completely reduced, resulting in a substrate to biocatalyst ratio of 60 g g^{-1}, environmental factor of 4.7 and 91.7% isolation yield at gram scale. This study provides guidance for the application of *Bc*AADH in the synthesis of chiral non-natural amino acids.

Introduction

Optically active non-natural amino acids are important compounds, and are widely used as vital building blocks for pharmaceuticals and agrochemicals.[1–4] Due to their multifunctional structure, non-natural amino acids could also be applied in the synthesis of structural motifs of peptides and peptidomimetics.[5–7] There has been an increasing demand for the non-natural amino acids, especially L-phenylglycine.[8] Ascribing this to their various functional groups (aromatic, carboxylic and chiral amine groups), chiral L-phenylglycine and derivatives are important blocks of antibiotics such as penicillin,[9] pristinamycin I,[10] and the antitumor Taxol,[11] antiplatelet inhibitors Clopidogrel.[12]

Various chemical routes have been developed for the synthesis of non-natural amino acids using asymmetric reactions. However, the utilization of environmentally harmful organic solvents and toxic reagents such as cyanides are disadvantageous for industrial production.[13,14] Moreover, the relatively low enantioselectivity could not satisfy the optical purity requirement for pharmaceuticals.[15] In comparison, biological methods, using microbes and enantioselective enzymes, have also been established and are preferable for the production of non-natural amino acid.[15,16] Through metabolic engineering and pathway recombination, L-phenylalanine and L-phenylglycine could be produced through fermentation.[17] However, the metabolic pathways is complex which might influence the normal growth of microbes, and the product L-phenylglycine is hard to be recovered from natural α-amino acids.[18,19] Enzymatic synthesis approach is a green alternative for the preparation of optically active non-natural amino acids considering its environmental benignity and high enantioselectivity.[20] Amino acid dehydrogenases are regarded as a group of enzymes capable of producing amino acids. Catalytic tandem reaction participated by various biocatalysts in one pot is an elegant way for the synthesis of enantiomeric pure compounds.[21,22] Asymmetric synthesis of chiral alcohols, amines and amino acids, as well as non-natural amino acid have been successfully accomplished using these multi-enzymatic cascades.[23–27] A three-enzyme cascade reaction has been developed by Fan and coworkers employing D-mandelate dehydrogenase from *Lactobacillus brevis*, mandelate racemase from *Pseudomonas* and leucine dehydrogenase from *Exiguobacterium sibircum* for the synthesis of L-phenylglycine.[24] Under optimized condition, 0.2 M mandelic acid was converted into L-phenylglycine, with 96.4% conversion and >99% *ee*. However, this bioprocess requires large loading of biocatalysts. Consequently, there is a constant demand for enzymes with high efficiency, stability and enantioselectivity for scale-up production of amino acids.[28–30]

In this study, a novel leucine dehydrogenase (*Bc*AADH) was identified and cloned from *Bacillus clausii*, with 50.6% identity

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to phenylalanine dehydrogenase from *Rhodococcus* sp. Recombinant *Bc*AADH was purified and the enzymatic properties such as optimum temperature and pH, substrate specificity and kinetic parameters were studied. To our knowledge, few AADHs have been applied in the preparation of L-phenylglycine. The potential of this newly identified *Bc*AADH in the synthesis of L-phenylglycine was also investigated. Our results suggest that *Bc*AADH could not only tolerate high substrate loading, but also display high substrate to biocatalyst ratio.

Results and discussion

Genome mining for amino acid dehydrogenases

To find efficient aryl amino acid dehydrogenases (AADHs), genome data mining was adopted. Four potential AADHs were selected from genome database using the amino acid sequence of phenylalanine dehydrogenase from *Rhodococcus* sp. (PheDH, Q59771.2) as probe. After heterogeneously expressed in *E. coli* BL21(DE3), the reductive amination activity of AADHs toward benzoylformic acid was investigated. One AADH from *Bacillus clausii* NRRL B-23342 (*Bc*AADH) under accession no. Q5WF72 was proved to be efficient in the asymmetric reduction reaction of benzoylformic acid. This newly identified AADH displays 50.6% identity with the amino acid sequence of PheDH. Sequence and phylogenetic analysis reveal that *Bc*AADH belongs to the Glu/Leu/Phe/Val dehydrogenase family and displays high similarity with leucine dehydrogenase from *Exiguobacterium sibiricum*[31] and valine dehydrogenase from *Bacillus badius*[33] as shown in Fig. 1. Alignment of *Bc*AADH and other amino acid dehydrogenases from different origins indicate that conserved residue and domains were also found in *Bc*AADH (Fig. S1†), including catalytic K80 and NADH binding domain of GVGNVAY (180–186).

Purification and characterization of *Bc*AADH

Recombinant *Bc*AADH with N-terminal His-taq was purified by nickel affinity chromatography. After desalting and concentration, purified *Bc*AADH was obtained and analyzed by SDS-PAGE. As shown in Fig. 2, *Bc*AADH was migrated a single band at about 45 kDa, in agreement with its theoretical value. In addition, gel

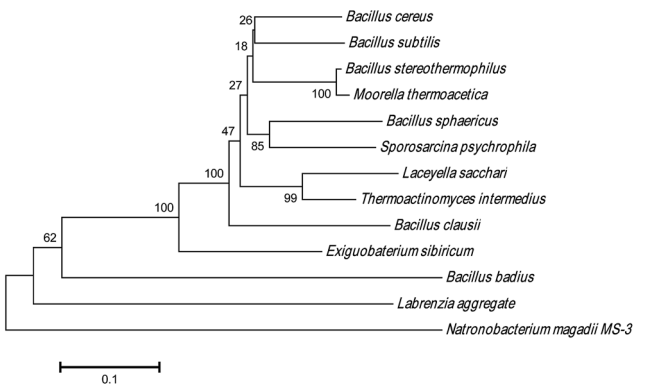


Fig. 1 Phylogenetic analysis of *Bc*AADH and other amino acid dehydrogenases.

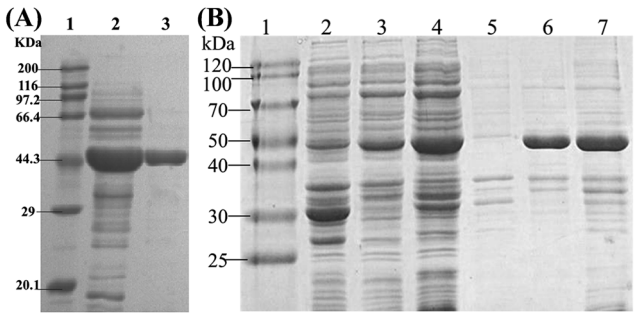


Fig. 2 SDS-PAGE of analysis of purified *Bc*AADH and coexpression of *Bc*AADH and *Bm*GDH. (A) Lane 1: protein molecular marker, lane 2: crude extract, lane 3: purified *Bc*AADH; (B) lane 1: protein molecular marker, lanes 2 & 5, 3 & 6, 4 & 7: supernatant and precipitant of recombinant *E. coli* BL21(DE3) harboring pACYCDuet-*Bm*GDH, pET28-*Bc*AADH and pACYCDuet-*Bm*GDH/pET28-*Bc*AADH.

exclusion chromatography revealed that the molecular weight was 90 kDa, indicating *Bc*AADH is a homodimeric enzyme consisting of two identical subunits. Specific activity of purified *Bc*AADH toward benzoylformic acid was 17.7 U mg^{-1}, 2.1 folds higher than that of the crude extract.

Enzyme activities at different temperatures of 20–50 °C were investigated. As illustrated in Fig. 3A, *Bc*AADH shows the highest activity at around 30 °C. Half-lives of *Bc*AADH were 315, 85.6 and 4.5 h at 30, 40 and 50 °C (Fig. S2†), respectively, indicating *Bc*AADH was stable than at operational temperature.[31] The half-lives of amino acid dehydrogenases from

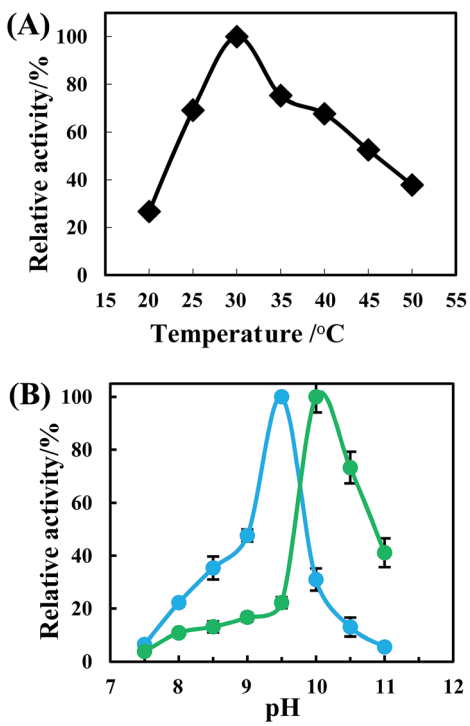


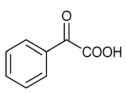
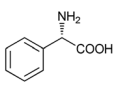
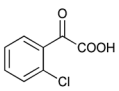
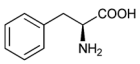
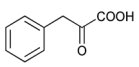
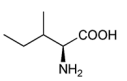
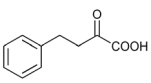
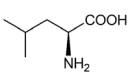
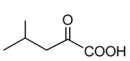
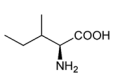
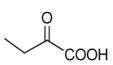
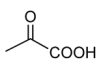
Fig. 3 Effects of temperature and pH on the activity of *Bc*AADH. (A) Temperature profile of *Bc*AADH. (B) pH profiles of the reductive and oxidative activities of *Bc*AADH, blue line: reductive profile, green line: oxidative profile.

Sporosarcina psychrophila DSM3 and *Exiguobacterium sibircum* ECU9271 at 50 °C were 0.5 h and 11 h.[31,33] Effect of pH ranging from 7.5 to 11.0 on the reductive amination and oxidative deamination activities of *Bc*AADH were also investigated (Fig. 3B). For reductive amination, the most suitable pH was pH 9.5. While for the oxidative deamination, *Bc*AADH displays the maximum activity at pH 10.5. Competing reductive and oxidative activities of *Bc*AADH indicate the dual-function of amino acid dehydrogenases in the synthesis of amino acids.

Effects of various metal ions on the activity of *Bc*AADH were also studied (Table S3†). The enzyme activity was obviously inhibited by the addition of 1 mM Li^+, Fe^{3+} and Ag^+, while Mg^{2+} and Co^+ could promote the activity of *Bc*AADH to 108.3% and 113.5% respectively. EDTA is a metal ions chelator, which could eliminate the metal ions from enzyme. As a result, the metal ion dependent enzyme could be deactivated or lose some activity. However, EDTA had none influence on the activity of *Bc*AADH. Consequently, *Bc*AADH is presumed to be a metal ion-independent amino acid dehydrogenase.

Substrate spectrum of *Bc*AADH toward various keto acids and L-amino acids with different substituents was investigated. As shown in Table 1, *Bc*AADH preferred aliphatic to aromatic keto acids in the asymmetrically reductive amination reaction. Moreover, most of the tested aromatic keto acids could be reduced by *Bc*AADH, especially benzoylformic acid (17.7 U mg^{-1}).

Table 1 Substrate specificity of purified *Bc*AADH

Reductive amination		Oxidative deamination	
Substrate	Relative activity [%]	Substrate	Relative activity [%]
	100[a]		100[b]
	5.88		<0.1
	<0.1		346
	0.23		318
	543		536
	322		<0.1
	2.86		
	8.96		

[a] Specific activity toward benzoylformic acid was regarded as 100%, 17.7 U mg^{-1}. [b] Specific activity toward L-phenylglycine was regarded as 100%, 0.11 U mg^{-1}.

The highest activity was observed with 4-methyl-2-oxopentanoic acid (96.1 U mg^{-1}), which could be reduced into L-leucine by *Bc*AADH. For the oxidative deamination, *Bc*AADH could also catalyse the oxidative deamination reaction of aromatic amino acids, such as L-phenylglycine (0.11 U mg^{-1}). However, higher activity was found with aliphatic amino acids, especially L-leucine (0.59 U mg^{-1}), which might be ascribed to its lower steric hindrance and similarity to leucine dehydrogenase from *Bacillus sphaericus* ATCC4525.[37] Our results indicate *Bc*AADH has a wide substrate spectrum.

Kinetic parameters of purified *Bc*AADH toward several preferred substrates were investigated (Table 2). The K_m and k_{cat} of *Bc*AADH toward aromatic amino acids, benzoylformic acid and 2-chlorobenzoylformic acid, were 13 mM and 65.2 s^{-1}, 6.33 mM and 1.56 s^{-1}, respectively. *Bc*AADH displays the highest affinity and catalytic efficiency on 4-methyl-2-oxopentanic acid (2.74 mM and 242 s^{-1}). However, with regard to the substrates with smaller side-chains, such as pyruvic acid and trimethylpyruvic acid, poor binding affinity was observed, resulting in low k_{cat}/K_m (0.088 s^{-1} and 0.423 s^{-1} respectively), which were similar to other amino acid dehydrogenases, such as *Es*AADH.[31]

Optimization of the asymmetric preparation of L-phenylglycine

To evaluate the potential of *Bc*AADH in the asymmetric preparation of L-phenylglycine, the reaction condition was optimized. Firstly, to drive the reaction, cofactor regeneration system was usually introduced to generate NADH for the reduction reaction. Glucose dehydrogenase (GDH) is usually used as cofactor regeneration system in the asymmetric bioreduction, due to its high stability and activity, low side reaction, low price and favorable thermodynamic.[38,39] By the reduction of NAD^+ along with the oxidation of glucose, NADH and gluconic acid are produced. Among various GDHs, the GDH from *Bacillus megaterium* (*Bm*GDH) displays excellent activity and high soluble expression level in *E. coli* BL21(DE3).[32] Consequently, *Bm*GDH was chosen for the regeneration of NADH. *Bm*GDH was coexpressed with *Bc*AADH in *E. coli* BL21(DE3) harboring both plasmids pET28-*Bc*LeuDH and pACYCDuet-*Bm*GDH. As illustrated in Fig. 2B, GDH and *Bc*AADH were successfully coexpressed in *E. coli* BL21(DE3). Activities of *Bc*AADH and *Bm*GDH in dry cells were determined to be 5.53 and 3.84 U mg^{-1} DCW, respectively.

Table 2 Steady-state kinetic constants of *Bc*AADH

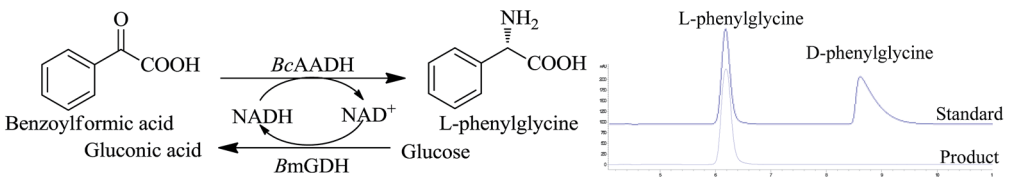
Substrate	K_m [mM]	k_{cat} [s^{-1}]	k_{cat}/K_m [s^{-1} mM^{-1}]
Benzoylformic acid	13.0	65.2	5.02
2-Chlorobenzoylformic acid	6.33	1.56	0.246
4-Methyl-2-oxopentanoic acid	2.74	242	88.3
2-Ketobutyric acid	13.9	153	11.0
Pyruvic acid	37.2	3.27	0.088
Trimethylpyruvic acid	25.7	10.9	0.423
NADH	0.373	100	268

To establish an efficient biocatalytic process, various influential factors, including temperature, substrate/biocatalyst loading and NAD^+ addition, were investigated. The reaction was optimized to improve the substrate loading and productivity as shown in entries 1–6 in Table 3. Within 0.5 h, 50 mM (7.5 g L^{-1}) benzoylformic acid could be fully reduced into L-phenylglycine using 10 g L^{-1} biocatalyst (dry cells) at 30 °C and 120 rpm with substrate to biocatalyst ratio of 0.75 g g^{-1} and >99.9% *ee*. Under the same condition, when substrate loading was increased to 200 mM (30 g L^{-1}), the conversion could only reach 88.9% even after 24 h (entry 3). Additionally, much L-phenylglycine was crystallized and attached to the reactor. Lower temperature is therefore presumed to be more suitable for the biocatalytic reaction, due to the higher stability of the enzymes. At 25 °C, 200 mM benzoylformic acid could be fully reduced. To test the maximum efficacy of *Bc*AADH, the substrate loading was further increased to 400 mM (60 g L^{-1}). Although 400 mM substrate could be completely reduced in 6 h under assistance of 0.3 mM NAD^+, too much L-phenylglycine crystals were attached to the reactor, leading to poor mass transfer. Consequently, further optimization was conducted aiming at improving the efficiency of *Bc*AADH and substrate to biocatalyst ratio (S/B) as shown in entries 7–11 of Table 3. Under 5 g L^{-1} biocatalyst, 200 mM benzoylformic acid could not be fully reduced even after 12 h, with 92.7% conversion. Addition of 0.3 mM NAD^+ could however accelerate the reaction, and 200 mM substrate could be reduced within 1 h (entry 8). The amount of biocatalyst was further reduced to 3, 1 and 0.5 g L^{-1}, the substrate could be fully reduced within 2, 6 and 12 h respectively (entries 9–11). The S/B reached as high as 60 g g^{-1} with >99.9% *ee*, ranking the highest level for the bioreductive preparation of L-phenylglycine.[34] Environmental factor (E factor) was calculated to be 4.7, demonstrating a green bioprocess catalyzed by *Bc*AADH.[35] Enzyme consumption number (e.c.n.) was calculated to be 0.018, illustrating an extremely low amount of enzyme used in the bioprocess.[36] In addition, the crystallized product L-phenylglycine could easily be isolated by adjusting the pH to dissolve the crystals, followed by boiling to remove the cells due to the low biocatalyst loading, and then subjected to rotary evaporation. To the best of our knowledge, this *Bc*AADH catalyzed bioprocess for L-phenylglycine is ranking one of most efficient bioprocess, considering its high substrate loading and biocatalysts utilization efficiency.[24,40] Therefore, this newly mined *Bc*AADH is highly potential in the asymmetric preparation of L-phenylglycine.

Preparation of L-phenylglycine at gram scale

A gram-scale preparation of L-phenylglycine was conducted using the recombinant whole cells of *E. coli* BL21(DE3)/pET28-*Bc*AADH/pACYCDuet-*Bm*GDH. In a 100 mL reaction system, 3.0 g benzoylformic acid, 50 mg DCW of whole cells, 0.3 mM NAD^+ (final concentration) and 7.2 g glucose were added. The reaction was performed at 25 °C with 60 rpm mechanical agitation. The conversion of benzoylformic acid reached 99% in 12 h. After rotary evaporation and crystallization, 2.73 g optically pure L-phenylglycine was harvested, with 91% isolation yield and >99.9% *ee*. The product was also confirmed to be L-phenylglycine by LC-MS, ^{1}H-NMR and ^{13}C-NMR as follows (Fig. S3–S5†): ^{1}H-NMR (400 MHz, D_2O), δ/ppm: 4.36 (s, 1H), 7.16–7.42 (m, 5H); ^{13}C-NMR (100 MHz, D_2O), δ/ppm: 60.5126.8, 127.5, 128.8, 181.0.

Table 3 Asymmetric synthesis of L-phenylglycine with recombinant whole cells coexpressing *Bc*AADH and *Bm*GDH



Entry	Substrate [g L^{-1}]	Biocatalyst [g L^{-1}]	S/B[a] [g g^{-1}]	Temperature [°C]	NAD^+ [mM]	Time [h]	Conversion[b] [%]	*ee*[c] [%]
1	7.5	10	0.75	30	0	0.5	>99	>99.9
2	15	10	1.5	30	0	2	>99	>99.9
3	30	10	3	30	0	24	88.9	>99.9
4	30	10	3	25	0	4	>99	>99.9
5	60	10	6	25	0	24	62.8	>99.9
6	60	10	6	25	0.3	6	>99	>99.9
7	30	5	6	25	0	12	92.7	>99.9
8	30	5	6	25	0.3	1	>99	>99.9
9	30	3	10	25	0.3	2	>99	>99.9
10	30	1	30	25	0.3	6	>99	>99.9
11	30	0.5	60	25	0.3	12	>99	>99.9

[a] S/B: substrate to biocatalyst ratio, g g^{-1}. [b] Conversion was analysed by HPLC equipped with Diamonsil C18 column at 220 nm and flow rate of 0.6 mL min^{-1} with 5% methanol, 95% KH_2PO_4 and 0.08% trifluoroacetic acid as mobile phase. [c] *ee* was determined by HPLC equipped with Chirobiotic T column at 220 nm and flow rate of 0.5 mL min^{-1} with 20% methanol, 80% ddH_2O and 0.1% TFA as mobile phase.

Experimental

Strains and chemicals

Escherichia coli DH5α and BL21(DE3) in this study were preserved in our laboratory and used as the cloning and expression hosts respectively. Plasmids pET28a and pACYCDuet-1 were commercially obtained from Novagen (Madison, WI, USA). Benzoylformic acid, D/L-phenylglycine and other substrates were all purchased from Shanghai CIVI Chemical Technology co. Ltd (shanghai, China).

Cloning, expression and purification of *Bc*AADH

Gene coding for *Bc*AADH was amplified from the genomic DNA of *Bacillus clausii* NRRL B-23342 by PCR using primers (F: CGCGGATCCATGGAATTATTTGCAAAGA, R: CCGCTCGAGTTAC TTTTTCCTCGAA) with *Bam*HI and *Xho*I restriction sites. PCR product and pET28a were double digested with *Bam*HI and *Xho*I. The resultant DNA fragments were recovered and ligated at 4 °C for 12 h. Then the constructed plasmid pET28-*Bc*AADH was transformed into *E. coli* DH5α. After verified by colony PCR, the recombinant plasmid was extracted and further transferred into *E. coli* BL21(DE3).

Recombinant strain *E. coli* BL21(DE3)/pET28-*Bc*AADH cell was cultivated in 30 mL LB medium containing 50 μg mL^{-1} kanamycin at 37 °C and 180 rpm, until optical density at 600 nm reached 0.6–0.8. Then 0.1 mM IPTG was added and the recombinant strain was further cultivated at 25 °C for 7 hours. Cells were collected by centrifuging at 8000 × *g* for 10 min. Then the cells were disrupted by a ATS engineering AH-BASIC II homogenizer (Shanghai, China). And the recombinant *Bc*AADH with N terminal his tag was purified as previously described.[31] Purified *Bc*AADH was added with 30% (v/v) glycerol after desalting and concentration, and subsequently stored at −80 °C.

Activity assays

The reductive amination and oxidative deamination activities of *Bc*AADH were determined by monitoring the change of NADH at 340 nm (ε = 6220 L M^{-1} cm^{-1}) using spectrophotometer. For the reductive amination activity, 200 μL reaction mixture was consisted of 0.5 mM NADH, 5 mM benzoylformic acid and appropriate amount of *Bc*AADH in NH_4Cl–$NH_3 \cdot H_2O$ (pH 9.5, 0.5 M). For the oxidative deamination activity, 200 μL reaction mixture was consisted of 0.5 mM NAD^+, 5 mM L-phenylglycine and appropriate amount of *Bc*AADH in NH_4Cl–$NH_3 \cdot H_2O$ (pH 9.5, 0.5 M). One unit of AADH or glucose dehydrogenase (GDH) was defined as the amount of enzyme that catalyzes the reduction of phenylglycine or oxidation of glucose to produce 1.0 μmol NAD^+ or 1.0 μmol NADH per minute. Activity of GDH was defined and determined as previously reported.[32] All the assay was performed in triplicate.

Enzymatic characterization of purified *Bc*AADH

Optimum pH and temperature and thermostability. The optimum pH was determined by above mentioned standard activity assay in different NH_4Cl–$NH_3 \cdot H_2O$ buffers (pH 7.5–11.0). The optimum temperature was determined at different temperature range from 20 °C to 50 °C. The thermostability of *Bc*AADH was investigated by incubating the purified *Bc*AADH (0.1 mg mL^{-1}) at 30, 40, and 50 °C for certain period of time in 0.5 M NH_4Cl–$NH_3 \cdot H_2O$ buffer (pH 9.5). The relative residual activity was detected by standard assay protocol. The half-lives of *Bc*AADH were calculated by the fitting curves of relative residual activity and time.

Effect of metal ions. Influence of metal ions on enzyme activity was studied by pre-incubating the purified *Bc*AADH with different metal ions (1 mM) and EDTA for 30 min at 30 °C. The enzyme activity was determined using the standard assay. Enzyme activity in the absence of metal ions was expressed as control (100%). The promotion or inhibition of metal ions were studied by comparison with the control. All the activities were carried out in triplicate.

Substrate spectrum. Substrate specificity of purified *Bc*AADH toward various prochiral keto acids and amino acids was measured as shown in Table 1. The specific activity of *Bc*AADH toward benzoylformic acid and L-phenylglycine was regarded as 100%.

Kinetic analysis. Kinetic parameters of purified *Bc*AADH toward various keto acids, L-phenylglycine, NADH and NAD^+ were measured using standard assay protocol. The concentrations were in range of 1–100 mM for keto acids and L-phenylglycine, 0.025–1.0 mM for NADH and NAD^+. The K_m and V_{max} were calculated according to the Lineweaver–Burk plot.

Co-expression of *Bc*AADH and GDH

Glucose dehydrogenase coding gene was cloned from *Bacillus megaterium* and inserted into the *Bam*HI and *Sal*I restriction sites of pACYCDuet using ClonExpressII one step clone kit (Vazyme Inc., Nanjing). The resultant plasmids pACYCDuet-*Bm*GDH and pET28-*Bc*AADH were simultaneously transformed into *E. coli* BL21(DE3) cells and spread on LB plates supplemented with kanamycin and chloramphenicol. *E. coli* BL21(DE3) harboring pET28-*Bc*AADH and pACYCDuet-*Bm*GDH was identified by colony PCR. Recombinant coexpression cells were cultured and induced as mentioned above. The cells was lyophilized to dry cells and stored at 4 °C for further use.

Optimization of the asymmetrically reductive amination of benzoylformic acid

Optimization of the asymmetric amination of benzoylformic acid was conducted in a 20 mL reactor and magnetically stirred at 120 rpm and 30 °C. Firstly, benzoylformic acid was dissolved by addition of diluted NaOH (0.5 M), then NH_4Cl and glucose were added into the reactor. The pH of reaction mixture was adjusted to 8.5 with 0.5 M $NH_3 \cdot H_2O$. Finally the lyophilized cells of the recombinant *E. coli* BL21(DE3) harboring pET28-*Bc*AADH and pACYCDuet-*Bm*GDH were added to start the reaction. Amount of enzyme, reaction temperature and NAD^+ were varied as shown in Table 3. Samples were withdrawn from the reaction mixture to determine the conversion and enantiomeric excess (ee) by HPLC analysis. Conversion was monitored by HPLC equipping with Diamonsil C18 column (250 mm × 4.6 mm, ID 5

mm, DIMKA), detecting at UV 220 nm and 30 °C in the mobile phase including methanol 5%, KH_2PO_4 95% and trifluoroacetic acid 0.08% at a flow rate of 0.6 mL min^{-1}. The ee of the L-phenylglycine was measured by HPLC with a chiral Astec column (Chirobiotic T, 150 mm × Φ 4.6 mm) at 220 nm, using mobile phase consisted of methanol, ddH_2O and TFA with a volume ratio of 20 : 80 : 0.001. The flow rate was 0.5 mL min^{-1}. Retention times of L- and D-phenylglycine were 6.1 min and 8.6 min, respectively.

Gram-scale synthesis of L-phenylglycine

In a 100 mL reaction system, 3.0 g benzoylformic acid was dissolved in deionized water and adjusted to pH 8.5 with 0.5 M $NH_3 \cdot H_2O$, then 2.675 g NH_4Cl, 0.19 g NAD^+, 7.2 g glucose and 0.05 g dry cells were added. The reaction pH was maintained at 8.5 by titrating 0.5 M $NH_3 \cdot H_2O$. The reaction was conducted at 120 rpm and 30 °C. Samples were withdrawn to analyze the conversion and ee as described above. Finally, reaction mixture was centrifuged (8000 × *g*, 15 minutes). The precipitation was dissolved with 1.0 M $NH_3 \cdot H_2O$ and subsequently boiled for 10 min to remove the biocatalysts. The product was collected by rotary evaporation and crystallization. The synthesized L-phenylglycine was verified by LC/MS, ^{1}H-NMR and ^{13}C-NMR.

Conclusions

In summary, a novel amino acid dehydrogenase (*Bc*AADH) was identified from *Bacillus clausii* NRRL B-23342 with high activity and enantioselectivity toward aromatic keto acids. *Bc*AADH belongs to Glu/Leu/Phe/Val dehydrogenase family and could catalyse reductive amination and oxidative deamination. The optimum pH for reduction and oxidation were 9.5 and 10.5. Specific activities of *Bc*AADH toward benzoylformic acid and L-phenylglycine were 17.7 and 0.11 U mg^{-1}. The K_m and k_{cat} were 13 mM and 65.2 s^{-1} toward benzoylformic acid. *Bc*AADH displays high efficiency in the asymmetric preparation of L-phenylglycine. When coexpressed with *Bm*GDH from *Bacillus megaterium*, 200 mM benzoylformic acid could be fully reduced using merely 0.5 g L^{-1} dry cells, with 91% isolation yield and >99.9% ee. The substrate to biocatalyst ratio and Environmental factor were 60 g g^{-1} and 4.7. Our results indicate that *Bc*AADH is a highly potential and robust enzyme for the industrial production of valuable non-natural amino acids.

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