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Identification of D-carbamoylase for biocatalytic cascade synthesis of D-tryptophan featuring high enantioselectivity

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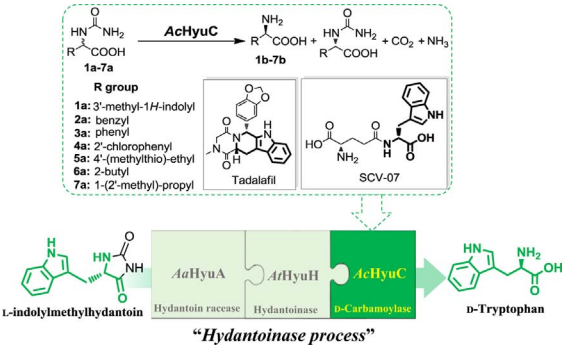
GRAPHICAL ABSTRACT



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ABSTRACT

In this study, an enantioselective D-carbamoylase (*Ac*HyuC) was identified from *Arthrobacter crystallopoietes* with optimum pH of 8.5, much more compatible with hydantoinase process than other reported D-*N*-carbamoylases. *Ac*HyuC has a substrate preference for aromatic carbamoyl-compounds. The dynamic kinetic resolution (DKR) cascade was developed by combining this *Ac*HyuC with hydantoin racemase from *Arthrobacter aurescens* (*Aa*HyuA) and D-hydantoinase from *Agrobacterium tumefaciens* (*At*HyuH) for enantioselective resolution of L-indolylmethylhydantoin into D-Trp. The optimum pH of DKR cascade reaction was determined to be 8.0, and PEG 400 could facilitate the reaction. As much as 80 mM L-indolylmethylhydantoin could be fully converted to D-Trp within 12 h at 0.5 L scale, with 99.4% yield, > 99.9% *e.e.* and productivity of 36.6 g L^{-1} d^{-1}. This study provides a new D-carbamoylase compatible with the DKR cascade for efficient production of optically pure D-Trp from L-indolylmethylhydantoin.

1. Introduction

D-Amino acids are widely exist in plants (Vranova et al., 2012), animals (Fujii, 2002) and microbes (Radkov and Moe, 2014) and can be widely used in pharmaceutical, food, and cosmetics industry. Among them, D-tryptophan (D-Trp) can be used to synthesize contryphans and Tadalafil (cialis), which are important drugs in treatment of erectile dysfunction or pulmonary arterial hypertension. D-Trp can also be used in the synthesis of some peptides for the treatment of dermatitis, for example, tyrocidines C and D, or Thymodepressin (γ-D-Glu-D-Trp) (Martínez-Rodríguez et al., 2010). In food additive industry, D-Trp can be used as nonnutritive sweetener, which is popular among people on diet (Gao et al., 2015).

Various approaches have been developed for the synthesis of D-Trp,

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including chemical or enzymatic or chemoenzymatic resolution, transamination, and reductive amination etc. D-Trp could be produced by D-amidase in selective hydrolyzing D-tryptophanamide, or L-amidase through selective resolution of D-tryptophanamide from racemic tryptophanamide, and then D-tryptophanamide was chemically hydrolyzed to give D-Trp (Yamamoto et al., 1998). Similar to the amidase method, L-aminoacylase/D-aminoacylase could also be used in preparation of D-Trp, in which only L-enantiomer of *N*-acetyl-DL-tryptophanamide was hydrolyzed and the remaining enantiomer can be chemically deacetylated to form D-Trp (Greenstein, 1957). Yamamoto et al. has identified an enantioselective tryptophanase that could degrade L-Trp from DL-Trp and give the D-Trp, whereas the by-products, pyruvic acid and indole, could inhibit the activity of tryptophanase and need to be removed (Kawasaki et al., 1995). All these methods suffer from disadvantages such as 50% theoretic yield, complicated procedures, low yield and enantioselectivity. Although the optically pure D-Trp can also be produced from indolylpyruvic acid and D-alanine by D-amino acid transaminase with 100% theoretical yield, the activity was low and the yield was merely 13% (Eadie et al., 1949).

Hydantoinase process is a classical three-enzyme cascade reaction, including hydantoin racemase, D-hydantoinase and D-carbamoylase. Various D-amino acids have been successfully synthesized at industrial scale by this dynamic kinetic resolution (DKR) cascade process, with 100% theoretical yield and high enantioselectivity. Among them, hydantoin racemase is responsible for racemization of L-hydantoin or D-hydantoin, D-Hydantoinase could hydrolyze D-hydantoin to give D-*N*-carbamoyl-amino acid and further be converted into D-amino acids by D-carbamoylase. DKR cascade has received increasing attentions due to advantages such as higher theoretical yield and no intermediate extraction and purification steps, and has widely applied for synthesis of amino acids (Luo et al., 2017; Yamaguchi et al., 2007), alcohols (Enoki et al., 2016), chiral alkenes (Wu et al., 2016) and some bio-based antioxidants (Gómez Baraibar et al., 2016; Zhou et al., 2016). As a result, the DKR cascade of hydantoinase process is one of the most promising strategy to achieve economic and enantioselective synthesis of D-amino acids (Runser et al., 1990; Martínez-Gómez et al., 2007; Liu et al., 2008a,b; Aranaz et al., 2015). However, there are several issues in the application of hydantoinase process, including sparing solubility of hydantoin substrates, insoluble protein expression (Sareen et al., 2001), susceptible to oxidative deactivation (Grifantini et al., 1996) and poor thermostability of D-carbamoylase (Wu et al., 2006), incompatible reaction conditions among three enzymes. Consequently, it is critical to identify an enantioselective D-carbamoylase with high solubility and compatibility, and establish an efficient DKR cascade for the preparation of D-amino acids from their corresponding L-hydantoins.

To establish DKR cascade for the preparation of D-Trp from L-indolylmethylhydantoin, D-carbamoylases were screened by genome mining for high enantioselectivity, activity, protein solubility, and reaction compatibility. A D-carbamoylase from *Arthrobacter crystallopoietes* was identified and designated as *Ac*HyuC. A DKR cascade was established by combining *Ac*HyuC with hydantoin racemase (*Aa*HyuA) from *Arthrobacter aurescens* (Wiese et al., 2001) and D-hydantoinase (*At*HyuH) from *Agrobacterium tumefaciens* (Clemente-Jiménez et al., 2003). Factors influencing the compatibility of the DKR cascade, and substrate and enzymes loadings, were investigated to achieve economic synthesis of D-Trp with high yield and enantioselectivity.

2. Materials and methods

2.1. Chemical reagents and synthesized compounds

D-Tryptophan (D-Trp), L-tryptophan (L-Trp), DL-tryptophan (DL-Trp), DL-Methionine, DL-phenylalanine, DL-leucine, DL-isoleucine and DL-2-chlorophenylglycine were purchased from Sinopharm Chemical Regent Co., Ltd. *N*-carbamoyl-DL-tryptophan (**1a**), *N*-carbamoyl-DL-phenylalanine (**2a**), *N*-carbamoyl-D-phenylglycine (**3a**), *N*-carbamoyl-DL-(2-chlorophenyl)glycine (**4a**), *N*-carbamoyl-DL-methionine (**5a**), *N*-carbamoyl-DL-leucine (**6a**), *N*-carbamoyl-DL-isoleucine (**7a**), DL-5-indolylmethylhydantoin (DL-IMHD) and L-5-indolylmethylhydantoin (L-IMHD) were synthesized according to reported method (Patching, 2011), and evaluated by ^{1}H NMR.

***N*-Carbamoyl-DL-tryptophan ($C_{12}H_{13}N_3O_3$, 1a)** ^{1}H NMR (400 MHz, DMSO-d_6): δ 12.56 (s, 1H), 10.88 s, 1H), δ 7.52 (d, J = 7.9 Hz, 1H), δ 7.33 (d, J = 8.0 Hz, 1H), δ 7.13–7.02 (m, 2H), δ 6.98 (t, J = 7.5 Hz, 1H), δ 6.13 (d, J = 8.1 Hz, 1H), δ 5.64 (s, 2H), δ 4.38 (q, J = 6.8 Hz, 1H), δ 3.11 (dd, J_1 = 14.7 Hz, J_2 = 5.3 Hz, 1H), δ 3.01 (dd, J_1 = 14.6 Hz, J_2 = 7.0 Hz, 1H).

***N*-Carbamoyl-DL-phenylalanine ($C_{10}H_{12}N_2O_3$, 2a)** ^{1}H NMR (400 MHz, DMSO-d_6): δ 12.64 (s, 1H), δ 7.91–6.60 (m, 5H), δ 6.16 (d, J = 6.8 Hz, 1H), δ 5.62 (s, 2H), δ 4.32 (dd, J_1 = 12.9 Hz, J_2 = 7.3 Hz, 1H), δ 2.92 (ddd, J_1 = 21.6 Hz, J_2 = 13.7 Hz, J_3 = 6.4 Hz, 2H).

***N*-Carbamoyl-DL-phenylglycine ($C_9H_{10}N_2O_3$, 3a)** ^{1}H NMR (400 MHz, DMSO-d_6): δ 12.51 (s, 1H), δ 6.26 (d, J = 7.4 Hz, 1H), δ 5.61 (s, 2H), δ 4.05 (p, J = 7.3 Hz, 1H), δ 3.43 (s, 1H), δ 1.21 (d, J = 7.2 Hz, 4H).

***N*-Carbamoyl-DL-2-chlorophenylglycine ($C_9H_9ClN2O_3$, 4a)** ^{1}H NMR (400 MHz, DMSO-d_6): δ 7.35 (d, J = 2.3 Hz, 1H), δ 6.79 (d, J = 7.8 Hz, 1H), δ 5.71 (s, 2H), δ 5.15 (d, J = 7.9 Hz, 1H), δ 3.39 (s, 4H).

***N*-Carbamoyl-DL-methionine ($C_6H_{12}N_2O_3S$, 5a)** ^{1}H NMR (300 MHz, DMSO-d_6): δ 12.59 (s, 1H), δ 6.35 (s, 1H), δ 5.61 (s, 2H),δ 4.22–4.15 (m, 1H), δ 2.49 (s, 1H), δ 2.44 (s, 1H), δ 2.04 (s, 3H), δ 1.92–1.90 (m, 1H), δ 1.76 (s, 1H).

***N*-Carbamoyl-DL-leucine ($C_7H_{14}N_2O_3$, 6a)** ^{1}H NMR (300 MHz, DMSO-d_6): δ 12.45 (s, 1H), δ 6.18 (d, J = 8.5 Hz, 1H), δ 5.56 (s, 2H), δ 4.07 (td, J_1 = 8.7 Hz, J_2 = 5.8 Hz, 1H), δ 1.76–1.54 (m, 1H), δ 1.54–1.32 (m, 2H), δ 0.88 (dd, J_1 = 8.5 Hz, J_2 = 6.5 Hz, 6H).

***N*-Carbamoyl-DL-isoleucine ($C_7H_{14}N_2O_3$, 7a)** ^{1}H NMR (300 MHz, DMSO-d_6): δ 12.50 (s, 1H), δ 6.15 (dd, J_1 = 17.5 Hz, J_2 = 9.1 Hz, 1H), δ 5.59 (s, 1H), δ 4.11 (ddd, J_1 = 14.2 Hz, J_2 = 9.1 Hz, J_3 = 4.6 Hz, 1H), δ 2.51 (dt, J_1 = 3.5 Hz, J_2 = 1.7 Hz, 1H), δ 1.88–1.68 (m, 1H), δ 1.46–1.24 (m, 1H), δ 1.10 (dt, J_1 = 20.2, J_2 = 6.6 Hz, 1H), δ 0.93–0.71 (m, 6H).

L-Indolylmethylhydantoin ($C_{12}H_{11}N_3O_3$) ^{1}H NMR (400 MHz, DMSO-d_6): δ 10.90 (s, 1H), δ 10.36 (s, 1H), δ 7.90 (s, 1H), δ 7.54 (d, J = 8.0 Hz, 1H), δ 7.32 (d, J = 8.1 Hz, 1H), δ 7.15–7.09 (m, 1H), δ 7.05 (t, J = 7.6 Hz, 1H), δ 6.96 (t, J = 7.5 Hz, 1H), δ 4.31 (t, J = 4.9 Hz, 1H), δ 3.06 (d, J = 4.8 Hz, 2H).

DL-Indolylmethylhydantoin ($C_{12}H_{11}N_3O_3$) ^{1}H NMR (400 MHz, DMSO-d_6): δ 10.91 (s, 1H), δ 10.36 (s, 1H), δ 7.90 (s, 1H), δ 7.54 (d, J = 7.9 Hz, 1H), δ 7.32 (d, J = 8.0 Hz, 1H), δ 7.12 (d, J = 2.4 Hz, 1H), δ 7.05 (t, J = 7.5 Hz, 1H), δ 6.96 (t, J = 7.4 Hz, 1H), δ 4.35–4.27 (m, 1H), δ 3.06 (d, J = 4.8 Hz, 2H).

2.2. Cloning, expression and purification of D-carbamoylase

General protocol on the cloning and expression of D-carbamoylase was exemplified with D-*N*-carbamoylase from *Arthrobacter crystallopoietes* CGMCC1.1926 (*Ac*HyuC). The *Ac*HyuC coding gene (Accession No. Q84FR7) was cloned by PCR amplification using the genomic DNA of *A. crystallopoietes* as template, and 5′-CAGCAAATGGGTCGCGGATCCTTGGCGAAAAACTTGATGCTC-3′ and 5′-TGCGGCCGCAAGCTTGTCGACTTAGTCATTCACGTTGAACGGG-3′ as primers (underline denotes the recognition sites of restriction endonuclease). Amplification was performed using PrimeSTAR polymerase (Takara Ltd., Shanghai) and the PCR procedure was set as initial denaturation at 94 °C for 10 min, 30 cycles of denaturation at 95 °C for 30 s, annealing at 65 °C for 30 s and elongation at 72 °C for 30 s, and followed by a final extension for 10 min at 72 °C. The PCR product was recovered and purified from agarose gel, and ligated into pET28a double digested with *Bam*H I and *Sal* I using Exnase II (Vazyme Ltd., Nanjing), resulting in pET28a-*AchyuC*. *Ac*HyuC overexpressing strain was constructed by

transforming pET28a-*AchyuC* into *Escherichia coli* BL21(DE3), spreading on LB agar plate and cultivating at 37 °C overnight. The recombinant strains were selected for verification by colony PCR and double digestion.

A single colony of *E. coli* BL21(DE3)/pET28a-*AchyuC* was picked up and cultivated in LB medium containing 50 μg/mL kanamycin at 37 °C and 180 rpm. When the OD_{600} reached 0.8, recombinant *E. coli* BL21(DE3)/pET28a-*AchyuC* was induced with 0.2 mM isopropyl-*β*-D-thiogalactoside (IPTG) and further cultivated at 25 °C and 180 rpm for 12 h. The cells were harvested by centrifugation at 8000*g* for 10 min and 4 °C. After washing with physiological saline, the cell pellets were resuspended in sodium phosphate buffer (PBS, pH 8.0, 10 mM) and disrupted by high pressure homogenizer at 600 bar for two times (AH-BASICI, ATS Engineering Inc, Shanghai). The supernatant was separated by centrifugation at 8000*g* for 10 min and 4 °C, then freezing lyophilized under vacuum (SCIENTZ-10N, Ningbo Scientz Biotechnology Co. Ltd., Ningbo) to obtain the crude enzyme powder. This crude enzyme power was stored at 4 °C for further use. The recombinant *Ac*HyuC was purified according to the reported method (Xu et al., 2016). Purified *Ac*HyuC was verified by SDS-PAGE and stored at −80 °C with 20% (v/v) glycerol for enzyme characterization.

2.3. Activity assay of D-carbamoylase

Standard protocol for activity assay of D-carbamoylase was performed in 1 mL reaction mixture consisted of 100 μL of 20 mM *N*-carbamoyl-DL-tryptophan (**1a**) dissolved in PBS (pH 8.0, 100 mM) and appropriate amount of *Ac*HyuC enzyme power suspended in PBS (pH 8.0, 100 mM) at 30 °C and 180 rpm for 30 min. Reaction was stopped by heating at 100 °C for 10 min. After centrifugation at 12,000*g* for 5 min, the supernatant was filtrated with 0.22 μm filter and analyzed by RE-HPLC. RE-HPLC analysis was conducted with mobile phase of 25% acetonitrile and KH_2PO_4 (v/v, pH 3.0) at a flow rate of 0.8 mL min^{-1} and 210 nm using Agilent Infinity 1260 (Agilent Technologies Ltd., United States) equipped with Diamonsil Plus C18 (25 cm × 4.6 mm, 5 μm). The retention times of **1a** and D-tryptophan were 4.97 and 2.86 min. The activity of *Ac*HyuC was defined as the amount of enzyme that catalyzed the formation of 1 μmol D-amino acid per minute at 30 °C.

2.4. Properties of D-carbamoylase

2.4.1. Effect of pH on AcHyuC

The pH profile of purified *Ac*HyuC was determined using the standard activity assay protocol except in the following buffers: sodium phosphate buffer (pH 6.0–8.0, 100 mM), Tris–HCl (pH 7.0–9.0, 100 mM), Glycine-NaOH (pH 8.5–10.0, 100 mM) and sodium carbonate-sodium hydrogen carbonate (pH 9.5–10.5, 100 mM). All the activities were assayed in triplicate.

2.4.2. Effect of temperature on AcHyuC

The optimum temperature of *Ac*HyuC was also measured under the above-mentioned standard condition at various temperatures (25–50 °C). Thermostability of *Ac*HyuC was investigated by incubating purified *Ac*HyuC solution (0.1 mg mL^{-1}) at 30, 40 and 50 °C. About 10 μL *Ac*HyuC was withdrawn from the mixture, and the residual activity was determined using the standard assay protocol. All the activities were assayed in triplicate.

2.4.3. Effect of metal ions on AcHyuC

The effects of various metal ions and additives (including Ni^{2+}, Mg^{2+}, Fe^{2+}, Zn^{2+}, Cu^{2+}, Ca^{2+}, Mn^{2+}, Co^{2+} and EDTA) on activity of *Ac*HyuC were examined by adding each compounds in the enzyme solution and incubated at 30 °C for 30 min. Afterwards the enzyme activities were measured under the standard condition. Control was performed in the absence of any tested compound. All the activities were assayed in triplicate.

2.4.4. Substrate spectrum and enantioselectivity analysis of AcHyuC

The specific activity of purified *Ac*HyuC toward various *N*-carbamoyl-amino acids including *N*-carbamoyl-DL-phenylalanine, *N*-carbamoyl-DL-methionine, *N*-carbamoyl-DL-leucine, *N*-carbamoyl-DL-isoleucine, *N*-carbamoyl-DL-2-chlorophenyl-glycine and *N*-carbamoyl-D-phenylglycine were measured using above mentioned method and analyzed by RE-HPLC. The enantioselectivity of purified *Ac*HyuC in preparation of D-amino acids was analyzed by chiral HPLC equipped with Astec CHIROBIOTIC™ T column (15 cm × 4.6 mm, 5 μm) using 40% methanol and 60% ddH_2O (v/v) as mobile phase at a flow rate of 1 mL min^{-1} and 210 nm. The retention times of D- and L-tryptophan were 9.4 and 8.5 min. The enantiomeric excess (*e.e.*) value was calculated according to the following formula:

$$\text{Enantiomeric excess}(e.\,e.) = \frac{\text{Conc}_{(\text{D-config.})} - \text{Conc}_{(\text{L-config.})}}{\text{Conc}_{(\text{D-config.})} + \text{Conc}_{(\text{L-config.})}} \times 100\%$$

$\text{Conc}_{(\text{D-config.})}$ and $\text{Conc}_{(\text{L-config.})}$ refer to the concentrations of D- and L-amino acids respectively.

The enantioselectivity (*E* value) of purified *Ac*HyuC was calculated according to the following formula:

$$E = \frac{\text{In}[1-c(1+ee_p)]}{\text{In}[1-c(1-ee_p)]}$$

c refers to the conversion of D-*N*-carbamoyl-amino acids and ee_p refers to the *e.e.* value of synthesized D-amino acids.

2.4.5. Determination of kinetic parameters of AcHyuC toward N-carbamoyl-DL-tryptophan

Kinetic parameters of *Ac*HyuC toward *N*-carbamoyl-DL-tryptophan were determined under the above-mentioned standard condition at different concentrations (0.1, 0.25, 0.5, 1.0, 2.0, 5.0 and 10.0 mM). K_m and V_{max} were calculated according to Lineweaver-Burk plot.

2.5. Construction and optimization of cascade reaction for synthesis of D-Trp

2.5.1. Cloning and expression of AaHyuA and AtHyuH to construct a cascade reaction

To construct a cascade reaction for producing D-Trp, the hydantoin racemase (*Aa*HyuA) from *A. aurescens* and D-hydantoinase (*At*HyuH) from *A. tumefaciens* which exhibited high activity and stereoselectivity toward aromatic 5′-monosubstituted hydantoins were selected. The cloning and expression of *AahyuA* and *AthyuH* were similar as *AchyuC*. These recombinant *Aa*HyuA and *At*HyuH were purified and enzymatic properties including effect of metal ions and kinetic constants were determined.

2.5.2. Effect of enzyme dosage on single step reaction

Effects of enzyme dosage of *Aa*HyuA, *At*HyuH and *Ac*HyuC on individual reaction were determined with 5 mM L-IMHD, 10 mM DL-IMHD and 10 mM *N*-carbamoyl-DL-tryptophan respectively dispersed with 10% (v/v) PEG400 in 9 mL pH 8.0 PBS (100 mM) using appropriate of lyophilized *Aa*HyuA (1–5 kU L^{-1}), *At*HyuH (1–20 kU L^{-1}) and *Ac*HyuC (1–5 kU L^{-1}). Reaction mixture was incubated at 30 °C and 180 rpm. Aliquots (100 μL) were withdrawn at 0 h, 0.5 h, 1.0 h, 1.5 h and 2.0 h, afterwards 100 μL ethanol for *Aa*HyuA or 900 μL ddH_2O for *At*HyuH and *Ac*HyuC were added and heated at 100 °C for 10 min to stop the reaction. Samples were analyzed using HPLC as above described. All the reaction were performed in triplicate.

2.5.3. Effect of pH on the cascade reaction

Considering the optimum pH of *Aa*HyuA, *At*HyuH and *Ac*HyuC were different, effects of three different pH buffers including pH 8.0 PBS (100 mM), pH 7.0 PBS (100 mM) and pH 9.0 Glycine-NaOH (100 mM) on cascade reaction were determined using lyophilized enzymes. The reaction mixture (10 mL) consisted of 5 mM L-IMHD, 5 kU L^{-1} of each

enzyme, 0.5 mM $MnCl_2$, and then supplemented with different buffers to a final volume of 10 mL. Reaction mixture was incubated at 30 °C and 180 rpm. Samples (100 μL) were withdrawn at 0 h, 0.5 h, 2 h, 4 h and 6 h, terminated and analyzed as above described. All the reaction were performed in triplicate.

2.5.4. Effect of co-solvents on the cascade reaction

Effects of different co-solvents including PEG400, Tween20, β-cyclodextrin, Triton X-100, cetyltrimethyl ammonium bromide (CTAB), ethanol, dimethyl sulfoxide (DMSO) and tetrabutylammonium bromide (TBAB), on the cascade reaction were determined using lyophilized enzymes. The reaction system consisted of 50 mM L-IMHD, 5 kU L^{-1} of each enzyme, 0.5 mM $MnCl_2$, 5% and 10% of above mentioned co-solvents and supplemented with PBS 8.0 to 1.0 mL. The reaction mixture was incubated at 30 °C and 180 rpm for 24 h. Samples (20 μL) were withdrawn, terminated and analyzed as above described. All the reaction were performed in triplicate.

2.5.5. Optimization cascade reaction for preparation of D-Trp

To establish an efficient one-pot cascade reaction, substrate and biocatalyst loadings were investigated. Different amount of L-IMHD (5–100 mM) and dosages of each enzyme (2–20 kU L^{-1} for *Aa*HyuA, 5 kU L^{-1} for *At*HyuH and 2–10 kU L^{-1} for *Ac*HyuC) were tested in the reaction mixture as above described. All the reaction were incubated at 30 °C and 180 rpm till the end of the reaction. Samples were intermittently withdrawn and analyzed using RE-HPLC as above described.

2.6. Preparation of D-Trp at gram scale

To test the potential of this newly established cascade reaction in the preparation of D-Trp, a 0.5 L reaction system was constructed, containing 9.16 g L-IMHD (80 mM), 0.4 g *Aa*HyuA, 1.25 g *At*HyuH, 5 g *Ac*HyuC, 0.5 mM $MnCl_2$ (final concentration) and 50 mL PEG400, and then supplemented with PBS (pH 8.0, 100 mM) to 0.5 L. The reaction was performed at 30 °C with 150 rpm mechanical agitation. Aliquots (20 μL) were taken at 0 h, 0.5 h, 2 h, 4 h, 6 h, 8 h, 12 h, then terminated and analyzed as above described.

Isolation of D-Trp from the reaction mixture was carried out as follows. The pH of the reaction mixture was adjusted to pH 2.0 with 3.0 M HCl to dissolve D-Trp as much as possible due to the high solubility of D-Trp in acidic condition. Then the mixture was filtrated to remove enzymes and other insoluble compounds. The supernatant was loaded on 732 cation exchange resin (120 g) to purify D-Trp. Ammonium hydroxide was used to elute D-Trp until no D-Trp was detected in the eluent. Afterwards elusion buffer was evaporated under vacuum at 70 °C and D-Trp was crystallized. Furthermore the resultant D-Trp was dissolved in water for recrystallization, and subjected to RE-HPLC, chiral HPLC and ^1H NMR analysis.

3. Results and discussion

3.1. Identification of AcHyuC

In order to develop a hydantoinase process for the synthesis of D-tryptophan, screening for D-carbamoylases with high pH compatibility, activity, enantioselectivity and solubility was conducted since D-carbamoylase is widely accepted as the rate-limiting step. D-carbamoylases have been mostly identified from *Agrobacterium* (Buson et al., 1996), *Pseudomonas* (Ikenaka et al., 1998), *Comamonas* (Ogawa et al., 1993) and *Sinorhizobium morelens* (Wu et al., 2005). Eleven potential D-carbamoylase producing strains preserved in our laboratory, including *Agrobacterium vitis*, *Alcaligenes faecalis*, *Cupriavidus necator*, *Mesorhizobium amorphae*, *Ralstonia pickettii*, *Loktanella hongkongensis*, *Pelagibaca bermudensis*, *Achromobacter xylosoxidans*, *Bradyrhizobium japonicum*, *Comamonas aquatic*, *Arthrobacter crystallopoietes*, were analyzed to identify putative D-carbamoylase. According to the phylogenetic analysis (Fig. 1A), all the putative D-carbamoylases displayed moderate identities (40–70%) to reported D-carbamoylases with hydrolytic activity toward *N*-carbamoyl-DL-tryptophan (**1a**). Eight of them were successfully ligated into pET28a and transformed into *E. coli* BL21(DE3). Only four D-carbamoylases genes were overexpressed in soluble or partially soluble form (E-supplementary), which were in consistence with the fact that it was often difficult to achieve soluble overexpression of carbamoylases in *E. coli* (Chen et al., 2005; Liu et al., 2008a,b). The D-carbamoylase from *Arthrobacter crystallopoietes* (accession No. AAO24770) was overexpressed mostly in soluble form. Activity assay of them revealed that the D-carbamoylases from *Comamonas aquatic* (accession No. EXU80228) and *A. crystallopoietes* displayed activity toward **1a**, with specific activity of 0.003 and 0.07 U mg^{-1} crude protein, respectively (E-supplementary). Considering the highest activity and solubility, the recombinant D-carbamoylase from *A. crystallopoietes* was designated as *Ac*HyuC and subjected to enzymatic characterization and evaluation in the hydantoinase process for potential preparation of D-tryptophan.

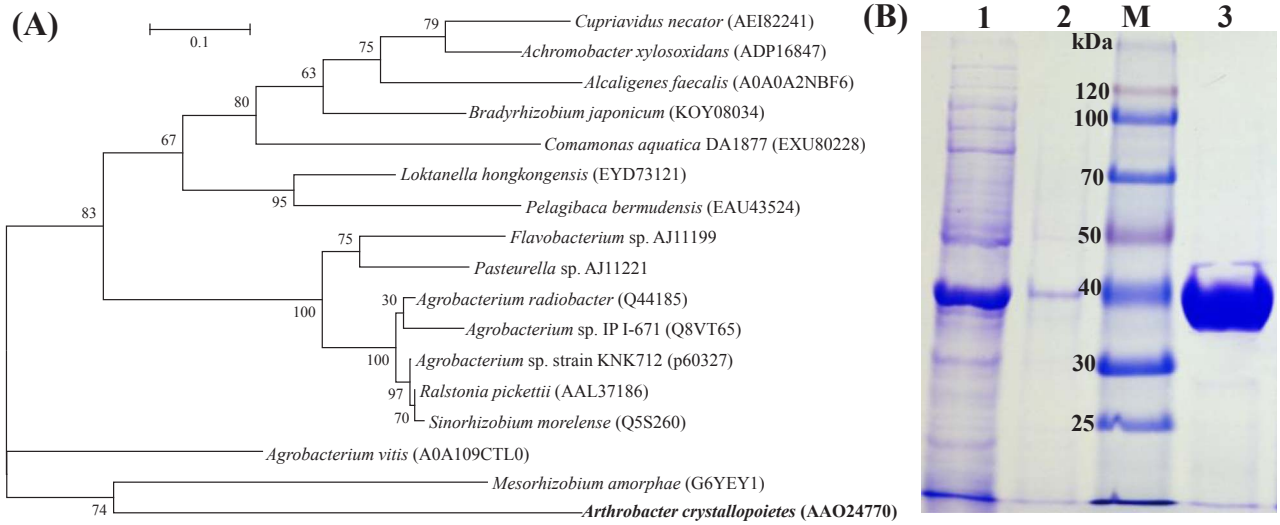

Fig. 1. (A) Phylogenetic analysis of *Ac*HyuC and d-carbamoylases from various microorganisms. (B) SDS-PAGE analysis of recombinant *Ac*HyuC expressed in *E. coli* BL21(DE3). The phylogenetic tree was constructed with MEGA 6.0 software by neighbor-joining method. The bootstrap values were based on 10,000 replicates. Lane 1: supernatant, Lane 2: precipitant, Lane 3: purified *Ac*HyuC, Lane M: protein molecular marker.

The *Ac*HyuC was classified into the class 6 of the larger nitrilase superfamily comprising nitrile- or amide-hydrolyzing enzymes and amide-condensing enzymes. The catalytic triad of *Ac*HyuC was presumed to be Glu46-Lys126-Cys171. The *Ac*HyuC is close to D-carbamoylase from *Mesorhizobium amorphae* and distant from other-reported D-carbamoylases with hydrolytic activity toward **1a** as illustrated in Fig. 1A. BLASTp analysis suggests that *Ac*HyuC displays the highest amino acid sequence identity of 50.7% to *N*-carbamoyl-D-amino acid amidohydrolase (PDB: 1FO6) from *Agrobacterium tumefaciens* (Wang et al., 2001). It has been reported that D-carbamoylases from *S. morelense* (*Sm*HyuC), *Agrobacterium* sp. KNK712 (Nanba et al., 1998), *Flavobacterium* sp. AJ11199 and *Pasteurella* sp. AJ11221 (Nozaki et al., 2005a,b) displayed activities toward **1a**. According to the multiple sequence alignment, amino acid sequence identities of 49.7%, 50.3%, 49.0% and 47.2% were observed with above mentioned D-carbamoylases respectively, indicating their differences in enzymatic structure and function.

3.2. Characterization of recombinant AcHyuC

3.2.1. Purification of AcHyuC

This recombinant *Ac*HyuC was purified to homogeneity by one-step nickel affinity chromatography. As shown in the SDS-PAGE (Fig. 1B), only one band was migrated at about 39 kDa, which was in good agreement with the theoretical molecular weight (38.28 kDa). Specific activity of purified *Ac*HyuC toward **1a** was 0.84 U mg^{-1}, 12-fold of the crude enzyme. Enantioselective analysis showed that only D-Trp was produced in the hydrolysis of racemic **1a**, suggesting this recombinant *Ac*HyuC was highly enantioselective. The *E* value was calculated to be 1057 toward **1a**.

3.2.2. Effect of temperature on the activity of AcHyuC

Effect of temperature on the activity of purified *Ac*HyuC was performed by determining the activity at different temperatures for 30 min. It was found that *Ac*HyuC was mesophilic and the optimum reaction temperature was 30 °C. Further increase or decrease in temperature led to significant decline in activity. Thermostability of *Ac*HyuC against 30 °C, 40 °C and 50 °C was determined by incubating at above temperatures and tracking the residual activity. *Ac*HyuC was stable at 30 °C, displaying a half-life of 14 h (Fig. 2A), and delicate at 40 °C and 50 °C. Most of the reported D-carbamoylases exhibit low thermostability, mainly because the cysteine in the catalytic center is liable to oxidization. The D-carbamoylase from *Agrobacterium radiobacter* NRRL B11291 (Grifantini et al., 1996) possesses five cysteines in its catalytic center, and was susceptible to aggregation under oxidizing conditions and highly sensitive to hydrogen peroxide. Based on site-directed mutagenesis and the crystal structure, Cys172 is confirmed to be one of the key residues responsible for the activity. When exposed to air, Cys172 could be oxidized and lead to inactivation of D-carbamoylase (Wang et al., 2001).

3.2.3. Effect of pH on the activity of AcHyuC

The pH preference of purified *Ac*HyuC was determined by measuring the activity in different buffer systems with pH ranging from 6.5 to 10.5 (Fig. 2B). The optimum pH for *Ac*HyuC was pH 8.5. However, *Ac*HyuC preferred sodium phosphate buffer (PBS) and displayed the highest activity (0.89 U mg^{-1}) in PBS (pH 8.0, 100 mM), which was 2.9- and 2.1-fold of those in pH 8.5 of Tris-HCl (0.31 U mg^{-1}) and Glycine-NaOH (0.43 U mg^{-1}). Considering the activity, PBS of pH 8.0 was regarded as the best condition for *Ac*HyuC. All the reported D-carbamoylases with hydrolytic activity toward **1a** exhibited the highest activity at pH 7.0. However, this neutral condition was not compatible with the activities of hydantoin racemase and hydantoinase, since their optimum pH was generally found to be basic (pH 8.0–9.5). As a result, *Ac*HyuC is more advantageous and compatible for the hydantoinase process.

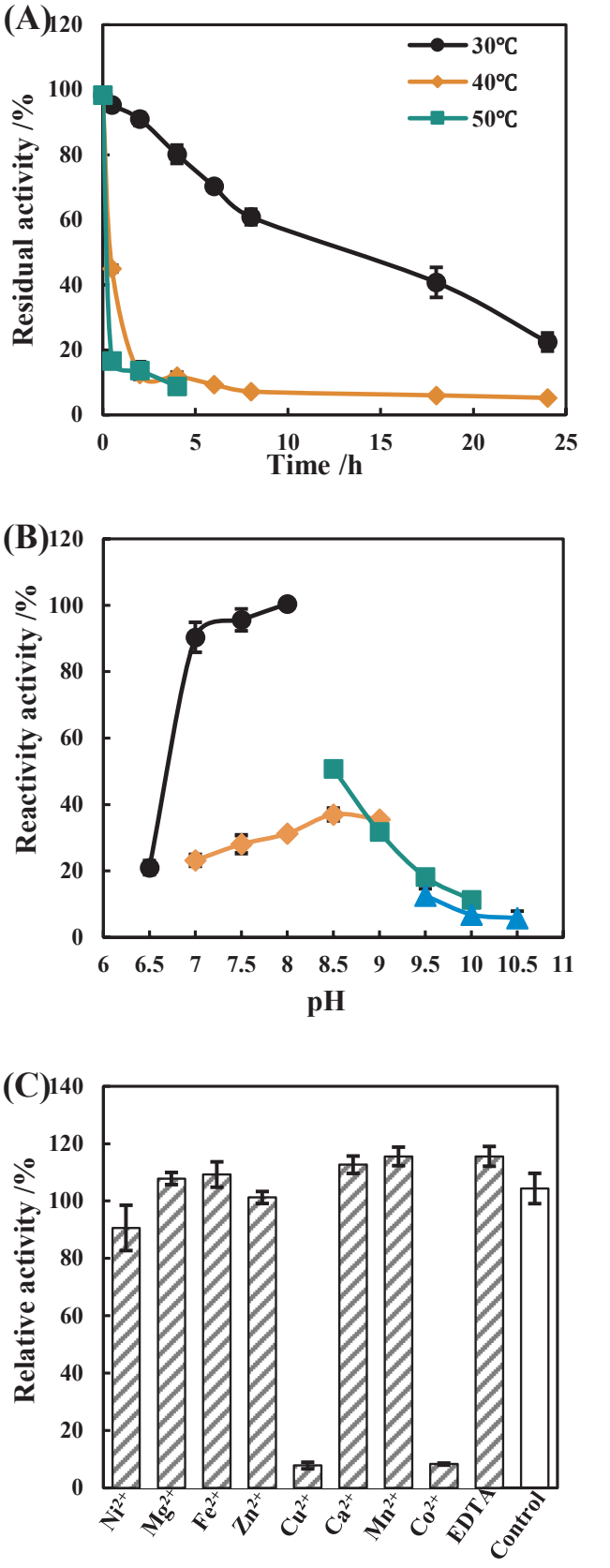

Fig. 2. Enzymatic properties of purified *Ac*HyuC. (A) Thermostability, (-●-): 30 °C, (-◆-): 40 °C, (-■-): 50 °C. (B) Optimum pH, (-●-): sodium phosphate buffer (pH 6.0–8.0, 100 mM), (-◆-): Tris–HCl (pH 7.0–9.0, 100 mM), (-■-): Glycine-NaOH (pH 8.5–10.0, 100 mM), (-▲-): sodium carbonate-sodium hydrogen carbonate (pH 9.5–10.5, 100 mM). (C) Metal ions and EDTA.

3.2.4. Effect of metal ions and EDTA on the activity of AcHyuC

Effects of various metal ions and EDTA on the activity of *Ac*HyuC were examined by incubation purified enzymes in PBS (pH 8.0, 100 mM) supplemented with additives (Fig. 2C). No significant change was found with divalent cations except for the addition of Co^{2+} and Cu^{2+} causing about 90% loss in activity of *Ac*HyuC. Since no influence was detected with EDTA, *Ac*HyuC was supposed to be non-metal ion dependent enzyme, which was similar as D-carbamoylases from *S. morelense*, *Agrobacterium* sp. KNK712, *Flavobacterium* sp. AJ11199 and *Pasteurella* sp. AJ11221.

3.2.5. Substrate spectrum

Substrate spectrum of *Ac*HyuC was investigated toward various *N*-carbamoyl-DL-amino acids including *N*-carbamoyl-DL-phenylalanine (**2a**), *N*-carbamoyl-DL-phenylglycine (**3a**), *N*-carbamoyl-DL-2-chlorophenylglycine (**4a**), *N*-carbamoyl-DL-methionine (**5a**), *N*-carbamoyl-DL-leucine (**6a**), *N*-carbamoyl-DL-isoleucine (**7a**). It was found that *Ac*HyuC showed no activity toward *N*-carbamoyl-L-amino acids and high specificity toward *N*-carbamoyl-D-amino acids. It preferred aromatic to aliphatic substituted *N*-carbamoyl-amino acids in the hydrolysis reaction and displayed the highest specific activity toward **1a** (0.84 U mg^{-1}). The activity of *Ac*HyuC toward **2a** (0.24 U mg^{-1}) and **3a** (0.042 U mg^{-1}) were lower than that of **1a**, which could be enantioselectively hydrolyzed into D-phenylalanine and D-phenylglycine. This is different from the recombinant D-carbamoylases from *S. morelens* S-5, *Flavobacterium* sp. AJ11199, *Pasteurella* sp. AJ11221 and *Agrobacterium* sp. KNK712, which displayed higher activity toward **2a** or **3a** than **1a**. No activity was detected with **4a**, suggesting the chloride substituent may affect its binding into the substrate pocket. It was surprisingly to found that *Ac*HyuC showed no detectable activity toward all tested aliphatic substituted *N*-carbamoyl-amino acids (**5a–7a**), which might be converted into D-methionine, D-leucine and D-isoleucine. The kinetic parameters of purified *Ac*HyuC was determined, and the K_m and V_{max} of purified *Ac*HyuC toward **1a** was 1.4 mM and 1.14 μmol min^{-1} mg^{-1}, which were at similar level to D-carbamoylases from *Flavobacterium* sp. AJ11199 and *Pasteurella* sp. AJ11221 with K_m and V_{max} of 0.13 mM and 0.43 μmol min^{-1} mg^{-1}, 0.37 mM and 2.27 μmol min^{-1} mg^{-1}, respectively. Although the specific activity with **1a** of this newly identified *Ac*HyuC was moderate compared with reported recombinant D-carbamoylases from *S. morelens* S-5 and *Agrobacterium* sp. KNK712, its alkaline pH optimum is desirable for dynamic kinetic resolution (DKR) hydantoinase process, which could be potential for the preparation of D-Trp.

3.3. Cloning and expression of AaHyuA and AtHyuH to construct a DKR cascade

To construct a DKR cascade system for the synthesis of D-Trp, a hydantoin racemase (*Aa*HyuA) from *A. aurescens* and a D-hydantoinase (*At*HyuH) from *A. tumefaciens* were also cloned and successfully overexpressed in *E. coli* BL21(DE3)Tab. *Aa*HyuA showed preference to aromatic substituted hydantoins and optimum pH of 8.0–9.0, while *At*HyuH displayed high activity toward D-IMHD and optimum pH of 9.5. It was also noticed that *Aa*HyuA was a non-metallic enzyme (E-supplementary), whereas *At*HyuH was a metalloenzyme and the specific activity could be improved for 6.37-fold in the presence of 0.5 mM Mn^{2+} (E-supplementary). Interestingly, Mn^{2+} does not have negative effect on the activities of *Ac*HyuC and *Aa*HyuA, which is undoubtedly beneficial for DKR reaction of hydantoinase process. The K_m and V_{max} of *Aa*HyuA and *At*HyuH toward L-IMHD and DL-IMHD were 2.13 and 1.17 mM, 24.0 and 3.96 μmol min^{-1} mg^{-1} respectively.

3.4. Optimization of DKR cascade for producing D-Trp

Generally, *in vivo* cascade by co-expression of several genes is often challenging, due to obstacles in harnessing the efficiency of natural pathway, such as kinetic imbalance, incompatibility and spatial arrangement etc, while *in vitro* cascade is advantageous in facile combination to break these hurdles (Guterl and Sieber, 2013). To develop a highly efficient and economic DKR process, enzymatic compatibility, including enzyme dosages, optimum pH and co-solvent tolerance etc, of *Aa*HyuA, *At*HyuH and *Ac*HyuC were evaluated.

3.4.1. Effect of pH and co-solvent on DKR cascade

First of all, effects of enzyme dosages on single step reaction were studied to understand the potential of *Aa*HyuA, *At*HyuH and *Ac*HyuC. It was observed that 2 kU L^{-1} *Aa*HyuA could convert 50% of L-IMHD (5 mM) into D-IMHD within 0.5 h, which was regarded as the appropriate dosage for *Aa*HyuA (E-supplementary). For *At*HyuH, 5 kU L^{-1} was sufficient for the hydrolysis of about 5 mM D-IMHD in 1.0 h (E-supplementary). With regard to *Ac*HyuC, 2 kU L^{-1} was adequate for the hydrolysis of 50% **1a** (10 mM) to give D-Trp within 1.0 h (E-supplementary). This DKR cascade was established employing appropriate dosages of *Aa*HyuA, *At*HyuH and *Ac*HyuC. Our results confirmed that 5 mM L-IMHD could be fully converted into D-Trp in 5 h with a yield of 99.5% (E-supplementary).

The pH compatibility is vital for the multi-enzyme reaction. In this hydantoinase process, the optimum pH of *Aa*HyuA, *At*HyuH and *Ac*HyuC was 8.0–9.0, 9.5 and 8.5 respectively. Then, effect of pH including 7.0 (PBS), 8.0 (PBS) and 9.0 (Glycine-NaOH) on the initial reaction rate and yield of the DKR cascade was investigated. As shown in Fig. 3A, initial reaction rates at pH 7.0, 8.0 and 9.0 were 0.057, 0.079 and 0.073 μmol min^{-1} mL^{-1} respectively. After 2.0 h of reaction, the yield of D-Trp at pH 7.0, 8.0 and 9.0 were 94.74%, 98.94% and 98.92%. The DKR cascade displayed higher initial reaction rate and yield at pH 9.0 than those at pH 7.0, mainly due to the basic preference of three

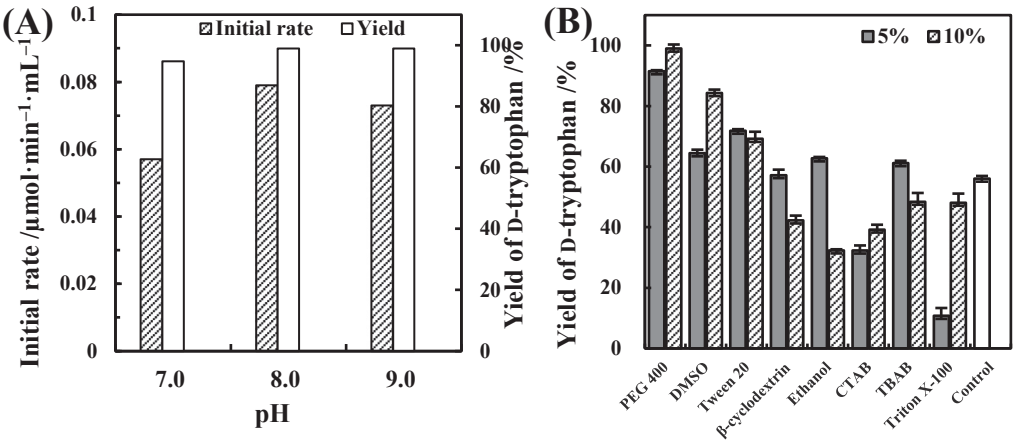

Fig. 3. Effect of pH and co-solvent on the cascade DKR reaction for the synthesis of D-tryptophan from L-IMHD. (A) pH. Reaction was performed with 5 mM L-IMHD, 5 kU L^{-1} of *Aa*HyuA, *At*HyuH and *Ac*HyuC in pH 7.0 (PBS, 100 mM), pH 8.0 (PBS, 100 mM) or pH 9.0 (Glycine-NaOH, 100 mM) at 30 °C for 6 h. (B) Co-solvents. Reactions were carried out with 50 mM L-IMHD, 5 kU L^{-1} of *Aa*HyuA, *At*HyuH and *Ac*HyuC, 0.5 mM $MnCl_2$, 5% or 10% co-solvents in PBS (pH 8.0, 100 mM) at 30 °C for 24 h. All the experiments were performed in triplicate.

enzymes and the higher spontaneous racemization of L-IMHD at pH 9.0 (Andújar-Sánchez et al., 2006). Since the highest initial reaction rate and yield were observed in pH 8.0 PBS, it was regarded as the suitable reaction buffer for the cascade reaction. 5′-Monosubstituted hydantoins, especially L-IMHD, are sparingly soluble in water, which severely limits the efficiency of reaction (Qian et al., 2012). To solve this issue, different additives were evaluated to improve the substrate solubility. They were divided into four categories: nonionic surfactant (including PEG 400, Tween 20, β-cyclodextrin and Triton X-100), cation surfactant cetyltrimethyl ammonium bromide (CTAB), common polar solvent (such as ethanol and DMSO), and phase transfer catalyst tetrabutylammonium bromide (TBAB). Considering the toxicity of additives on enzymes, a lower (5%, v/v) and a higher amount (10%, v/v) of additives were tested with 50 mM L-IMHD in the DKR cascade. In Fig. 3B, 5% and 10% of PEG400 can effectively facilitate substrate conversion, resulting in 91.5% and 98.9% yield of D-Trp at 24 h, representing 35.5% and 42.9% increases than that without additives. Addition of ethanol and TBAB at 5% (v/v) could slightly improve the yield of D-Trp, while reduced yield of 32.3% and 48.4% were noted at 10% (v/v), ascribing to the toxicity of additives. Supplementation with 10% DMSO and 10% Tween 20 rendered increased D-Trp yields of 84.3% and 69.1%, indicating the excellent solubilization effect of DMSO and Tween 20, and their good compatibility. No positive effect was found with *β*-cyclodextrin, Triton X-100 and CTAB. Consequently, 10% PEG 400 was selected as the co-solvent for the DKR cascade.

3.4.2. Optimization of substrate/enzyme loadings in DKR cascade

To evaluate the potential of this DKR cascade in preparation of D-Trp, substrate and biocatalyst loadings were investigated (Table 1). Employing 2 kU L^{-1} *Aa*HyuA, 5 kU L^{-1} *At*HyuH and 2 kU L^{-1} *Ac*HyuC, 5 mM (1.15 g L^{-1}) L-IMHD could be completely converted within 4 h with D-Trp yield of 99.5% (entry 1). Under the same condition, at substrate loadings of 10 mM (2.3 g L^{-1}) (entry 2) and 20 mM (4.6 g L^{-1}) (entry 3), it took 6 h and 12 h to reach 99.7% conversion, and D-Trp yields of 98.3% and 98.1%. Further increasing the concentration of L-IMHD to 40 mM (9.2 g L^{-1}), the conversion could reach 98.5% at 12 h, but the yield of D-Trp was reduced to 86.7% (entry 4) and more intermediate (*N*-carbamoyl-DL-tryptophan) was accumulated. It was speculated that the dosage of *Ac*HyuC was insufficient. As shown in entries 5–7, yields of D-Trp increased from 86.7% to 97.0% along with the increase of *Ac*HyuC from 2 to 5 kU L^{-1}. Short of *Aa*HyuA might be another reason, since inadequate *Aa*HyuA could limit the formation of D-IMHD, hence reduce the following reaction rates, and elongate the reaction time which further lead to the inactivation of *Ac*HyuC. When *Aa*HyuA was increased from 2 to 5 kU L^{-1} (entries 8 and 9), the conversion and yield of D-Trp were increased to 100% and 99.5% as expected. Under the same condition, 50 mM L-IMHD (11.5 g L^{-1}) could be transformed with 99.6% conversion and 97.8% yield. Further increasing the amount of *Aa*HyuA to 10 kU L^{-1} resulted in 99.7% conversion and 98.6% yield at 50 mM L-IMHD (entry 11). The substrate concentration was further increased to 80 mM (18.4 g L^{-1}). With the same amount of enzymes as entry 11, the conversion and yield were significantly reduced to 93.2% and 83.4% (entry 12). Enzyme dosages of *Aa*HyuA, *At*HyuH and *Ac*HyuC and the reaction time were further adjusted to realize complete conversion (entries 13–15). In entry 15, doubled dosage of *Ac*HyuC gave 99.5% conversion and 98.4% yield at extended reaction time of 24 h, further confirming the importance of D-carbamoylase in this DKR cascade as above mentioned. Although further increasing substrate loading to 100 mM (22.9 g L^{-1}) resulted in slightly decreased conversion of 98.0% and yield of 93.4% at 24 h (entry 16), optimization of the ratio and dosage of enzymes would also facilitate to achieve full conversion (data not shown).

3.5. Preparation of D-Trp at gram scale

This one-pot DKR cascade was up-scaled for the preparation of D-Trp at gram scale. In a 0.5 L reaction system, 9.16 g L-IMHD (80 mM), 5 kU *Aa*HyuA, 2.5 kU *At*HyuH and 5 kU *Ac*HyuC, 0.25 mmol $MnCl_2$ were mixed and magnetically agitated at 150 rpm and 30 °C. As illustrated in Fig. 4, L-IMHD was rapidly racemized and enantioselectively hydrolyzed into D-Trp within 6 h, resulting a conversion of 80%. Then, the conversion rate gradually decreased in the following 6 h, and the L-IMHD was completely converted into D-Trp with a molar yield of 99.4% at 12 h. It was noteworthy that merely 5 mM intermediate accumulated in the first 6 h, which was almost hydrolyzed into D-Trp by *Ac*HyuC in the following 6 h, and only 0.52 mM was remained at the end of reaction. After the reaction, D-Trp was isolated and purified. The pH of the reaction mixture was adjusted to pH 2.0, then centrifuged and filtered to remove the proteins. Then supernatant was loaded on cation exchange resin activated by 2.0 M NaOH for 2 h, and the D-Trp was eluted by ammonium hydroxide. After rotary evaporation and crystallization, about 6.75*g* optically pure D-Trp was harvested with over 99.9% *e.e.* and 84.3% isolation yield. Product was verified by ^{1}H NMR and enantioselective analysis. Only one peak retained at 9.4 min in the chiral HPLC spectra, which is consistent with the retention time of the standard D-Trp. D-Trp ($C_{11}H_{12}N_2O_2$, 1b) ^{1}H NMR (400 MHz, DMSO-d_6): *δ*

Table 1
Cascade DKR reaction for the synthesis of D-Trp from L-indolylmethylhydantoin.

Entry	Substrate (mM)	*Aa*HyuA (kU L^{-1})	*At*HyuH (kU L^{-1})	*Ac*HyuC (kU L^{-1})	Time (h)	Conversion[a] (%)	Yield[b] (%)	*e.e.*[c] (%)
1	5	2	5	2	4	100	99.5	> 99
2	10	2	5	2	6	99.7	98.3	> 99
3	20	2	5	2	12	99.7	98.1	> 99
4	40	2	5	2	12	98.5	86.7	> 99
5	40	2	5	3	12	98.5	86.9	> 99
6	40	2	5	4	12	99.4	94.2	> 99
7	40	2	5	5	12	99.7	97.0	> 99
8	40	4	5	3	12	99.2	92.1	> 99
9	40	5	5	5	12	100	99.5	> 99
10	50	5	5	5	12	99.6	97.8	> 99
11	50	10	5	5	12	99.7	98.6	> 99
12	80	10	5	5	12	93.2	83.4	> 99
13	80	20	5	5	24	98.7	94.0	> 99
14	80	10	10	5	24	99.2	95.7	> 99
15	80	10	5	10	24	99.5	98.4	> 99
16	100	10	5	10	24	98.0	93.4	> 99

[a] Conversion was determined according to the decrease of L-IMHD and was analyzed by RE-HPLC equipped with Diamonsil C18 column at 30 °C, 210 nm and flow rate of 0.8 mL min^{-1} with 25% acetonitrile and 75% KH_2PO_4 as mobile phase.
[b] Yield was calculated according to the production of D-Trp.
[c] *e.e.* was determined by HPLC equipped with Chirobiotic T column at 30 °C, 210 nm and flow rate of 0.5 mL min^{-1} with 40% methanol and 60% H_2O as mobile phase.

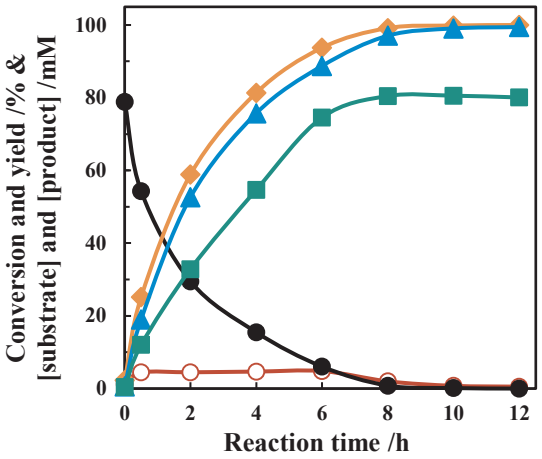

Fig. 4. Time course of one-pot DKR reaction for the preparation of D-tryptophan from L-IMHD at gram scale. (-●-): concentration of L-IMHD, (-○-) concentration of *N*-carbamoyl-D-tryptophan, (-■-): concentration of D-tryptophan, (-◆-): conversion of substrate, (-▲-): yield of D-tryptophan. The 0.5-L reaction system containing 0.4 g *At*HyuH, 5 g *Ac*HyuC, 0.25 mM $MnCl_2$ and 9.16 g L-IMHD (80 mM) in PBS (pH 8.0, 100 mM) was conducted at 30 °C and 150 rpm with mechanical agitation.

10.97 (s, 1H), δ 7.86–6.71 (m, 6H), δ 3.48–3.37 (m, 2H), δ 3.31 (d, J = 15.1 Hz, 2H), δ 2.97 (dd, J_1 = 14.9 Hz, J_2 = 9.0 Hz, 1H).

Hydantoinase process has been widely used in the synthesis of D-amino acids. For example, D-*p*-hydroxyphenylglycine was prepared from 130 mM DL-5-*p*-hydroxyphenylhydantoin with molar yield of 94% at 30 h (Jiang et al., 2007); D-methionine was prepared from 300 mM DL-5-methylthioethylhydantoin with molar yield of 100% at 6 h (Martínez-Gómez et al., 2007). However, few has been reported on the production of D-Trp. A *in vivo* cascade reaction has been constructed by co-expression of HyuA from *Microbacterium liquefaciens* AJ3912, D-HyuH and D-HyuC from *Flavobacterium* sp. AJ11199 and employed in the preparation of D-Trp. At 110 mM DL-IMHD, D-Trp yield and productivity of 96% and 12.0 g L^{-1} d^{-1} were attained at 48 h (Nozaki et al., 2005a,b). The hydantoinase process developed in this study was applied in the dynamic kinetic resolution of L-IMHD, with a productivity of 36.6 g L^{-1} d^{-1}, much more efficient than the previous report. This study provides a newly identified D-carbamoylase from *Arthrobacter crystallopoietes* for the DKR preparation of D-Trp with high yield and enantioselectivity.

4. Conclusion

In summary, an enantioselective D-carbamoylase was identified from *Arthrobacter crystallopoietes* and designated as *Ac*HyuC. The optimum pH of *Ac*HyuC was 8.5, higher than those of reported D-carbamoylases. Recombinant *Ac*HyuC displayed high D-enantioselectivity and preferred aromatic to aliphatic substrates. A three-enzyme DKR cascade was developed for the synthesis of D-tryptophan from L-indolylmethylhydantoin. As much as 80 mM L-indolylmethylhydantoin could be completely converted into D-tryptophan within 12 h in a 0.5-L system, reaching a yield of 99% and productivity of 36.6 g L^{-1} d^{-1}. This study provides an efficient and feasible process for the production of optically pure D-Trp.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.biortech.2017.09.162.

References

Andújar-Sánchez, M., Martínez-Rodríguez, S., Las Heras-Vázquez, F.J., Clemente-Jiménez, J.M., Rodríguez-Vico, F., Jara-Pérez, V., 2006. Binding studies of hydantoin racemase from *Sinorhizobium meliloti* by calorimetric and fluorescence analysis. Biochim. Biophys. Acta 1764, 292–298.

Aranaz, I., Acosta, N., Fernandez-Valle, M.E., Heras, A., 2015. Optimization of D-amino acid production catalyzed by immobilized multi-enzyme system in polyelectrolyte complex gel capsules. J. Mol. Catal. B: Enzym. 121, 45–52.

Buson, A., Negro, A., Grassato, L., Taqliaro, M., Basaqlia, M., Grandi, C., Fontana, A., Nuti, M.P., 1996. Identification, sequencing and mutagenesis of the gene for a D-carbamoylase from *Agrobacterium radiobacter*. FEMS Microbiol. Lett. 145, 55–62.

Chen, H.M., Lin, K.Y., Lu, C.H., 2005. Refolding and activation of recombinant *N*-carbamoyl-D-amino acid amidohydrolase from *Escherichia coli* inclusion bodies. Process Biochem. 40, 2135–2141.

Clemente-Jiménez, J.M., Martínez-Rogríguez, S., Mingorance-Cazorla, L., De La Escalera-Hueso, S., Las Heras-Vázquez, F.J., Rodríguez-Vico, F., 2003. Catalytic analysis of a recombinant D-hydantoinase from *Agrobacterium tumefaciens*. Biotechnol. Lett. 25, 1067–1073.

Eadie, G.S., Bernheim, F., Bernheim, M.L., 1949. The partial purification and properties of animal and plant hydantoinases. J. Biol. Chem. 181, 449–458.

Enoki, J., Meisborn, J., Müller, A.C., Kourist, R., 2016. A multi-enzymatic cascade reaction for the stereoselective production of γ-oxyfunctionalized amino acids. Front. Microbiol. 7, 425.

Fujii, N., 2002. D-amino acids in living higher organisms. Orig. Life Evol. Biosph. 32, 103–127.

Gao, X., Ma, Q., Zhu, H., 2015. Distribution, industrial applications, and enzymatic synthesis of D-amino acids. Appl. Microbiol. Biotechnol. 99, 3341–3349.

Gómez Baraibar, Á., Reichert, D., Mügge, C., Seger, S., Gröger, H., Kourist, R., 2016. A one-pot cascade reaction combining an encapsulated decarboxylase with a metathesis catalyst for the synthesis of bio-based antioxidants. Angew. Chem. Int. Ed. 55, 14823–14827.

Greenstein, J.P., 1957. Resolution of DL mixtures of α-amino acids. Methods Enzymol. 3, 554–570.

Grifantini, R., Pratesi, C., Galli, G., Grandi, G., 1996. Topological mapping of the cysteine residues of *N*-carbamyl-D-amino-acid amidohydrolase and their role in enzymatic activity. J. Biol. Chem. 271, 9326–9331.

Guterl, J.K., Sieber, V., 2013. Biosynthesis "debugged": novel bioproduction strategies. Eng. Life Sci. 13, 4–18.

Ikenaka, Y., Nanba, H., Yamada, Y., Yajima, K., Takano, M., Takahashi, S., 1998. Screening, characterization, and cloning of the gene for *N*-carbamyl-D-amino acid amidohydrolase from thermotolerant soil bacteria. Biosci. Biotechnol. Biochem. 62, 882–886.

Jiang, M., Shang, L., Wei, P., Yu, R., Shen, N., Ouyang, P., Chang, H.N., 2007. Pilot-scale production of D-*p*-hydroxyphenylglycine from DL-5-*p*-hydroxyphenylhydantoin by *Burkholderia cepacia* JS-02. Enzym. Microb. Technol. 41, 407–412.

Kawasaki, K., Yokota, A., Tomita, F., 1995. Enzymatic synthesis of l-tryptophan by *Enterobacter aerogenes* tryptophanase highly expressed in *Escherichia coli*, and some properties of the purified enzyme. Biosci. Biotechnol. Biochem. 59, 1938–1943.

Liu, Y., Li, Q., Hu, X., Yang, J., 2008a. Construction and co-expression of polycistronic plasmid encoding D-hydantoinase and D-carbamoylase for the production of D-amino acids. Enzym. Microb. Technol. 42, 589–593.

Liu, Y., Li, Q., Hu, X., Yang, J., 2008b. Using native hydantoinase promoter to induce D-carbamoylase soluble expression in *Escherichia coli*. Biochem. Eng. J. 41, 12–16.

Luo, H., Zhu, L., Chang, Y., Liu, X., Liu, Z., Sun, H., Li, X., Yu, H., Shen, Z., 2017. Microenvironmental pH changes in immobilized cephalosporin C acylase during a proton-producing reaction and regulation by a two-stage catalytic process. Bioresour. Technol. 223, 157–165.

Martínez-Gómez, A.I., Martínez-Rodríguez, S., Clemente-Jiménez, J.M., Pozo-Dengra, J., Rodríguez-Vico, F., Las Heras-Vázquez, F.J., 2007. Recombinant polycistronic structure of hydantoinase process genes in *Escherichia coli* for the production of optically pure D-amino acids. Appl. Environ. Microbiol. 73, 1525–1531.

Martínez-Rodríguez, S., Martínez-Gómez, A.I., Rodríguez-Vico, F., Clemente-Jiménez, J.M., Heras-Vázquez, L., Javier, F., 2010. Natural occurrence and industrial applications of D-amino acids: an overview. Chem. Biodivers. 7, 1531–1548.

Nanba, H., Ikenaka, Y., Yamada, Y., Yajima, K., Takano, M., Takahashi, S., 1998. Isolation of *Agrobacterium* sp. strain KNK712 that produces *N*-carbamyl-D-amino acid amidohydrolase, cloning of the gene for this enzyme, and properties of the enzyme. Biosci. Biotechnol. Biochem. 62, 875–881.

Nozaki, H., Kira, I., Watanabe, K., Yokozeki, K., 2005a. Purification and properties of D-hydantoin hydrolase and *N*-carbamoyl-D-amino acid amidohydrolase from *Flavobacterium* sp. AJ11199 and *Pasteurella* sp. AJ11221. J. Mol. Catal. B: Enzym. 32, 205–211.

Nozaki, H., Takenaka, Y., Kira, I., Watanabe, K., Yokozeki, K., 2005b. D-Amino acid production by *E. coli* co-expressed three genes encoding hydantoin racemase, D-hydantoinase and *N*-carbamoyl-D-amino acid amidohydrolase. J. Mol. Catal. B: Enzym. 32, 213–218.

Ogawa, J., Shimizu, S., Yamada, H., 1993. *N*-Carbamoyl-D-amino acid amidohydrolase from *Comamonas* sp. E222c purification and characterization. FEBS J. 212, 685–691.

Patching, S.G., 2011. Efficient syntheses of ^{13}C-and ^{14}C-labelled 5-benzyl and 5-indolylmethyl L-hydantoins. J. Labelled Compd. Radiopharm. 54, 110–114.
Qian, J., Chen, C., Liu, M., Lv, B., 2012. Bioconversion producing D-*p*-hydroxyphenylglycine from DL-p-hydroxyphenylhydantoin by *Pseudomonas putida* in aqueous two-phase system. Indian J. Biotechnol. 11, 445–452.
Radkov, A.D., Moe, L.A., 2014. Bacterial synthesis of D-amino acids. Appl. Microbiol. Biotechnol. 98, 5363–5374.
Runser, S., Chinski, N., Ohleyer, E., 1990. D-*p*-Hydroxyphenylglycine production from DL-5-*p*-hydroxyphenylhydantoin by *Agrobacterium* sp. Appl. Microbiol. Biotechnol. 33, 382–388.
Sareen, D., Sharma, R., Vohra, R.M., 2001. Chaperone-assisted overexpression of an active D-carbamoylase from *Agrobacterium tumefaciens* AM 10. Protein Exp. Purif. 23, 374–379.
Vranova, V., Zahradnickova, H., Janous, D., Skene, K.R., Matharu, A.S., Rejsek, K., Formanek, P., 2012. The significance of D-amino acids in soil, fate and utilization by microbes and plants: review and identification of knowledge gaps. Plant Soil 354, 21–39.
Wang, W.C., Hsu, W.H., Chien, F.T., Chen, C.Y., 2001. Crystal structure and site-directed mutagenesis studies of *N*-carbamoyl-D-amino-acid amidohydrolase from *Agrobacterium radiobacter* reveals a homotetramer and insight into a catalytic cleft. J. Mol. Biol. 306, 251–261.
Wiese, A., Syldatk, C., Mattes, R., Altenbuchner, J., 2001. Organization of genes responsible for the stereospecific conversion of hydantoins to α-amino acids in *Arthrobacter aurescens* DSM 3747. Arch. Microbiol. 176, 187–196.
Wu, S., Liu, Y., Zhao, G., Wang, J., Sun, W., 2006. Thermostable D-carbamoylase from *Sinorhizobium morelens* S-5: purification, characterization and gene expression in *Escherichia coli*. Biochimie 88, 237–244.
Wu, S., Yang, L., Liu, Y., Zhao, G., Wang, J., Sun, W., 2005. Enzymatic production of D-*p*-hydroxyphenylglycine from DL-5-*p*-hydroxyphenylhydantoin by *Sinorhizobium morelens* S-5. Enzym. Microb. Technol. 36, 520–526.
Wu, S., Zhou, Y., Wang, T., Too, H.P., Wang, D.I., Li, Z., 2016. Highly regio- and enantioselective multiple oxy- and amino-functionalizations of alkenes by modular cascade biocatalysis. Nat. Commun. 7.
Xu, G.C., Li, L., Han, R.Z., Dong, J.J., Ni, Y., 2016. Characterization and soluble expression of D-hydantoinase from *Pseudomonas fluorescens* for the synthesis of D-amino acids. Appl. Biochem. Biotechnol. 179, 1–15.
Yamaguchi, S., Komeda, H., Asano, Y., 2007. New enzymatic method of chiral amino acid synthesis by dynamic kinetic resolution of amino acid amides: use of stereoselective amino acid amidases in the presence of α-amino-ε-caprolactam racemase. Appl. Environ. Microbiol. 73, 5370–5373.
Yamamoto, H., Mitsuhashi, K., Matsuyama, A., Tomita, F., 1998. Method for producing D-tryptophan. European Patent, EP0853128A1.
Zhou, Y., Wu, S., Li, Z., 2016. Cascade biocatalysis for sustainable asymmetric synthesis: from biobased L-phenylalanine to high-value chiral chemicals. Angew. Chem. Int. Ed. 128, 11819–11822.