



# Characterization of a new nitrilase from *Hoeflea phototrophica* DFL-43 for a two-step one-pot synthesis of (*S*)- $\beta$ -amino acids

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## Abstract

A nitrilase from *Hoeflea phototrophica* DFL-43 (HpN) demonstrating excellent catalytic activity towards benzoylacetone nitrile was identified from a nitrilase tool-box, which was developed previously in our laboratory for (*R*)-*o*-chloromandelic acid synthesis from *o*-chloromandelonitrile. The HpN was overexpressed in *Escherichia coli* BL21 (DE3), purified to homogeneity by nickel column affinity chromatography, and its biochemical properties were studied. The HpN was very stable at 30–40 °C, and highly active over a wide range of pH values (pH 6.0–10.0). In addition, the HpN could tolerate against several hydrophilic organic solvents. Steady-state kinetics indicated that HpN was highly active towards benzoylacetone nitrile, giving a  $K_M$  of 4.2 mM and a  $k_{cat}$  of 170 s<sup>-1</sup>, the latter of which is ca. fivefold higher than the highest record reported so far. A cascade reaction for the synthesis of optically pure (*S*)- $\beta$ -phenylalanine from benzoylacetone nitrile was developed by coupling HpN with an  $\omega$ -transaminase from *Polaromonas* sp. JS666 in toluene-water biphasic reaction system using  $\beta$ -alanine as an amino donor. Various (*S*)- $\beta$ -amino acids could be produced from benzoylacetone nitrile derivatives with moderate to high conversions (73–99%) and excellent enantioselectivity (> 99% *ee*). These results are significantly advantageous over previous studies, indicating a great potential of this cascade reaction for the practical synthesis of (*S*)- $\beta$ -phenylalanine in the future.

**Keywords** Biocatalysis · Nitrilase ·  $\omega$ -Transaminase · Cascade reaction · (*S*)- $\beta$ -Phenylalanine

## Introduction

Optically pure  $\beta$ -amino acids occur in numerous natural products with potent biological activities and represent an important class of chiral building blocks for a variety of pharmaceuticals, agrochemicals, and other fine chemicals (Jin et al. 2006; Ruf et al. 2012). They can also be incorporated into proteins and peptide mimics to improve their stability and therapeutic properties (Steer et al. 2002). Due to the significance of enantiomerically pure  $\beta$ -amino acids, great efforts have been made towards their efficient synthesis (Weiner et al. 2010). However, chemical methods are usually performed in kinetic resolution mode whose theoretical yield is only 50% and typically require the acquisition of chiral precursors, high loads of

expensive catalysts or chiral auxiliaries, and careful control of the reaction conditions. All of the abovementioned drawbacks limit the practical application of chemical methods.

As an alternative, biocatalytic methods are attracting tremendous attention because of the environmentally benign reaction conditions and high selectivities (chemoselectivity, regioselectivity, and enantioselectivity) (Bornscheuer et al. 2012; Xu et al. 2017). Therefore, several enzymatic routes for the synthesis of optically pure  $\beta$ -amino acids have been developed, including kinetic resolution of racemic  $\beta$ -amino acids or their ester/amide derivatives (Bea et al. 2011; Li et al. 2013; Weise et al. 2017; Wu et al. 2010; You et al. 2013) and asymmetric amination/transamination of respective keto acids or asymmetric addition of ammonia to acrylates (Kim et al. 2007; Weise et al. 2015; Zhang et al. 2015a). In the case of kinetic resolution, the theoretical yield of only 50% limits their practical application, while in the case of asymmetric synthesis, the biocatalysts involved suffer from either low activity (Kim et al. 2007; Weise et al. 2015; Zhang et al. 2015a) or poor regioselectivity/enantioselectivity (Weise et al. 2015), resulting in low yields of the target product. Additionally, the preparation of racemic  $\beta$ -amino acids and

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their derivatives are relatively complicated and highly expensive, which would significantly increase the cost of the process. Therefore, there is still great need for the development of new methods for the asymmetric synthesis of optically pure  $\beta$ -amino acids from cheap and easily available starting materials.

Nitrilase, which catalyzes the hydrolysis of nitriles to carboxylic acids, is considered as one of the most promising industrial-relevant biocatalysts and has been employed for the production of optically pure (*R*)-(-)-mandelic acid at BASF in an industrial scale (Ress-Loschke et al. 2005). Nitrilases from different origins have also been applied for the synthesis of various bulk chemicals and fine chemicals, such as acrylic acid, lactams, glycolic acid, nicotinic acid, (*R*)-2-chloromandelic acid, (*R*)/(*S*)-4-cyano-3-hydroxybutyric acid, *S*-ibuprofen, (3*S*)-3-cyano-5-methyl hexanoic acid, (*R*)-ethyl-3-hydroxyglutarate, as well as (*R*)/(*S*)- $\beta$ -hydroxy acids (Ankati et al. 2009; DeSantis et al. 2003; Dong et al. 2010; Gavagan et al. 1998; Nagasawa et al. 1990; Stepanek et al. 1999; Wu et al. 2008; Xie et al. 2006; Yamamoto et al. 1990; Zhang et al. 2012). The synthetic applications and future perspectives of nitrilases were reviewed elsewhere (Gong et al. 2017; Martinkova and Kren 2010; Martinkova and Mylerova 2003). Recently, we also reported the application of nitrilases from *Alcaligenes* sp. ECU0401 and *Labrenzia aggregata* for the efficient synthesis of (*R*)-(-)-mandelic acid and (*R*)-*o*-chloromandelic acid, respectively (Zhang et al. 2010, 2012, 2015b).

In the present study, a nitrilase from *Hoeflea phototrophica* DFL-43, which shows very high activity towards benzoylacetoneitrile, was identified by screening the nitrilase tool-box previously developed in our laboratory for (*R*)-*o*-chloromandelic acid production from *o*-chloromandelonitrile (Zhang et al. 2012), and its biochemical properties were characterized in detail. In addition, a new strategy for the asymmetric synthesis of optically pure  $\beta$ -phenylalanine from benzoylacetoneitrile was developed by coupling the nitrilase-catalyzed benzoylacetoneitrile hydrolysis with an  $\omega$ -transaminase-mediated transamination of benzoylacetic acid (Scheme 1). Although  $\beta$ -amino acids could also be produced from  $\beta$ -nitriles by a one-pot two-step process employing a nitrilase and an amino acid dehydrogenase, however, a third enzyme (glucose dehydrogenase) is needed to regenerate the

expensive NADPH (Zhang et al. 2015a). Recently, a similar process coupling a nitrilase and an  $\omega$ -transaminase for the synthesis of  $\beta$ -amino acids from  $\beta$ -nitriles was reported (Mathew et al. 2017). The weakness of this process is that the expensive amino donor, optically pure (*S*)-phenethylamine, was needed to achieve a moderate conversion. In addition, the byproduct acetophenone served as a strong inhibitor for  $\omega$ -transaminase, which would reduce the total yield of the product (Bea et al. 2011; Kim et al. 2007).

## Materials and methods

### Materials

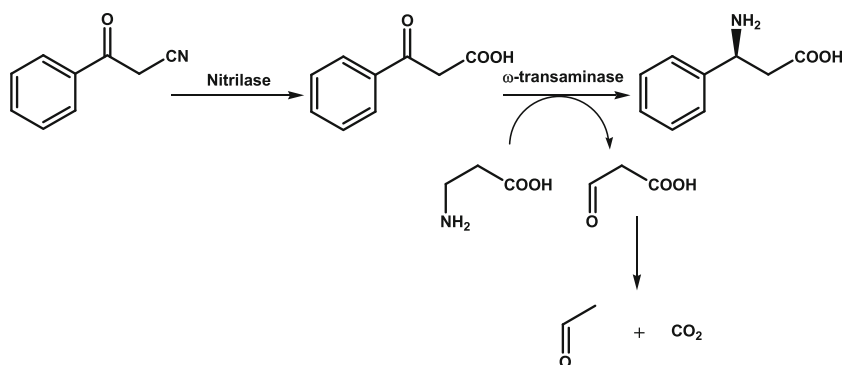
Benzoylacetoneitrile (98%) was obtained from Energy Chemicals (Shanghai, China), racemic  $\beta$ -phenylalanine (99%) was purchased from Accela ChemBio Co., Ltd. (Shanghai, China),  $\beta$ -alanine (98%), pyridoxal 5'-phosphate (PLP, 98%), and 2,3,4,6-tetra-*O*-acetyl- $\beta$ -D-glucopyranosyl isothiocyanate (GITC, 98%) were provided by Aladdin (Shanghai, China). Cation resin D001 was kindly donated by Shanghai Huazhen Sci. & Tech. Co., Ltd. All other chemicals with the highest purity were also obtained commercially and used without further purification unless otherwise stated.

The cloning and overexpression of nitrilases from different origins were based on our previous studies (Zhang et al. 2012). Gene sequence of the  $\omega$ -transaminase from *Polaromonas* sp. JS666 (GenBank accession number: ABE43415.1) as described previously (Bea et al. 2011) was synthesized by Genscript (Nanjing, China) and cloned into the vector pET28a (+). The  $\omega$ -transaminase was overexpressed in *E. coli* BL21 (DE3) as described previously (Bea et al. 2011).

### Purification of recombinant nitrilase HpN

Recombinant *E. coli* cells expressing HpN were harvested by centrifugation (8000 $\times$ g) at 4 °C for 10 min, washed twice by physiological saline, and resuspended in buffer A (20 mM sodium phosphate, 500 mM NaCl, 10 mM imidazole, pH

**Scheme 1** Two-step one-pot synthesis of (*S*)- $\beta$ -phenylalanine from benzoylacetoneitrile



8.0), then the cells were disrupted by sonication with an ultrasonic oscillator (JY92-II, Scientz Biotech. Co., Ltd.). The cell debris was removed by centrifugation (12,000×g) at 4 °C for 40 min. Then the supernatant was loaded onto a His-trap Ni-NTA FF column (5 mL, GE Healthcare Corp.) pre-equilibrated with buffer A, and the proteins were eluted with an increasing gradient of imidazole from 10 to 500 mM in buffer A at a flow rate of 5 mL/min. The fractions containing the target protein were concentrated to about 1 mL and exchanged with potassium phosphate buffer (20 mM, pH 8.0; 4 × 10 mL) for reducing the imidazole concentration using centrifugal filter units with 10 kDa cutoff (Millipore, USA). Protein purity was confirmed by SDS-PAGE analysis, and protein concentration was determined by the Bradford method using bovine serum albumin as the standard (Bradford 1976).

### Activity assay

For nitrilase: the reaction mixture (1 mL) containing 10 mM benzoylacetoneitrile (200 mM stock solution in DMSO), 10 µg of enzyme in potassium phosphate buffer (100 mM, pH 8.0), was incubated at 30 °C and 1000 rpm on a Thermomixer Compact (Eppendorf, Germany). Samples (100 µL) were withdrawn at 10 min, and the reaction was stopped by adding 10 µL HCl (2 M) and extracted with 500 µL ethyl acetate for GC analysis. The conversion was determined by measuring the amount of benzoylacetoneitrile consumed in the reaction. One unit of enzyme activity was defined as the amount of enzyme consuming 1 µmol of benzoylacetoneitrile per minute under the standard assay conditions.

For ω-TA: the reaction mixture (10 mL) containing 20 mM benzoylacetoneitrile in 5 mL toluene, 0.1 mM PLP, 200 mM β-alanine, 50 mg lyophilized nitrilase powder, and 150 mg of lyophilized transaminase powder in 5 mL potassium phosphate buffer (100 mM, pH 8.0) was incubated at 37 °C and 180 rpm. The aqueous layer (100 µL) was withdrawn at 30 min for HPLC analysis. The conversion was determined by measuring the amount of β-phenylalanine produced in the reaction. One unit of enzyme activity was defined as the amount of enzyme producing 1 µmol of β-phenylalanine per minute under the coupled reaction conditions.

### Effect of temperature and pH on the activity of HpN and stability

The effects of temperature and pH on HpN were determined by measuring the enzyme activity at different temperatures (20–60 °C) and in buffers with different pH values (pH 5.0–10.0): 100 mM sodium citrate buffer, pH 5.0–6.0; 100 mM potassium phosphate buffer, pH 6.0–8.0; 100 mM Tris-HCl buffer, pH 8.0–9.0; and 100 mM glycine-NaOH, pH 9.0–10.0.

Thermostability of the nitrilase was investigated by measuring the residual activity under the standard assay conditions

after being incubated at different temperatures (30, 40, 50, and 60 °C) for a required period of time.

### Influence of metal ions and EDTA on nitrilase activity

The influences of different metal ions or EDTA on nitrilase activity were studied by assaying the enzyme activity in the presence of specific metal ion or EDTA at final concentrations of 1 and 10 mM. The activity measured in the absence of metal ions and EDTA was taken as 100% and used as a reference.

### Effect of organic solvents on nitrilase activity

The effects of various organic solvents on nitrilase activity were investigated by measuring the enzyme activity in the presence of specific organic solvent at a final concentration of 5% (v/v). The activity measured in the absence of organic solvent was taken as 100% and used as a reference.

### Substrate spectrum

For substrate specificity investigation, the nitrilase activity was assayed under the standard assay conditions with 10 mM each substrate (from 200 mM stock solution in DMSO). The conversion was determined by measuring the ammonia formed in the reaction as described previously (Fawcett and Scott 1960). A control experiment, in which the nitrilase was replaced by reaction buffer, was performed for each substrate, and the ammonia produced in the control experiment was subtracted from the value found in the enzyme reaction.

### Kinetic constants

To determine the steady-state kinetic constants of the nitrilase towards benzoylacetoneitrile, the reactions (1 mL) were carried out at 50 °C and 1000 rpm with substrate concentrations ranging from 1 to 20 mM using purified HpN in potassium phosphate buffer (100 mM, pH 8.5). Aliquots (100 µL) were taken at fixed time intervals, and the reactions were stopped by adding 10 µL HCl (2 M) and extracted with 500 µL ethyl acetate for GC analysis. The best fit of the data to the Michaelis-Menten equation found using the solver function of Microsoft excel gave the respective kinetic constants.

### Two-step one-pot synthesis of (S)-β-phenylalanine from benzoylacetoneitrile

Asymmetric synthesis of (S)-β-phenylalanine from benzoylacetoneitrile was carried out by a cascade reaction coupling the nitrilase with an ω-transaminase. The reaction mixture (10 mL) containing 200 mM β-alanine, 1 mM PLP,

50 mg lyophilized nitrilase powder (0.6 U, determined in the biphasic system), and 150 mg lyophilized  $\omega$ -transaminase powder (0.75 U, based on the coupled reaction) in 5 mL potassium phosphate buffer (100 mM, pH 8.0), and 20 mM benzoylacetoneitrile in 5 mL toluene was incubated at 37 °C and 180 rpm. At different time intervals, samples were withdrawn for HPLC and GC analysis. For preparative synthesis, the reaction was performed in 100-mL scale under the same reaction conditions described above. The product was isolated by cation exchange resin column as described previously using D001 instead (Wu et al. 2010).

## Analytic methods

Quantitative analysis of  $\beta$ -phenylalanine was analyzed by HPLC with an ODS-C18 column (5  $\mu$ m, 4.6  $\times$  200 mm, Shimadzu, Japan), eluted with methanol:water:trifluoroacetic acid (55:45:0.1, v/v/v) at a flow rate of 0.8 mL/min and detected at 254 nm. For enantiomeric excess (*ee*) determination, (*S*)- $\beta$ -phenylalanine was derivatized by GITC as described previously (Ito et al. 1992) before HPLC analysis. Conversion of benzoylacetoneitrile and the concentration of byproduct acetophenone were determined by GC analysis with Rxi®-5Sil MS Capillary Column (0.25  $\mu$ m, 0.25 mm  $\times$  30 m, RESTEK, USA) using n-dodecane as an internal standard. The temperature for injector and detector was 280 °C, and the column temperature was 100 °C for 2 min, rise to 160 °C at 10 °C/min and hold for 2 min.

## Results

### Screening of nitrilases for benzoylacetoneitrile hydrolysis

To identify nitrilases capable of catalyzing benzoylacetoneitrile hydrolysis, a nitrilase tool-box previously developed in our laboratory for the synthesis of (*R*)-*o*-chloromandelic acid from *o*-chloromandelonitrile (Zhang et al. 2012) was subjected to a primary screening using benzoylacetoneitrile as a substrate (10 mM) under the standard assay conditions. As shown in Table 1, a nitrilase from *Hoeflea phototrophica* DFL-43, abbreviated as HpN, showed the highest hydrolytic activity towards benzoylacetoneitrile with a relatively high activity of 2.35 U mg<sup>-1</sup> cell-free extract. Three other nitrilases (SpwN, LaN, and ShwN) showed moderate to low activities, ranging from 0.02 to 0.92 U mg<sup>-1</sup> cell-free extract. In contrast, three more nitrilases (ArN, EbN, and JpE) could not accept benzoylacetoneitrile as substrate at all. It is noteworthy that all these nitrilases could be expressed very well in *E. coli* in soluble form. Therefore, HpN was chosen as a promising nitrilase for benzoylacetoneitrile hydrolysis and was used for the following research.

## Enzyme purification

Due to the *N*-terminal His-tag, HpN was easily purified to homogeneity by nickel column affinity chromatography (Fig. 1), and the purified enzyme migrated as a single band on SDS-PAGE with a molecular size of about 40 kDa, which is consistent with that predicted from the amino acid sequence. The purified nitrilase showed a very high specific activity of 35 U mg<sup>-1</sup> protein towards benzoylacetoneitrile, indicating that the enzyme was purified by 15-fold from the cell-free extract.

## Effects of pH and temperature

The activity of the purified HpN was investigated at various pH values ranging from 5.0 to 10.0. Interestingly, the nitrilase was active over a wide range of pH values from 6.0 to 10.0, and more than 70% of the maximum activity was retained between pH 6.0 and 9.0. The maximum activity was observed at 100 mM Tris-HCl buffer with a pH value of 8.5 (Fig. 2a). The optimum temperature of HpN was 50 °C, lower or higher temperatures would significantly decrease the nitrilase activity, indicating that the nitrilase was highly temperature-sensitive, especially at higher temperatures (Fig. 2b).

Thermostability of the purified nitrilase was then determined at temperatures of 30, 40, 50, and 60 °C, respectively, in potassium phosphate buffer (100 mM, pH 8.0). It should be noted that the enzyme was very stable at 30 °C, and no activity loss was observed after 24-h incubation (Fig. 3). The nitrilase was relatively stable at 40 °C with a half-life of 12 h, while at 50 and 60 °C, the enzyme was more labile, giving half-lives of only 3 and 1 h, respectively.

## Influences of metal ions and EDTA

The effects of metal ions and EDTA on the activity of HpN were investigated at concentrations of 1 and 10 mM. As shown in Table 2, the nitrilase activity was obviously inhibited by the thiol-binding metal ions, such as Ag<sup>+</sup>, Hg<sup>2+</sup>, and Cu<sup>2+</sup>, and as low as 1 mM Ag<sup>+</sup> or Hg<sup>2+</sup> could result in completely loss of enzyme activity, confirming that the thiol group is essential for nitrilase activity; similar results were also observed by previous studies (Zhang et al. 2011a, 2012). Meanwhile, Ni<sup>2+</sup> and Fe<sup>2+</sup> could also serve as effective inhibitors. The other metal ions and the metal chelating agent EDTA did not show obvious effect on the nitrilase activity.

## Effect of organic solvents

Since most of the nitriles are highly hydrophobic, and in the bioreaction process, organic solvents are usually added to help

**Table 1** Screening of nitrilase for benzoylacetone nitrile hydrolysis

Nitrilase	Microorganism <sup>a</sup>	GenBank accession number	Activity (U/mg crude enzyme) <sup>b</sup>
AnN	<i>Arsenophonus nasoniae</i> DSM 15247	FN545161.1	N.D.
ArN	<i>Agrobacterium radiobacter</i> CGMCC 1.2556	NC_011987.1	N.D.
EbN	<i>Erwinia billingiae</i> DSM 17872	NC_014305.1	N.D.
HpN	<i>Hoeflea phototrophica</i> DFL-43 DSM 17068	NZ_ABIA02000005.1	2.35 ± 0.05
JpN	<i>Alcaligenes</i> sp. CGMCC 2009	FJ943638	N.D.
LaN	<i>Labrenzia aggregata</i> DSM 13394	NZ_AAUW01000019.1	0.34 ± 0.03
ShwN	<i>Shewanella woodyi</i> ATCC 51908	NC_010506.1	0.02 ± 0.00
SpwN	<i>Sphingomonas wittichii</i> RW1 DSM 6014	NC_009511.1	0.92 ± 0.06

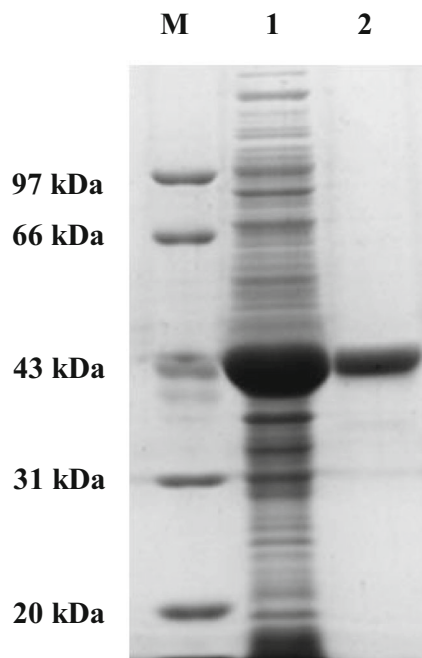
N.D., not detected

<sup>a</sup> The microorganisms were obtained from DSMZ, ATCC, or CGMCC

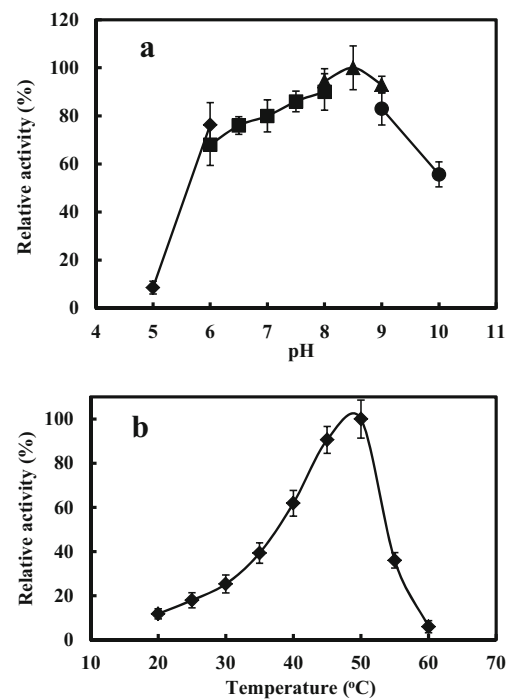
<sup>b</sup> The activities of crude enzymes were determined with 10 mM benzoylacetone nitrile under the standard assay conditions. One unit of enzyme activity was defined as the amount of enzyme required for the consumption of 1 μmol benzoylacetone nitrile under the standard assay conditions

the dispersion of substrate in the reaction mixture. However, organic solvents especially highly hydrophilic organic solvents have also been proven to be detrimental to biocatalysts (Zhang et al. 2011b). Therefore, the effects of various organic solvents on the nitrilase activity were examined with a concentration of 5% (v/v). As shown in Fig. 4, most of the organic solvents tested displayed good biocompatibility with the

nitrilase, and more than 80% of the activity could be retained. The only exception was acetone, in which more than 60% of the nitrilase activity was inhibited, demonstrating that acetone

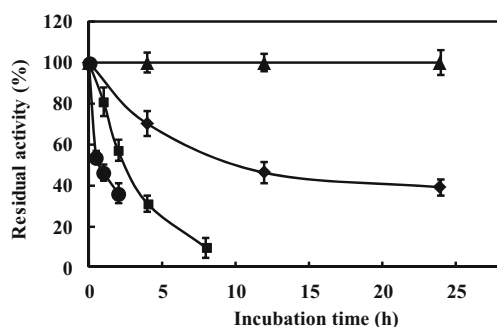


**Fig. 1** SDS-PAGE analysis of the purified HpN. M, protein markers; Lane 1, cell-free extract; Lane 2, purified enzyme



**Fig. 2** The pH and temperature optima of the purified enzyme. (a) pH optima. *Diamond*, 100 mM sodium citrate buffer (pH 5.0–6.0); *Square*, 100 mM potassium phosphate buffer (pH 6.0–8.0); *Triangle*, 100 mM Tris-HCl buffer (pH 8.0–9.0); *Circle*, 100 mM glycine-NaOH buffer (pH 9.0–10.0). (b) Temperature optima: The enzyme activity was measured at various temperatures (20–60 °C) in 100 mM potassium phosphate buffer (pH 8.0)





**Fig. 3** Thermal stability of the purified enzyme. The purified enzyme was incubated in potassium phosphate buffer at different temperatures for a required period and the residual activity was measured. *Triangle*, 30 °C; *Diamond*, 40 °C; *Square*, 50 °C; *Circle*, 60 °C

might not be a suitable organic solvent for the nitrilase-catalyzed reaction.

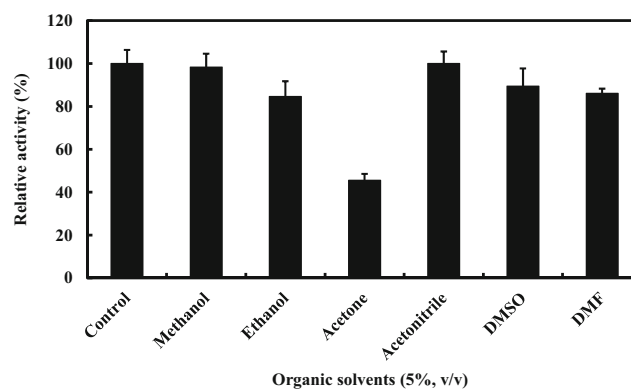
### Substrate spectrum

The substrate specificity of the purified nitrilase HpN towards a series of nitriles including arylacetoneitriles, aromatic nitriles, and aliphatic nitriles were investigated (Table 3). It is not surprising that the nitrilase showed moderate to high activities towards arylacetoneitriles since the nitrilase tool-box from which HpN was identified was developed previously in our laboratory using an arylacetoneitrilase gene sequence as a template for blasting in NCBI database (Zhang et al. 2012). The HpN exhibited very high activity towards phenylacetoneitrile and benzoylacetoneitrile. However, substitution on the

**Table 2** Effects of metal ions and EDTA on the nitrilase activity

Reagent	Relative activity (%) <sup>a</sup>	
	1 mM	10 mM
Control	100 ± 3	100 ± 5
Mg <sup>2+</sup>	81 ± 2	85 ± 4
Ca <sup>2+</sup>	108 ± 6	100 ± 5
Fe <sup>2+</sup>	51 ± 4	41 ± 3
Cu <sup>2+</sup>	13 ± 2	6 ± 1
Ag <sup>+</sup>	0 ± 0	0 ± 0
Hg <sup>2+</sup>	0 ± 0	0 ± 0
Mn <sup>2+</sup>	85 ± 5	76 ± 3
Zn <sup>2+</sup>	80 ± 2	80 ± 2
Li <sup>+</sup>	94 ± 3	102 ± 5
Pb <sup>2+</sup>	89 ± 3	85 ± 2
Ni <sup>2+</sup>	34 ± 2	17 ± 1
EDTA	95 ± 4	80 ± 3

<sup>a</sup>The enzyme activity was assayed under the standard assay conditions with one of the metal ions or EDTA. Control enzyme activity was assayed in the absence of any tested additive



**Fig. 4** Effect of various organic solvents on the activity of nitrilase

aromatic ring of phenylacetoneitrile and benzoylacetoneitrile significantly decreased the nitrilase activity, probably due to steric hindrance. It should be noted that the HpN displayed very low activities towards aliphatic nitriles with the exception of diaminomaleonitrile, which could not be hydrolyzed by HpN. In addition, the nitrilase HpN does not accept aromatic nitriles as substrates.

### Steady-state kinetics

The steady-state kinetic constants of the purified nitrilase towards benzoylacetoneitrile were studied with substrate concentrations ranging from 1 to 20 mM, giving  $V_{max}$ ,  $k_{cat}$

**Table 3** Substrate spectrum of HpN

Substrate	Relative activity (%) <sup>a</sup>
Benzoylacetoneitrile	100 ± 5
3-Chlorobenzoylacetoneitrile	43 ± 3
4-Chlorobenzoylacetoneitrile	16 ± 1
3-Methoxybenzoylacetoneitrile	38 ± 4
4-Methoxybenzoylacetoneitrile	10 ± 1
Mandelonitrile	32 ± 3
<i>o</i> -Chloromandelonitrile	7 ± 1
Phenylacetoneitrile	160 ± 12
<i>o</i> -Chlorophenylacetoneitrile	38 ± 3
Benzonitrile	N.D.
3-Cyanopyridine	N.D.
2,3-Dicyanopyrazine	N.D.
Acetonitrile	12 ± 2
Glycolonitrile	18 ± 2
Diaminomaleonitrile	N.D.

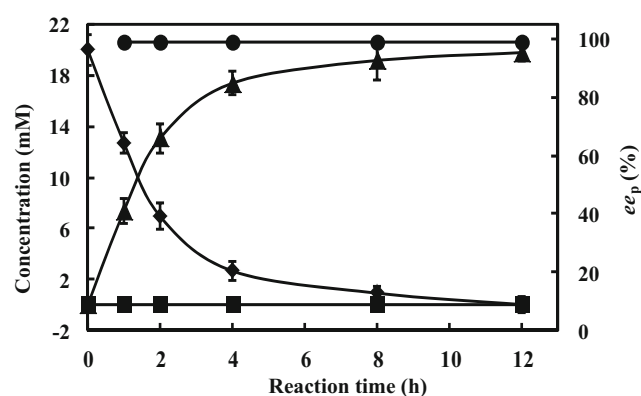
N.D., not detected

<sup>a</sup>The enzyme activity was measured under the standard assay conditions with 10 mM substrate. The conversion was determined by measuring the amount of ammonia produced as described previously (Fawcett and Scott 1960). The activity towards benzoylacetoneitrile was taken as 100%

and  $K_M$  of  $256 \text{ U mg}^{-1}$ ,  $170 \text{ s}^{-1}$ , and  $4.2 \text{ mM}$ , respectively. Although nitrilases have found applications in the synthesis of various pharmaceuticals, agrochemicals, and fine chemicals, their activity levels ( $V_{\max}$ ) if available are usually below  $50 \text{ U mg}^{-1}$  (Banerjee et al. 2006; Zhang et al. 2011a; Zhu et al. 2007), indicating that the nitrilase presented in this study represents a highly active and promising biocatalyst.

### Two-step one-pot synthesis of (*S*)- $\beta$ -amino acids from benzoylacetoneitrile derivatives

Due to the significant inhibitory effect of carbonyl compounds on  $\omega$ -transaminase (Bea et al. 2011; Kim et al. 2007) and the poor solubility of benzoylacetoneitrile derivatives in aqueous buffer, a toluene-aqueous biphasic reaction system was adopted for the two-step one-pot synthesis of (*S*)- $\beta$ -amino acids from benzoylacetoneitrile derivatives since this biphasic system has been proven very useful in relieving substrate inhibition (Zhang et al. 2011a, 2012). Meanwhile, toluene is also a good solvent for benzoylacetoneitrile derivatives. In the toluene-aqueous biphasic reaction system (1:1, v/v), 20 mM benzoylacetoneitrile in the organic phase could be completely transformed into (*S*)- $\beta$ -phenylalanine within 12 h with an optical purity of  $>99\%$  ee (Fig. 5 and Table 4). However, only moderate conversions (73–89%) of (*S*)- $\beta$ -amino acids were obtained from benzoylacetoneitrile derivatives even though the biocatalyst loading was twofold higher than that for benzoylacetoneitrile, and the reaction time was also extended to 24 h, which was ascribed to the much lower specific activity of the nitrilase towards benzoylacetoneitrile derivatives (Table 3). A preparative scale reaction (100-mL) was then carried out under the same reaction conditions, and enantiomerically pure (*S*)- $\beta$ -phenylalanine was obtained with an isolated yield of 82 and  $>99\%$  ee



**Fig. 5** Time course for the two-step one-pot synthesis of (*S*)- $\beta$ -phenylalanine from benzoylacetoneitrile in a biphasic system. *Triangle*, concentration of (*S*)- $\beta$ -phenylalanine; *Diamond*, concentration of benzoylacetoneitrile; *Square*, concentration of acetophenone; *Circle*, the ee of (*S*)- $\beta$ -phenylalanine

**Table 4** Biocatalytic two-step one-pot synthesis of various  $\beta$ -amino acids from benzoylacetoneitrile derivatives

Entry	Substrate (X =)	Time (h)	Conversion (%)	ee (%)
1	H <sup>a</sup>	12	>99	>99
2	3-Cl <sup>b</sup>	24	89	>99
3	4-Cl <sup>b</sup>	24	77	>99
4	3-OMe <sup>b</sup>	24	86	>99
5	4-OMe <sup>b</sup>	24	73	>99

<sup>a</sup>The biocatalyst loadings were 10 mg/mL lyophilized nitrilase powder and 30 mg/mL lyophilized  $\omega$ -transaminase powder

<sup>b</sup>The biocatalyst loadings were 20 mg/mL lyophilized nitrilase powder and 60 mg/mL lyophilized  $\omega$ -transaminase powder

after purification by column chromatography using a cation exchange resin (D001).

### Discussion

Due to the important role of optically pure  $\beta$ -amino acids in synthetic organic chemistry and the common limitations including poor catalytic activity, unsatisfactory selectivity, and the low product yield of currently available biocatalytic synthesis methods (Bea et al. 2011; Kim et al. 2007; Li et al. 2013; Weise et al. 2015, 2017; Wu et al. 2010; You et al. 2013; Zhang et al. 2015a), there is still a great need to develop new synthetic routes, especially asymmetric synthetic methods starting from cheap raw materials.  $\omega$ -Transaminase has been shown to be a group of versatile biocatalyst in the synthesis of chiral amines or amino acids due to its widespread in nature and broad substrate spectrum (Koszelewski et al. 2010). Some progresses have been made towards the asymmetric amination of  $\beta$ -ketoacids for the synthesis of optically pure  $\beta$ -phenylalanine; however, the inherent inhibitory effect of  $\beta$ -ketoacids on  $\omega$ -transaminase and the spontaneous decarboxylation to acetophenone and carbon dioxide would greatly reduce the product yield (Bea et al. 2011; Kim et al. 2007). Although this could be partially alleviated by coupling the transamination with lipase, in which the more stable  $\beta$ -ketoacid esters were used instead of the labile  $\beta$ -ketoacids (Bea et al. 2011; Kim et al. 2007), the preparation of the ester precursors is relatively complicated and costly, since either harsh reaction conditions (e.g.,  $-78 \text{ }^\circ\text{C}$ ) (Galliford and Scheidt 2008) or highly expensive catalysts (e.g., palladium) (Chen et al. 2013) were required.

To this end, we focused on the cascade reaction coupling nitrilase with  $\omega$ -transaminase, in which the cheap and easily available benzoylacetoneitrile was hydrolyzed by nitrilase into benzoylactic acid which was then aminated by  $\omega$ -transaminase forming  $\beta$ -phenylalanine (Scheme 1). Although nitrilases have been employed for the synthesis of

various carboxylic acids from nitriles, there was only one nitrilase (NitBJ) reported to be active towards benzoylacetone nitrile so far. Most importantly, the specific activity of NitBJ towards benzoylacetone nitrile was merely  $0.36 \text{ U mg}^{-1}$  protein (Mathew et al. 2017; Zhang et al. 2015a). By screening the nitrilase tool-box developed in our laboratory (Zhang et al. 2012), a *Hoeflea phototrophica* nitrilase (HpN) was identified, exhibiting excellent catalytic activity towards benzoylacetone nitrile with a  $V_{\max}$  of  $256 \text{ U mg}^{-1}$  protein, which is about fivefold higher than the highest nitrilase activity reported so far (Zhu et al. 2007). Substrate spectrum investigation showed that HpN was highly active towards arylacetone nitriles, while very low activities were observed with aliphatic nitriles, and aromatic nitriles could not be hydrolyzed by HpN. A BLASTp search revealed that the amino acid sequence of HpN shows 67% identity to a nitrilase from an uncultured organism (GenBank accession number: AAR97377.1) (Robertson et al. 2004), which is the highest among the homologous nitrilases, indicating that HpN might represent a novel nitrilase. The nitrilase HpN was then chosen for the following research due to its highest activity and good expression level in *E. coli*.

Biochemical property characterization indicated that the nitrilase was very stable between 30 and 40 °C (Fig. 3), which are frequently employed temperatures for the biotransformation process. It should be noted that the nitrilase was highly active over a wide pH range (6.0–10.0), with an optimum pH around 8.5 (Fig. 2a). The optimum temperature (37 °C) and pH (8.5) of  $\omega$ -transaminase from *Polaromonas* sp. JS666 (Bea et al. 2011) are similar to those of HpN, which is beneficial for the cascade reaction since coordination of the inter-compatibility between different enzymes in the cascade process is a great challenge. In addition, the nitrilase could tolerate against a wide range of hydrophilic organic solvents (Fig. 4), which is very important for the biotransformation of highly hydrophobic nitriles, in which organic solvents are usually

added to the reaction mixture to help the distribution of substrates.

A cascade reaction coupling nitrilase with  $\omega$ -transaminase for the enzymatic synthesis of optically pure  $\beta$ -phenylalanine from benzoylacetone nitrile was thereby firstly performed in an aqueous monophasic system. However, only little amount of  $\beta$ -phenylalanine was detected, while benzoylacetone and acetophenone accounted the majority of products (data not shown), indicating that benzoylacetone nitrile was successfully hydrolyzed by nitrilase to form benzoylacetone, and acetophenone was formed by the spontaneous decarboxylation of benzoylacetone. The low yield of  $\beta$ -phenylalanine might be ascribed to the inhibitory effect of carbonyl compounds on  $\omega$ -transaminase, which was also observed in previous studies (Bea et al. 2011; Kim et al. 2007). In addition, when benzoylacetone nitrile or acetophenone was used as the substrate for the  $\omega$ -transaminase-catalyzed transamination, neither decrease of substrate nor formation of new product was detected, demonstrating that these two compounds could not be accepted as substrates by the  $\omega$ -transaminase.

To relieve the substrate inhibition, a toluene-water biphasic system (1:1, v/v) was then adopted for the cascade reaction. Meanwhile, excess amount of  $\omega$ -transaminase as compared with nitrilase was added to make sure that benzoylacetone produced at the first step could be transformed into  $\beta$ -phenylalanine by  $\omega$ -transaminase as soon as its formation and the spontaneous decarboxylation to acetophenone could be avoided. Under these reaction conditions, various optically pure (*S*)- $\beta$ -amino acids could be produced from benzoylacetone nitrile derivatives in the cascade reaction employing nitrilase and  $\omega$ -transaminase with moderate to high yields (Table 4). Although similar cascade reactions coupling lipase/nitrilase with  $\omega$ -transaminase have also been employed for the production of a series of (*S*)- $\beta$ -amino acids, only low to moderate yield of the product could be obtained

**Table 5** Comparison of different cascades for the synthesis of (*S*)- $\beta$ -phenylalanine

Substrates	Substrate loading (mM)	Reaction time (h)	Conversion (%)	Ref.
Ethyl 3-oxo-3-phenyl propionate	10	24	20 <sup>a</sup>	Kim et al. (2007)
	10	24	18 <sup>b</sup>	Bea et al. (2011)
	10	24	52 <sup>c</sup>	Bea et al. (2011)
	50	24	96 <sup>d</sup>	Mathew et al. (2016a)
	20	18	43.6 <sup>d</sup>	Mathew et al. (2016b)
Benzoylacetone nitrile	10	24	72 <sup>d</sup>	Mathew et al. (2017)
	20	12	>99 <sup>c</sup>	This study

<sup>a</sup> 3-Aminobutyric acid was used as the amino donor

<sup>b</sup> L-Alanine was used as the amino donor

<sup>c</sup>  $\beta$ -Alanine was used as the amino donor

<sup>d</sup> (*S*)-phenethylamine was used as the amino donor



(Table 5), probably due to the much lower catalytic activity of the nitrilase towards the nitrile compounds than that used in this study (Bea et al. 2011; Kim et al. 2007; Mathew et al. 2016a, b, 2017). Another possible reason might be the aqueous-organic biphasic reaction system adopted in this work, in which the organic phase would serve as a reservoir of the keto nitriles and regulating the substrate concentration around the biocatalyst and minimizing the substrate inhibition. The only limitation of the aqueous-toluene biphasic system might be the mass transfer problem (partially due to high biocatalyst loading in this study); therefore, selection of suitable organic solvents for the biphasic system or using extraction reagents is still needed in the future studies. It should also be noted that the much cheaper amino donor  $\beta$ -alanine was used in this study instead of the highly expensive (*S*)-phenethylamine employed in the previous studies (Bea et al. 2011; Kim et al. 2007; Mathew et al. 2016a, b, 2017), which would significantly reduce the cost of the bioprocess.

In summary, a novel nitrilase from *Hoeflea phototrophica* was identified from a home-made nitrilase tool-box, with an excellent activity towards benzoylacetone nitrile which enables the development of a cascade reaction coupling nitrilase with  $\omega$ -transaminase for the enzymatic synthesis of optically pure (*S*)- $\beta$ -phenylalanine from the cheap and widely available benzoylacetone nitrile. It should be noted that an in-depth optimization of the reaction conditions or protein engineering of the involved nitrilase and especially  $\omega$ -transaminase to improve their catalytic efficiencies is still needed to further enhance the productivity of the cascade enzymatic process. However, the much better results in this work than the previous studies indicate that the cascade reaction we developed in this study might serve as a promising alternative for the green production of optically pure (*S*)- $\beta$ -phenylalanine in the future.

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## Compliance with ethical standards

**Conflict of interest** The authors declare that they have no conflict of interest.

**Ethical approval** This article does not contain any studies with human participants or animals performed by any of the authors.

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