

A novel amidase from *Brevibacterium epidermidis* ZJB-07021: gene cloning, refolding and application in butyrylhydroxamic acid synthesis

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Abstract A novel amidase gene (*bami*) was cloned from *Brevibacterium epidermidis* ZJB-07021 by combination of degenerate PCR and high-efficiency thermal asymmetric interlaced PCR (hiTAIL-PCR). The deduced amino acid sequence showed low identity ($\leq 55\%$) with other reported amidases. The *bami* gene was overexpressed in *Escherichia coli*, and the resultant inclusion bodies were refolded and purified to homogeneity with a recovery of 22.6 %. Bami exhibited a broad substrate spectrum towards aliphatic, aromatic and heterocyclic amides, and showed the highest acyl transfer activity towards butyramide with specific activity of 1331.0 ± 24.0 U mg^{-1} . Kinetic analysis demonstrated that purified Bami exhibited high catalytic efficiency (414.9 $\text{mM}^{-1} \text{s}^{-1}$) for acyl transfer of butyramide, with turnover number (K_{cat}) of 3569.0 s^{-1} . Key parameters including pH, substrate/co-substrate concentration, reaction temperature and catalyst loading were investigated and the Bami showed maximum acyl transfer activity at 50 °C, pH 7.5. Enzymatic catalysis of 200 mM butyramide with 15 $\mu\text{g mL}^{-1}$ purified Bami was completed in 15 min with a BHA yield of 88.1 % under optimized conditions. The

results demonstrated the great potential of Bami for the production of a variety of hydroxamic acids.

Keywords Acyl transfer activity · Amidase · *Brevibacterium epidermidis* · Butyrylhydroxamic acid · Refolding

Introduction

Amidases (EC 3.5.1.4) are a class of enzymes catalyzing the hydrolysis of a variety of amides to their corresponding acids and ammonia. Based on their specific amino acid homology, amidases were classified into two families: the amidase signature (AS) family and the nitrilase superfamily [10, 14]. The amidase signature family is characterized by the presence of the GGSSGG motif in a conserved stretch of approximately 130 amino acids [10, 28]. They are widespread in both eukaryotic and prokaryotic organisms. Due to excellent regio-, chemo- and enantioselective properties, AS amidases are increasingly recognized as attractive tools for the preparation of optically active compounds [12, 24, 30, 43]. Although some AS amidases have been cloned and overexpressed in *E. coli* [11, 23, 39, 41], high-level expression of amidases often leads to formation of insoluble inclusion bodies (IBs) in the cytoplasm [18, 25, 37]. In many cases, optimization of culture conditions, such as temperature, pH and autoinduction media could not eliminate the formation of IBs, and the amidase expression efficiency was unsatisfactory [32, 35, 42]. Therefore, it is necessary to establish an efficient refolding protocol to obtain bioactive amidases.

Besides their amide hydrolysis ability, most amidases were reported to exhibit acyl transfer activity and received great attention for synthesis of pharmaceutically important hydroxamic acids in the recent years [2, 5, 31]. The

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fatty hydroxamic acids have been studied as inhibitors of cyclooxygenase and 5-lipoxygenase with a potent anti-inflammatory activity, and thus, were recommended for the treatment of ureaplasma and anemia [17, 20]. Butyrylhydroxamic acid (BHA) is an efficient histone deacetylase inhibitor of erythropoiesis and shows great potential for β -chain hemoglobinopathies therapeutics [9]. Moreover, BHA has been proved to be capable to enhance memory in rodent behavioral models and could be further used as a potent memory enhancer and mood stabilizer [13]. Generally, BHA was prepared through chemical route by N-acylation of O-substituted hydroxylamine with butyric acid chloride as substrate [3]. However, it was very difficult to prevent further acylation during the reaction and the yield of BHA was unsatisfactory in most cases.

In our previous study, an amidase-producing strain *Brevibacterium epidermidis* ZJB-07021 was isolated and characterized [22]. Characterization of the purified amidase from *B. epidermidis* ZJB-07021 suggested that the enzyme exhibited both hydrolysis and acyl transfer activity, and showed the highest hydrolysis activity of 391.5 ± 2.0 U mg^{-1} towards butyramide [34]. In this paper, the gene encoding *B. epidermidis* amidase (*bami*) was cloned and expressed in *E. coli*. The refolding, purification and application of Bami in biosynthesis of BHA was investigated.

Materials and methods

Chemicals, strains and plasmids

Brevibacterium epidermidis ZJB-07021 was isolated in our laboratory and deposited at the China Center for Type Culture Collection (CCTCC) under the accession number of CCTCC M207076. *E. coli* BL21 (DE3) (Novagen, Germany) was used as the host for recombinant protein expression. Plasmid pGEM-T (Promega, USA) and pET-28b(+) (Novagen, Germany) were used as vectors for the cloning and expression, respectively. LA *Taq* DNA polymerase was purchased from Takara (Dalian, China). Restriction enzymes and T4 DNA ligase were supplied by Thermo scientific Co., Ltd (Shanghai, China). Primer synthesis and DNA sequencing were conducted by Sunny Biological Co., Ltd. (Shanghai China). The DNA gel extraction, plasmid miniprep and PCR cleanup kits were purchased from Axygen (Hangzhou, China). All chemicals used were of analytical grade and commercially available.

Cloning of *bami* gene from *B. epidermidis* ZJB-07021

The strategy used for cloning the complete *bami* gene was illustrated in Fig. 1. A pair of degenerate primers (5'-CGCBDTCCGTCCTCRGCCAATGGCGTC-3' and

5'-GTCYGTGACCTGCTGYAAGNCGTGAC-3') were used to clone the partial fragment of *bami* from the genomic DNA of *B. epidermidis* ZJB-07021. Degenerate primers were designed according to the partial amino acid sequence of purified Bami identified by MALDI-TOF/TOF-MS (RLPSSANGVTGLKPTWGRV), and the conserved fragment was obtained by alignment and comparative analysis of several amidase sequences from *Brevibacterium* sp. (VLAAGHAFQQVTD). The PCR products were cloned into pGEM-T and sequenced. To amplify the flanking fragments of the *bami*, high-efficiency thermal asymmetric interlaced PCR (hiTAIL-PCR) was applied according to Liu et al. [26]. The primers used in hiTAIL-PCR were listed in Table 1. The PCR products were purified and cloned into pGEM-T and sequenced.

The full-length *bami* was amplified by PCR using the specific primers *bami*-F (5'-CATGCCATGGGCATGCAGGGAGGCATGATGACCACAC-3') and *bami*-R (5'-CGGGATCCTCAGAGCCCGGCCGGGTGGCTGGT-3'), which contained artificial *Nco*I and *Bam*HI restriction enzyme sites (underlined). The amplified fragment with the expected size was purified and double-digested with *Nco*I and *Bam*HI, and then inserted into *Nco*I-*Bam*HI-digested pET-28b(+). The resultant recombinant plasmid pET28b-*bami* was transformed into *E. coli* BL21 (DE3) competent cells, and spread onto solid Luria–Bertani (LB) medium supplemented with 50 $\mu\text{g mL}^{-1}$ kanamycin. The positive clones were confirmed by DNA sequencing.

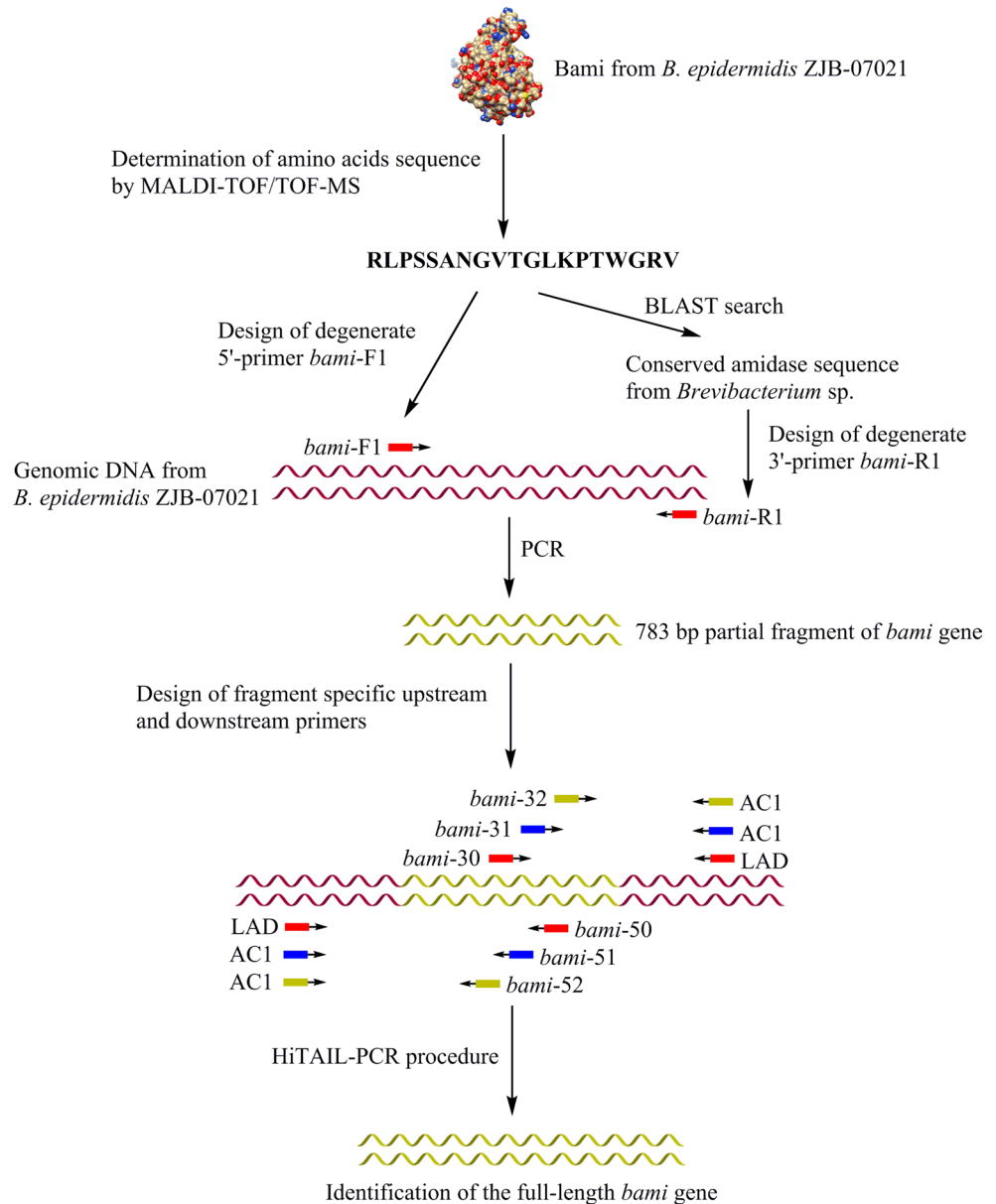
Expression of recombinant amidase and isolation of IBs

The positive colonies were grown at 37 °C in Luria–Bertani (LB) medium containing 50 $\mu\text{g mL}^{-1}$ kanamycin. When the optical density at 600 nm reached 0.6, isopropyl- β -D-thiogalactopyranoside (IPTG) (final concentration of 0.1 mM) was added and the cells were grown at 28 °C for another 8 h. The resultant cells (1 L) were harvested by centrifugation at 9000 $\times g$, 4 °C for 10 min. The cell pellet was resuspended in Tris–HCl buffer (100 mM, pH 7.5), and disrupted by ultrasonication (200 W, pulse 1 s, pause 1 s) for 20 min at 4 °C. Sonicated cells were centrifuged at 14,000 $\times g$, 4 °C for 10 min and the pellet containing recombinant amidase as IBs was separated. To remove contaminating membrane proteins, pellets were further washed twice at 4 °C with wash buffer composed of 100 mM Tris–HCl, pH 7.5, 2 M urea, 50 mM NaCl and 2 % (v/v) Triton X-100. The washed IBs were harvested by centrifugation at 9000 $\times g$, 4 °C for 10 min, and used for solubilization and refolding.

Solubilization and refolding of amidase from IBs

The washed IBs were solubilized in 5 mL Tris–HCl buffer (100 mM, pH 7.5) containing 8 M urea, 50 mM NaCl,

Fig. 1 Cloning strategy for full-length *bami* gene. The specific primers *bami*-50, 51, 52 and *bami*-30, 31, 32 were designed according to the known 783-bp partial *bami* gene. The primers LAD and AC1 were designed according to Liu et al. [26], which were universally applicable for various organisms



5 mM dithiothreitol (DTT) and kept for 4 h at room temperature. Solubilized amidase was centrifuged at $14,000\times g$, $4\text{ }^{\circ}\text{C}$ for 20 min and the supernatant was filtered through $0.22\text{ }\mu\text{m}$ filter (Millipore, USA). Refolding was performed by step-wise dialysis against refolding buffer (100 mM Tris-HCl, pH 7.5, 50 mM NaCl, 1 mM DTT) containing 6, 4, 2 and 0.5 M urea, respectively. Refolding proceeded for approximately 36 h at $4\text{ }^{\circ}\text{C}$ (without stirring). Refolded sample was centrifuged at $14,000\times g$, $4\text{ }^{\circ}\text{C}$ for 20 min, and the supernatant was pooled and used for purification.

Purification of refolded recombinant amidase

Refolded recombinant amidase was applied to a DEAE anion ion-exchange column ($1.5 \times 20\text{ cm}$, Bio-Rad

laboratories, USA) previously equilibrated with Tris-HCl buffer (100 mM, pH 7.5). After washing, elution was carried out using a linear gradient of NaCl (0–1.0 M) in Tris-HCl buffer (100 mM, pH 7.5) at a flow rate of 1 mL min^{-1} . The active fractions were eluted at a conductivity of $50\text{--}58\text{ mS cm}^{-1}$, dialyzed against Tris-HCl buffer (100 mM, pH 7.5) at $4\text{ }^{\circ}\text{C}$ for 24 h, and analyzed by 12 % SDS polyacrylamide gel electrophoresis (SDS-PAGE). Protein concentration was determined by Bradford method using bovine serum albumin (BSA) as the standard [7].

Acyl transfer reaction and amidase activity assay

Acyl transfer activity of Bami was determined according to the method developed by Brammar and Clarke [8].

Table 1 Primers used in the cloning of *bami* gene

Primers and reactions	Names	Sequence (5' → 3') ^a
Arbitrary degenerate primer	LAD1	ACGATGGACTCCAGAGVNVNNGGAA
	LAD2	ACGATGGACTCCAGAGBNBNNNGGTT
	LAD3	ACGATGGACTCCAGAGVVNVNCCAA
	LAD4	ACGATGGACTCCAGAGBDNBNNCGGT
	AC1	ACGATGGACTCCAGAG
The upstream hiTAIL-PCR	<i>bami</i> -50	CAGTCGGGCGACGACGGTGGCAT
	<i>bami</i> -51	ACGATGGACTCCAGTCCCGTGCAGATCGCCGAGCCAGTT
	<i>bami</i> -52	ATGCGTTCGAGCATCGCTTCGGTGA
The downstream hiTAIL-PCR	<i>bami</i> -30	CGACCTGTCCGGTCTCATCGA
	<i>bami</i> -31	ACGATGGACTCCAGTCATGGCCGATATCGATCTGCTCCTGCT
	<i>bami</i> -32	ATCGACAACCTGCGGAATGCCGTGCGAT
Degenerate primers	<i>bami</i> -F1	CGCBDTCCGTCCCTCRGCCAATGGCGTC
	<i>bami</i> -R1	GTCYGTGACCTGCTGYAAGNCGTGAC
Full-length amplification	<i>bami</i> -F	CATGCCATGGGCATGCAGGGAGGCATGATGACCACAC
	<i>bami</i> -R	CGGGATCCTCAGAGCCCCGGCCGGGTGGCTGGT

^a B (C/G/T), D (A/G/T), N (A/C/G/T), R (A/G), V (A/C/G), Y (C/T)

The standard reaction mixture (1 mL) comprised 690 μ L Tris–HCl buffer (100 mM, pH 7.5), 100 μ L butyramide solution [1.0 M in Tris–HCl buffer (100 mM, pH 7.5)], 200 μ L hydroxylamide hydrochloride solution (5.0 M, pH was adjusted to 7.5 with 10 M NaOH) and 10 μ L diluted refolded Bami. The reaction was carried out at 50 °C, unless otherwise stated. Aliquots (1 mL) of reaction mixture was stopped at regular intervals and mixed with 2 mL of acidic FeCl₃ solution (356 mM FeCl₃ in 0.65 M HCl). The formation of BHA/Fe(III) complex was determined spectrophotometrically at 500 nm. One unit of acyl transfer activity was defined as the amount of enzyme that catalyzed the production of 1 μ mol BHA per min.

Analytical methods

The molar extinction coefficient (ϵ_M) of BHA/Fe(III) complex was determined spectrophotometrically at 500 nm. To form a BHA/Fe(III) complex, 2 mL of FeCl₃ solution was added into 1 mL aqueous BHA solution with concentrations from 0.5 to 2.0 mM. The ϵ_M of BHA/Fe(III) was determined from the slope of a calibration curve. The concentration of BHA, butyramide and butyric acid in the reaction mixture was determined by Agilent 6890 N GC system equipped with a capillary column AT-FFAP (0.25 mm ID \times 30 m, 0.33 μ m film thickness) and a FID detector with N₂ as carrier gas. The flow rate of carrier gas was 1.0 mL min⁻¹, and the temperatures of the oven, injector and detector were set at 170, 250 and 250 °C, respectively. The retention times of BHA, butyric acid and butyramide were 2.4, 3.0, and 5.2 min, respectively.

Effects of pH and temperature on Bami activity

To investigate the pH dependency of acyl transfer reaction, Bami activity was determined at various pHs (4.0–10.0) with Citric acid–Sodium citrate buffer (4.0–6.0), Na₂HPO₄–NaH₂PO₄ buffer (6.0–7.5), Tris–HCl buffer (7.0–8.5) and Glycine–NaOH buffer (8.5–10.0). The optimal reaction temperature was determined under the standard conditions at various temperatures (20–60 °C).

Substrate specificity of Bami and bi-substrate kinetic analysis

To examine substrate specificity of the acyl transfer activity of recombinant Bami, various aliphatic, aromatic and heterocyclic amides were tested as substrates (final concentration 10 mM).

For kinetic analysis, enzyme assays were carried out under the standard assay conditions. The concentration of butyramide was varied from 2 to 10 mM at different hydroxylamine hydrochloride concentrations ranging from 40 to 200 mM. The kinetic parameters were calculated from the initial-velocity double-reciprocal plot.

Bioprocess development for the production of BHA

To work out the optimal combination of the substrate and co-substrate, the reactions were carried out with butyramide concentrations from 50 to 500 mM and hydroxylamine hydrochloride concentrations ranging from 100 to 1000 mM. To obtain high yield of BHA, time course of the bioprocess at different temperatures (30, 40, and 50 °C)

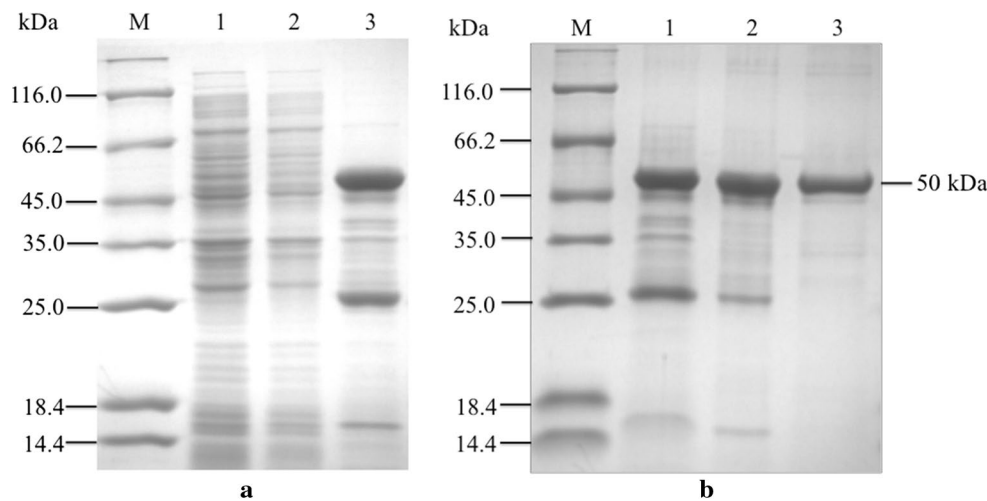


Fig. 2 SDS-PAGE analysis of expression and purification of recombinant Bami from *E. coli* IBs. Samples were analyzed on 12 % SDS polyacrylamide gel. *Lane M*, low molecular weight marker proteins in **a** and **b**. **a** SDS-PAGE analysis of recombinant Bami. *Lane 1* the cell lysate of uninduced recombinant strain; *Lane 2* soluble extract

of induced recombinant strain; *Lane 3* insoluble fraction of induced recombinant strain. **b** SDS-PAGE analysis of the refolding and purification of IBs. *Lane 1* the precipitate of IBs; *Lane 2* the refolded Bami; *Lane 3* eluted fraction of Bami from DEAE chromatography

was investigated. Samples were withdrawn at regular intervals to monitor the progress of the reaction. The influence of Bami loading on the formation of BHA was also examined. Varied amounts of purified Bami ($5, 10, 15 \mu\text{g mL}^{-1}$) were used to study optimum enzyme concentration for high conversion.

Nucleotide sequence accession number

The nucleotide sequence of the gene encoding Bami from *B. epidermidis* ZJB-07021 was available from GenBank under Accession Number KT807799.

Results

Cloning of *bami* gene from *B. epidermidis* ZJB-07021

A 783-bp fragment was amplified from *B. epidermidis* ZJB-07021 genomic DNA by PCR with the degenerate primers, designed on the basis of the amidase amino acid sequences obtained from MALDI-TOF/TOF-MS and conserved fragment of *Brevibacterium* sp. amidases. Amplification of unknown flanking regions of the *bami* gene by hiTAIL-PCR, a 3225-bp amplicon was obtained by assembling partial and flanking fragments. Nucleotide sequence analysis showed that the full-length *bami* gene consisted of an open reading frame (ORF) of 1410 bp, coding for a protein of 469 amino acids with a calculated isoelectric point of pH 4.98 and molecular mass of 49,574 Da. Based on the deduced amino acid sequence analysis, Bami was classified

into the AS family of amidase. In the region located seven nucleotides upstream of the start codon *atg*, a putative ribosome-binding site (GGAG) was detected. Moreover, the sequence of 19 amino acids obtained by MALDI-TOF/TOF-MS was also involved in the deduced amino acid sequence.

Expression, refolding and purification of Bami

As shown in Fig. 2a, recombinant Bami was not observed in soluble fractions (lane 2) but only in the insoluble IBs (lane 3). Although efforts towards soluble expression were made through optimization of expression conditions (e.g., expression plasmid, induction temperature, IPTG concentration and induction time) (data not shown), most of recombinant Bami still accumulated as IBs. Therefore, insoluble aggregate of the IBs was renatured and applied to a DEAE anion ion-exchange column. As illustrated in Fig. 2b, a single band corresponding to the molecular mass of about 50 kDa was obtained, which was in accordance with the predicted molecular mass of Bami. Approximately, 56.6 mg of purified active Bami was recovered from 1 L culture broth. The overall yield of purified refolded Bami from the IBs was about 22.6 % (Table 2).

Effects of pH and temperature on acyl transfer activity

To quantify BHA formed in the acyl transfer activity, the molar extinction coefficient (ϵ_M) of BHA/Fe(III) complex of $10.16 \times 10^2 \text{ L mol}^{-1} \text{ cm}^{-1}$ was calculated from the slope of calibration curve.

Table 2 Purification of recombinant *E. coli* Bami from IBs (culture volume 1 L)

Fractions	Protein (mg)	Specific activity (U mg ⁻¹) ^a	Yield (%)	Purification fold ^b
Total cell protein	684.8	–	–	–
IBs	250.4	–	100	–
Solubilized protein	201.0	–	80.3	–
Refolded protein	75.6	301.1	30.2	1
DEAE fraction	56.6	421.2	22.6	1.4

^a Acyl transfer activity was assayed at 35 °C under the standard conditions. Butyramide was used as the substrate

^b Purification fold was calculated after refolding as the ratio of specific activity at given step of purification to specific activity of refolded sample

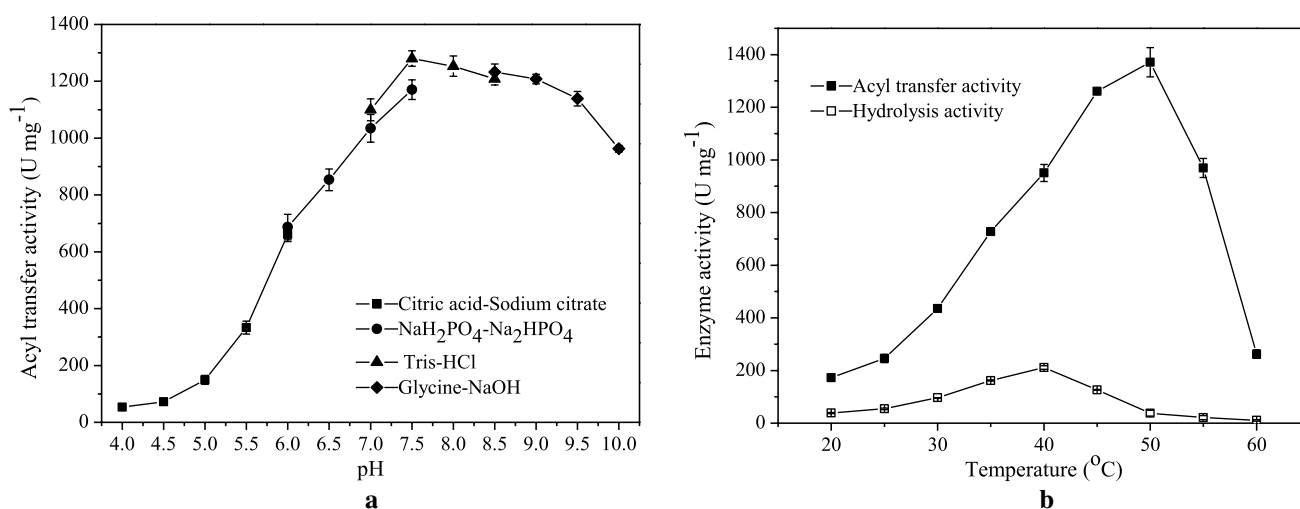


Fig. 3 Effects of pH and temperature on amidase. **a** Optimal pH for acyl transfer activity. The enzyme activity was determined in different buffer systems at various pH values by the standard assay method. **b** Optimal temperature for hydrolysis and acyl transfer activity. The

The effect of pH on Bami-mediated acyl transfer reaction was tested at various pHs (4.0–10.0). Figure 3a revealed that Bami showed maximum activity at pH 7.5 in 100 mM Tris-HCl buffer. The effect of temperature on acyl transfer activity was studied from 20 to 60 °C. As presented in Fig. 3b, the highest acyl transfer activity of 1370.7 ± 55.5 U mg⁻¹ was observed at 50 °C and the enzymatic activity was then rapidly dropped with further increase of temperature. In comparison, Bami showed highest hydrolysis activity at 40 °C.

Substrate specificity and kinetic parameters

The substrate spectrum of acyl transfer activity of Bami was investigated. As summarized in Table 3, Bami showed acyl transfer activity towards a broad spectrum of aliphatic, aromatic and heterocyclic amides. For linear aliphatic monoamides, the increase in carbon chain length led to a

considerable increase of Bami acyl transfer activity. The highest activity was observed with butyramide as substrate and the specific activity decreased progressively towards propionamide, acetamide and formamide. Although Bami exhibited moderate activity towards nicotinamide and isonicotinamide, surprisingly no activity was observed for 2-, 5-, and 6-chloride-substituted nicotinamides.

The kinetic parameters of Bami were determined from the initial-velocity double-reciprocal plots. As shown in Fig. 4, the acyl transfer reaction was catalyzed by the Ping-Pong Bi-Bi mechanism [16]. V_{max} , Km_{amide} and Km_{NH_2OH} values were calculated as $4322.7 \mu\text{M min}^{-1} \text{mg}^{-1}$, 8.6 mM and 128.5 mM, respectively. Its turnover number (K_{cat}) and catalytic efficiency (K_{cat}/Km_{amide} , K_{cat}/Km_{NH_2OH}) were 3569.0 s^{-1} , $414.9 \text{ mM}^{-1} \text{ s}^{-1}$ and $27.9 \text{ mM}^{-1} \text{ s}^{-1}$, respectively. The Bami exhibited 15.6 times higher K_{cat} value for butyramide and hydroxylamine than the amidase from *Rhodococcus* sp. R312 (229 s^{-1}) [16].

Table 3 Substrate specificity of acyl transfer activity of purified refolded Bami


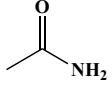
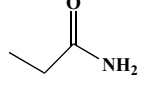
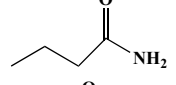
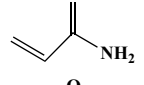
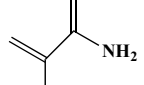
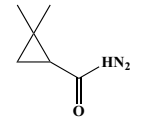
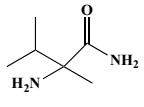
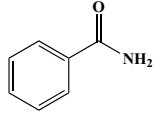
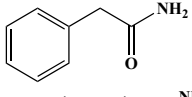
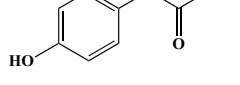
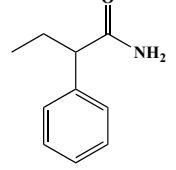
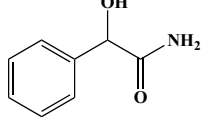
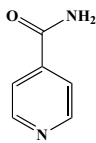
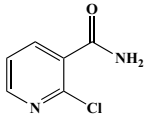
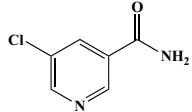
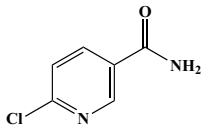
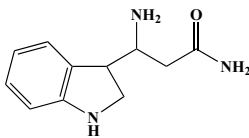
Compound		Structural formula	Acyl transfer activity (U mg ⁻¹ protein) ^a	
Aliphatic	Formamide		30.3 ± 2.8	
	Acetamide		103.3 ± 4.6	
	Propionamide		141.0 ± 6.6	
	Butyramide		1331.0 ± 24.0	
	Acrylamide		61.6 ± 6.0	
	Methacrylamide		82.6 ± 4.2	
	2,2-Dimethylcyclopropanecarboxamide		21.2 ± 0.9	
	2-Amino-2,3-dimethylbutyramide		24.7 ± 2.0	
	Aromatic	Benzamide		1.0 ± 0.2
		2-Phenylacetamide		31.0 ± 1.0
4-Hydroxybenzeneacetamide			318.0 ± 7.1	
2-Phenylbutyramide			ND ^b	
Mandelamide			112.8 ± 6.9	

Table 3 continued

Compound	Structural formula	Acyl transfer activity (U mg ⁻¹ protein) ^a
Isonicotinamide		36.9 ± 1.3
2-Chloronicotinamide		ND ^b
5-Chloronicotinamide		ND ^b
6-Chloronicotinamide		ND ^b
Tryptophanamide		23.8 ± 2.4

^a Acyl transfer activity was carried out at 50 °C under the standard conditions. The final concentration of hydroxamic acid was 1 M. All activities were measured in three separate experiments and expressed as means ± standard deviations

^b ND, not detected

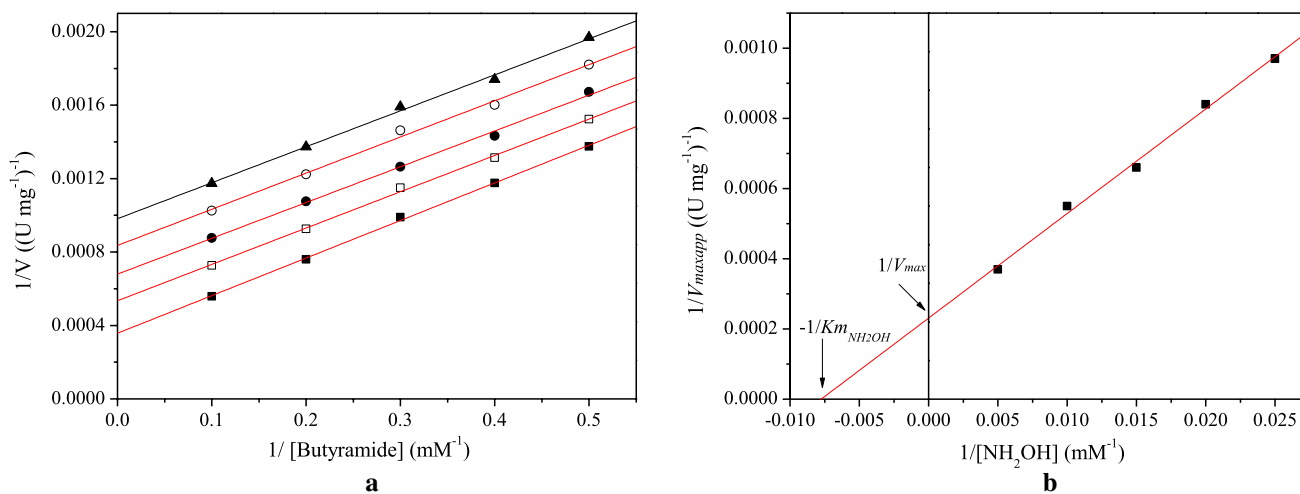


Fig. 4 Reciprocal plots obtained from a series of concentration of hydroxylamine and butyramide. **a** $1/v$ versus $1/[\text{Butyramide}]$ at different hydroxylamine concentrations. Symbols: 40 mM (filled square);

50 mM (open square); 66.6 mM (filled circle); 100 mM (open circle); 200 mM (filled triangle). **b** $1/v$ -axis intercept replot

Bioprocess development for the production of BHA

As Bami showed different K_m values for butyramide and hydroxylamine, the effect of substrate/co-substrate ratio on the reaction was studied. The highest activity of $1706.5 \pm 42.7 \text{ U mg}^{-1}$ was detected with the reaction mixture composed of 200 mM butyramide and 500 mM hydroxylamine hydrochloride (Fig. 5). Therefore, a substrate/co-substrate ratio of 2:5 was adopted for the following study.

Temperature is one of the most important factors controlling biochemical reactions. As shown in Fig. 6, the concentration of BHA reached 169.7 mM with the highest conversion of 84.9 % at 50 °C, while it decreased significantly when the reaction was performed at 30 and 40 °C. In addition, the by-product butyric acid was remarkably accumulated under lower reaction temperatures compared to the biotransformation performed at 50 °C. Therefore, the temperature of 50 °C was adopted as the operating temperature in the study.

The influence of Bami loading on the production of BHA was also examined. As shown in Fig. 7, the yield of BHA was improved apparently with the increase of Bami concentration from 5 to 10 $\mu\text{g mL}^{-1}$ in the reaction mixture. However, at 15 $\mu\text{g mL}^{-1}$ concentration, maximum yield 87.5 % was obtained in 15 min, which was slightly higher than that of 84.9 % achieved at 10 $\mu\text{g mL}^{-1}$ in 20 min. This indicated that the additional biocatalyst could not improve the conversion drastically. To obtain higher yield of BHA, 15 $\mu\text{g mL}^{-1}$ purified

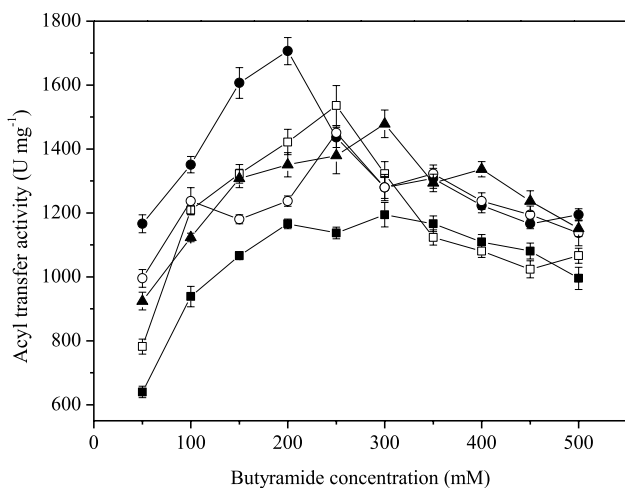


Fig. 5 Optimization of the combination of substrates. The concentration of butyramide was varied from 50 to 500 mM. Concentration of hydroxylamine hydrochloride: 100 mM (filled square); 250 mM (open square); 500 mM (filled circle); 750 mM (open circle); 1000 mM (filled triangle). The enzyme activity was determined in pH 7.5 Tris–HCl buffer at 50 °C. All activities were measured in three separate experiments and expressed as means \pm standard deviations

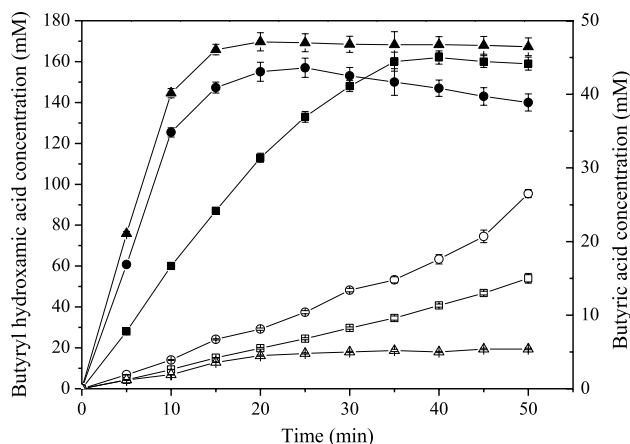


Fig. 6 The concentration of butyrylhydroxamic acid formed at different temperatures. Reaction conditions: 100 mM pH 7.5 Tris–HCl buffer, butyramide (200 mM), hydroxylamine (500 mM), 10 $\mu\text{g mL}^{-1}$ purified Bami. Butyrylhydroxamic acid at 30 °C (filled square), 40 °C (filled circle), 50 °C (filled triangle), butyric acid at 30 °C (open square), 40 °C (open circle), 50 °C (open triangle). All activities were measured in three separate experiments and expressed as means \pm standard deviations

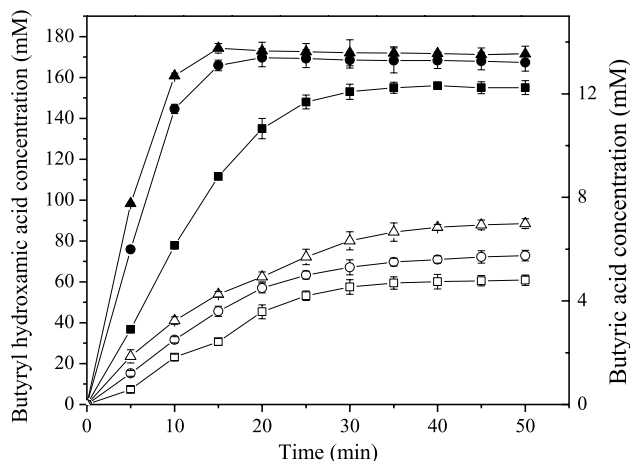


Fig. 7 The concentration of butyrylhydroxamic acid formed with different biocatalyst loadings. Reaction conditions: 100 mM pH 7.5 Tris–HCl buffer, butyramide (200 mM), hydroxylamine (500 mM), 50 °C. Butyrylhydroxamic acid formed with 5 $\mu\text{g mL}^{-1}$ (filled square), 10 $\mu\text{g mL}^{-1}$ (filled circle), 15 $\mu\text{g mL}^{-1}$ (filled triangle) Bami, butyric acid formed with 5 $\mu\text{g mL}^{-1}$ (open square), 10 $\mu\text{g mL}^{-1}$ (open circle), 15 $\mu\text{g mL}^{-1}$ (open triangle) Bami. All activities were measured in three separate experiments and expressed as means \pm standard deviations

Bami was selected as the optimal amount of catalyst in this study.

To further evaluate the potential of the bioprocess for practical use, a 100 mL reaction was carried out in a 200 mL stirred-tank reactor. Under the condition of substrate/co-substrate ratio 2:5, pH 7.5 (100 mM Tris–HCl

buffer), temperature 50 °C and purified refolded Bami loading 15 µg mL⁻¹, a BHA yield of 88.1 % was achieved from 200 mM butyramide within 15 min.

Discussion

In this study, a novel amidase gene was cloned from *B. epidermidis* ZJB-07021 which was previously isolated in our laboratory. Taking advantage of amino acid sequence of

the peptide fragment identified by MALDI-TOF/TOF–MS and the conserved sequence of *Brevibacterium* sp. amidases, a 783-bp fragment was amplified by PCR, and the sequenced fragment was further utilized with the hiTAIL-PCR strategy to amplify the complete *bami* gene. A homology search of NCBI database revealed that Bami exhibited a low sequence identity with other reported amidases. The most related proteins were D-amino acid amidase from *D. acidovorans* (GenBank Accession No: BAE87102) [21], D-amidase from *V. paradoxus* (GenBank Accession

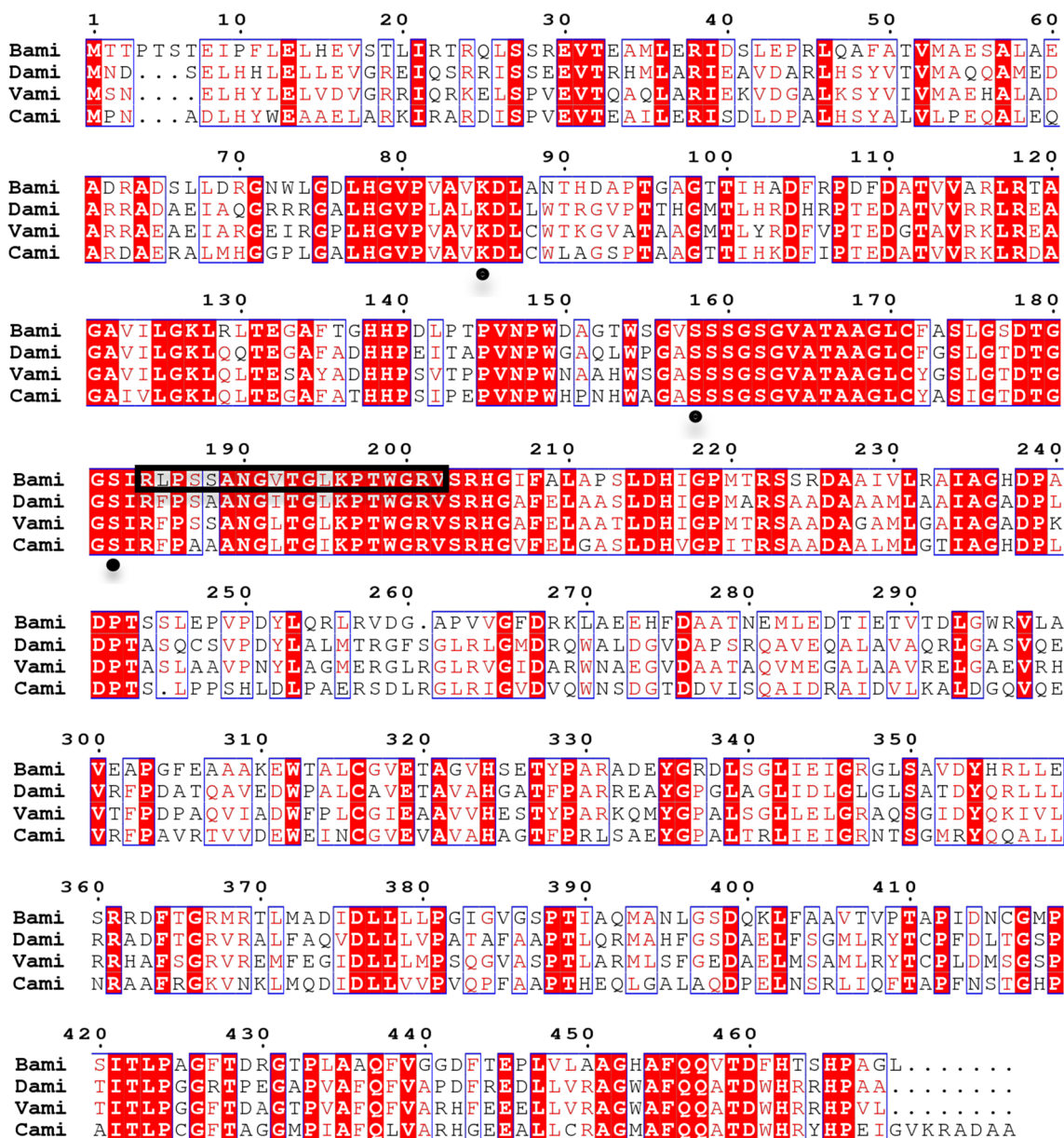


Fig. 8 Multiple alignment of the amino acid sequences of Bami and other *R*-stereospecific amidases. Abbreviations: Bami, *R*-stereoselective amidase from *B. epidermidis* ZJB-07021 (GenBank Accession No: KT807799); Dami, D-amino-acid amidase from *D. acidovorans* (GenBank Accession No: BAE87102); Vami, D-amidase from *V.*

paradoxus (GenBank Accession No: CAE06273); Cami, *R*-enantioselective amidase from *C. acidovorans* KPO-2771-4 (GenBank Accession No: E12107). The putative catalytic triad K-S-S was annotated with filled circles. The framed region represented the corresponding sequences obtained from MALDI-TOF/TOF–MS

No: CAE06273) [25] and *R*-amidase from *C. acidovorans* KPO-2771-4 (GenBank Accession No: E12107) [19], which shared 52–55 % of identity with Bami. Interestingly, all of them exhibited *R*-stereospecificity. As shown in Fig. 8, the alignment of the four *R*-amidases revealed that they formed a sub-family which shared G(A, V) SSSG (residues 156–161), a slight deviation from the characteristic motif of the AS family (GGSSGG) found in other enzymes. In addition, the *R*-amidases shared some other conserved regions, including LHGVPXAXKDL (residues 77–87), GXDTGGSIR (residues 176–184) and KPTWGRVSRHG (residues 196–206). The putative catalytic triad of K⁸⁵-S¹⁵⁸-S¹⁸² was also involved in the conserved regions.

Most of the recombinant Bami was overexpressed as insoluble IBs, which were devoid of biological activity, and therefore, solubilization and mild refolding to recover the desired enzyme was necessary. The IBs showed unique characteristics of high expression of protein, resistance to protease attack and simple purification procedure of the denatured protein [29]. However, one of the key issues for making full use of the IBs was to develop a refolding procedure with an ideal yield. In the current work, the IBs of Bami were firstly purified by detergent washing and then solubilized with 8 M urea solution. Combined with an efficient refolding method and one-step purification, 22.6 % of IBs was refolded into bioactive amidase. The yield of Bami (56.6 mg purified Bami from 1 L culture) was much higher than the final yield of *Sulfolobus tokodaii* strain 7 amidase (6.3 mg from 1 L culture) processed with another folding procedure [38].

The optimal pH of the recombinant Bami was similar to most of the reported amidases [1, 4, 33], which showed the

maximum acyl transfer activity around neutral pH. It has been proved that both amidase-catalyzed hydrolysis and acyl transfer reactions shared the same mechanism [27]. However, the reaction temperature had different effects on Bami-catalyzed hydrolysis and activity. The Bami showed the maximum acyl transfer activity at 50 °C, whereas its hydrolysis activity was hardly detected at the same temperature. Hydroxylamine is a better acyl acceptor than water, and the acyl transfer reaction with hydroxylamine proceeds faster than the hydrolysis of the same substrate [14]. Hydrolysis activity might be more dependent on the stable conformation of the active pocket of amidase using water as acceptor, and was prone to be inactive at high temperature. This characteristic of Bami was employed to reduce the formation of by-product butyric acid, resulting in a higher yield of BHA. Several bioprocesses for the synthesis of hydroxamic acid were summarized in Table 4. Interestingly, high yields (>90 %) of hydroxamic acids were usually obtained at high temperatures (>45 °C), which verified the conclusion that hydrolysis activity of amidase was more sensitive to high temperature compared with acyl transfer activity. It was reported that linear molecules were preferred substrate for acyl transfer reaction while bulky side-chain-containing amides exhibited close $K_{cat_{hydrolysis}}$ and $K_{cat_{acyl transfer}}$ values [16]. In this paper, it was also observed that butyramide (linear molecule) was hydrolyzed more slowly compared to its acyl transfer reaction under the same conditions (<40 °C). In addition, the substrate specificity study indicated that Bami had potential applications for the production of various high-value hydroxamic acids.

The biosynthesis of BHA from butyramide was studied using the purified recombinant Bami. To our

Table 4 Comparison of different properties of Bami with earlier studies

Enzyme or strain	Bami	<i>P. putida</i> BR-1	<i>Alcaligenes</i> sp. MTCC 10674	<i>G. pallidus</i> BTP-5x MTCC 9225	<i>Bacillus</i> sp. APB-6	<i>Bacillus smithii</i> Strain IITR6b2	<i>Rhodococcus</i> sp. R312	<i>R. erythropolis</i> A4
Buffer and pH	Tris–HCl, 100 mM, pH 7.5	Sodium phosphate, 100 mM, pH 7.5	Sodium phosphate, 100 mM, pH 7.0	Sodium phosphate, 100 mM, pH 7.0	Glycine–NaOH, 100 mM, pH 7.5	Sodium phosphate, 100 mM, pH 7.0	Sodium phosphate, 20 mM, pH7.0	Tris–HCl, 50 mM, pH 8.0
Reaction temperature (°C)	50	50	50	65	45	30	30	28
Substrate	Butyramide	Nicotinamide	Benzamide	Acetamide	Acetamide	Nicotinamide	Nicotinamide	Benzamide
Concentration of amide and hydroxylamine (mM)	200 500	200 1000	100 200	100 500	300 800	200 250	50 100	15 500
Conversion (%)	88.1	95.0	>90.0	93.0	94.0	94.5	40.0	40.0
References	This study	[6]	[5]	[36]	[31]	[2]	[15]	[40]

knowledge, molar ratio of substrate and co-substrate was a crucial parameter for higher yield in enzymatic synthesis of hydroxamic acid. A 2:5 molar ratio of butyramide to hydroxylamine was selected so that acyl group of amide was transferred more efficiently to hydroxylamine, leading to the formation of corresponding hydroxamic acid [15]. It was also observed that the increased concentration of butyramide and hydroxylamine resulted in substrate inhibition, which was similar to amidase from *Alcaligenes* sp. MTCC 10674 [5] and *Bacillus smithii* strain IITR6b2 [2]. There were several undesirable reactions accompanying the biosynthesis of hydroxamic acids from amides, and by-products would be irreversibly produced by the initial amides hydrolysis and final hydroxamic acids hydrolysis [16]. In this study, both of the undesirable hydrolysis reactions were evidently observed at 30 and 40 °C (Fig. 6). However, the by-product formation by Bami was significantly relieved at 50 °C as compared to lower temperatures, and the by-product concentration did not increase as the reaction proceeded. It was probably caused by the inactivation of amidase hydrolysis activity ahead of acyl transfer activity with increase of temperature.

In this paper, the sequence of the *R*-amidase gene from *B. epidermidis* ZJB-07021 has been elucidated by the hiTAIL-PCR approach. After assembling partial and flanking fragments, the complete gene was cloned from genomic DNA and transferred into *E. coli*. The strategies for heterologous expression, refolding and purification of the Bami were established successfully. An efficient biocatalytic process for the synthesis of BHA by the Bami was also developed with high conversion in a short period of reaction time. Increase of temperature was observed to prevent the undesired hydrolysis reactions while had no effect on the acyl transfer activity. These unique characteristics, together with the broad substrate spectrum, make Bami a potential catalyst for the production of BHA and other valuable hydroxamic acids.

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