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ORIGINAL ARTICLE

Characterization of an acid-stable catalase KatB isolated from *Bacillus altitudinis* SYBC hb4

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Abstract In recent decades, many microorganisms have been selected and bred for industrial production of alkali-stable catalases. Given the diversity and excellent properties of *Bacillus* catalases, the aim of this work was to screen potent acid-stable catalases from *Bacillus* strain(s). A strain with higher production of catalase activity was identified and designated as *Bacillus altitudinis* SYBC hb4 based on phenotypic properties, 16S rRNA and *gyrB* gene analyses. Its four catalase genes were cloned successfully and designated as *katX*, *katB*, *katN1* and *katN2*, respectively. Three distinct catalases were detected by isozyme zymography; however, only one (KatB) was successfully identified by MALDI-TOF. KatB had an estimated molecular mass of 228 kDa, and consisted of four identical subunits of 57 kDa. KatB displayed optimal activity under conditions of pH 5.0, 30 °C and 25 mM H_2O_2. The apparent K_m and V_{max} were calculated to be 62 mM and 33.4 mol min^{-1} mg^{-1}, respectively. KatB can maintain at least 60 % relative activity after 6 h under conditions of pH 5.0 and 30 °C. Its activity and stability were higher compared to bovine liver catalase under slightly acid conditions (pH 5.0). Thus, *B. altitudinis* SYBC hb4 KatB might represent a potential acid-stable catalase used in acidic conditions.

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Introduction

Catalases (EC 1.11.1.6) are used widely for removal of excess hydrogen peroxide (H_2O_2) as a bleaching or microbicidal agent in the paper, food, textile, and semiconductor industries (Yumoto et al. 1999). Many microorganisms have been used in the industrial production of catalases to date (Xu et al. 2014); however, past endeavors were focused mainly on screening of thermo and/or alkali stable catalases (Paar et al. 2001; Ebara and Shigemori 2008) due to the massive demand for catalases in the textile-dying and paper-making industries. Less attention has been paid to acid-stable catalases. Nevertheless, acid-stable catalases are indispensable in some practical areas. For example, catalases can be used to remove H_2O_2 generated by glucose oxidase in the conversion of glucose into gluconic acid (da Silva et al. 2011). Similarly, the conversion rate of cellobiose dehydrogenase converting lactose to lactobionic acid was facilitated by catalases (Gutierrez et al. 2012). Both glucose oxidase and cellobiose dehydrogenase exhibited optimal conversion rate under acidic conditions (pH 4–5). Auxiliary catalases should also have higher activity and stability under acidic conditions; however, commercial catalases are generally alkali-stable and with higher activity under alkaline conditions. Thus, screening of acid-stable catalases will be unavoidable in practice.

As mentioned, in recent decades, many microorganisms have been selected and bred for industrial production of alkali-stable catalases (Xu et al. 2014), while there have been very few reports on screening of strains producing acid-stable catalases. *Bacillus* strains are reported to possess a variety of catalases with excellent enzymatic characteristics (Yumoto

et al. 1990; Hicks 1995). Therefore, in this work, we focused on screening of *Bacillus* strains and assessing their potential for producing acid-stable catalases. We thought that *Bacillus* strains inhabiting acidic environments would have greater potential for producing acid-stable catalases. Honey is well known for its antibacterial activity, which stems from its acidic condition (pH 3.5–4.5), high concentration of sugar (60–70 %) and trace amounts of H_2O_2 (Saxena et al. 2010; Zhu et al. 2010). However, in the past, various *Bacillus* strains have been isolated from different honey samples (Ibarguren et al. 2010; Aween et al. 2012; Kwakman and Zaat 2012; Sinacori et al. 2014). Therefore, in this work, honey was chosen to screen for *Bacillus* strain(s) producing acid-stable catalases. All *Bacillus* strains isolated from different honey samples were assessed for their production of catalases. High-yielding *Bacillus* strains would be used preferentially for isolation and characterization of potential acid-stable catalases.

Materials and methods

Isolation and cultivation

Twenty-five honey samples were collected from several apiaries located in the Guangxi province of China. All samples were kept at room temperature in the shade. Each honey sample was diluted by a gradient dilution method. Diluted honey samples (200 μL; 10^{-5} dilution) were spread on a nutrient broth (NB) agar plate (g L^{-1}): beef extract 5.0, peptone 10.0, NaCl 5.0, and agar 20.0; pH 7.0–7.2. The plates were incubated at 30 °C until bacterial colonies grew to sufficient size. The purity of the bacterial colony was assessed through colony morphology and microscopy. The pure bacteria were inoculated on a NB agar slant and cultivated at 30 °C for 24 h. The basic fermentation medium for catalase production was prepared as nutrient broth (NB) medium (pH 7.0). Each flask (250 mL) contained 50 mL fermentation medium with 2 % (v/v) inoculum size. The inoculated medium was incubated aerobically at 30 °C and 200 rpm for 36 h.

Bacterial growth curve and enzyme assay

The bacterial growth curve was measured by the turbidimetric method at 600 nm. Crude enzyme was prepared as follows: 35 mL bacterial cultures were collected by centrifugation for 15 min at 6,000 rpm and 4 °C; the precipitated cells were resuspended in 1 mL K_2HPO_4-KH_2PO_4 buffer (pH 7.0; 50 mM) and pre-cooled on ice; the bacterial suspension was broken apart by the intermittent ultrasonic crushing method for 10 min; the resulting suspension was centrifuged to collect the supernatant at 10,000 rpm and 4 °C for 15 min. The supernatant was crude enzyme containing intracellular catalases. The total catalase activity of bacterial crude enzyme was determined according to the method described by Xu et al. (2014). The total reaction volume of 4 mL, containing 0.100 mL crude enzyme, 0.1 M phosphate buffer (pH 7.0), 50 mM H_2O_2, was kept warm at 30 °C for 30 min. One unit of catalase activity (U) was defined as the amount of enzyme required to decompose 1 μmol H_2O_2 per minute. The activity of bacterial intracellular catalases was measured in terms of U g^{-1} dry cell weight (DCW). Bacterial DCW was measured by weighing using an electronic balance.

Isozyme zymography of catalases was conducted by 8 % (w/v) polyacrylamide gel electrophoresis (PAGE) of crude enzyme under non-denaturing conditions. The reactive staining protocol for catalases was improved from the activity assay method using a chromogenic probe involving isoniazid (INH) and pyrocatechol (PC) (Shivakumar et al. 2011). Briefly, the native gel was incubated in 60 mL staining solution containing 0.218 mM INH, 0.756 mM PC, 0.218 mM INH, 14.1 mM H_2O_2 and 16.6 mM Tris buffer (pH 7.0). The staining solution was replaced every 5 min until isoenzyme bands were clear.

Bacterial identification

Phenotypic characteristics of the bacteria were determined according to *Bergey's Manual of Systematic Bacteriology*. Physiological and biochemical experiments included starch hydrolysis, catalase, urease, denitrification, V-P test, M-R test, gelatin liquefaction test, sugar fermentation tests and utilizing citric acid salt. Molecular identification of bacteria was performed by phylogenetic analyses of the 16S rRNA and *gyrB* genes. After bacterial genomic DNA was extracted, the 16S rRNA gene and *gyrB* (encoding DNA gyrase beta subunit) fragments were amplified by PCR with the primers shown in Table 1. PCR was carried out in a 50-μL reaction mixture containing 5 μL 10XPCR buffer (plus 20 mM MgSO4),

Table 1 Primers used for gene cloning in this study

Gene	Span (bp)	Primer
16S rRNA gene	1423	27F: 5′-AGAGTTTGATCCTGGCTCAG-3′
		1492R: 5′-ACGGTTACCTTGTTACGACTT-3′
gyrB	1923	F: 5′-GTGGCAATGGAACAGCAAC-3′
		R: 5′-CTAAATATCAAGATTTTTTAC-3′
cat1	1608	F: 5′-TTGAAGGAGGGATTCGAATTG-3′
		R: 5′-TTAATAAGGATCTGATGG-3′
cat2	1476	F: 5′-ATGACAAATTCAAATCAT-3′
		R: 5′-TTATTTCATGTTTCCTTG-3′
cat3	831	F: 5′-ATGTTTTATCACATTAAAGAAC-3′
		R: 5′-TTATTCGCCTTTTTTCGG-3′
cat4	897	F: 5′-ATGATCAAACGTGACAAG-3′
		R: 5′-TTATAAACCAGCAGATCTC-3′

4 μL dNTP mixture (2.5 mM), 1 U *Taq* polymerase, 20 μL primers (10 μM), and 20 ng template DNA. The PCR thermal profile was as follows: an initial denaturation at 95 °C for 5 min; 34 cycles each consisting of 94 °C for 30 s, 37 °C for 30 s, and 72 °C for 90 s; and a final extension at 72 °C for 10 min. The PCR products were electrophoresed on a 1 % agarose gel (plus Gold View as dye) at 10 V cm^{-1} for 1 h and photographed on a UV transilluminator (Huang and Fu 2005). The sequences of 16S rRNA and *gyrB* genes were aligned using Clustalx 1.81 software and used to construct phylogenetic trees with MEGA 4.0 software (Tamura et al. 2007).

Catalase gene cloning and phylogenetic analysis

Catalase genes were amplified by PCR with specific primers (Table 1) designed based on the genome of *Bacillus pumilus* SAFR-032 (NC_009848.1) (Gioia et al. 2007). The PCR process was the same as that described above for PCR-amplification of the 16S rRNA and *gyrB* genes. The PCR products were gel-purified and ligated to pET-28a (+) vector (Takara Bio, Dalian, China). The recombinant plasmids were then transformed into competent cells of *Escherichia coli* JM109. The transformed cells were selected on LB agar plates supplemented with 0.1 mg L^{-1} ampicillin. The positive clones were validated by colony PCR using the corresponding primers. Colony PCR was conducted as follows: the potential clones were numbered and each individual colony was picked with a sterilized tip; the tip with bacterial cells was touched on the LB agar plates lightly to make a colony and the same tip was then submerged in the 20 μL reaction mixture; the composition of the reaction mixture was the same as the 50-μL PCR reaction mixture above, with the bacterial cells washed from the tip acting as the template for the PCR reaction instead of DNA. The PCR thermal profile was also the same as with the above-mentioned PCR-amplification of 16S rRNA and *gyrB* genes.

Positive recombinants were screened roughly using the H_2O_2 bubbling test (Zeng et al. 2011). Clones with abundant foam were chosen as positive clones for further sequencing. The recombinant plasmids of positive clones were extracted and sequenced by Majorbio (Shanghai, China). Sequencing data were utilized to retrieve homologous sequences using the BLASTN program (http://www.ncbi.nlm.nih.gov). Catalase genes were identified by phylogenetic analysis and sequence conservation of homologous genes. A phylogenetic tree was constructed using homologous catalase genes by Clustalx 1.81 and MEGA 4.0 software (Tamura et al. 2007). Conservation analyses of the deduced catalase amino acid sequences were conducted with the BLASTP program.

Identification and characterization of catalases

Proteins of bacterial crude enzyme were separated on 12 % (w/v) gels by SDS-PAGE. The protein bands were dyed clearly by Coomassie Brilliant Blue G-250. The clear bands were extracted and analyzed by MALDI-TOF by Sangon Biotech Co. (Shanghai, China). The mass spectrometry analysis was completed by ABI 4800 tandem mass spectrometry. Secondary mass spectrometry, on the basis of peptide fingerprint mass spectrometry, was conducted for the ten peaks with maximum intensity. The molecular weight (MW) of peptides and debris was used to analyze mass spectrometry data and to retrieve homologous proteins using the GPS 3.6 (Applied Biosystems, Foster City, CA) and Mascot 2.1 (Matrix Science, http://www.matrixscience.com) programs.

Bacillus altitudinis SYBC hb4 catalases were purified and characterized according to the method described by Zeng et al. (2011). Briefly, the main procedures were as follows: the pooled crude enzyme was precipitated by 50 % and 60 % (w/v) saturation with ammonium sulfate; precipitates with catalase activity were collected and dissolved in an appropriate amount of 50 mM K_2HPO_4–KH_2PO_4 buffer (pH 7.0); the re-dissolved supernatants were desalted using Sephadex G-25 (GE Healthcare, Piscataway, NJ) and concentrated in an ultrafiltration centrifuge tube (Millipore, Bedford, MA); the desalted supernatants were subjected to an anion exchange column (HiTrap DEAE FF, 5 mL; GE Healthcare); fractions eluted with activity were collected and loaded on a Superdex 200 column (10/300 GL; GE Healthcare); fractions with activity were electrophoresed on 12 % SDS-PAGE; the equilibration buffer was prepared as 50 mM K_2HPO_4–KH_2PO_4 buffer (pH 7.0) at each purification step. The elution buffer used for anion exchange and Superdex 200 columns was prepared as 50 mM K_2HPO_4-KH_2PO_4 buffer (pH 7.0) plus 1 and 0.15 M NaCl, respectively.

The effects on the purified catalase of pH value and temperature were assessed as follows: effect of pH value on catalase activity was measured by incubating catalase in 50 mM Na_2HPO_4-citric acid buffer (pH 3.0–8.0) at 30 °C; effect of temperature on catalase activity was measured at 20 °C to 45 °C under the optimal pH value; effect of pH (3.0–8.0) on catalase stability was measured by incubating catalase at 30 °C for 180 min, and the initial activity was regarded as 100 %; stability of catalase was represented by catalase activity under optimal temperature and pH; effect of H_2O_2 concentration (5, 10, 15, 20, 25, 30, 35 mM) on catalase activity was evaluated in 50 mM Na_2HPO_4-citric acid buffer (pH 5.0) at 30 °C; catalase activity was defined as relative activity and the highest activity was regarded as 100 % in each determination. Michaelis-Menten constant (K_m) and maximal reaction velocity (V_{max}) were estimated by linear regression from double-reciprocal plots according to Lineweaver and Burk.

Statistical analysis and graphics

All experiments were performed in at least three repetitions. Data was evaluated statistically with SPSS base 11.0 software. All graphics were drawn by Origin 8.0 software.

Results

Isolation of bacteria with high catalase production

A total of 127 bacterial strains (numbered hb1–127) were isolated from 25 honey samples. These bacteria all tested positive for catalase. Enzyme assay showed that 19 bacteria were detected evidently with catalase activity in crude enzyme (Fig. 1). Strains hb4, 25, 36 and 58 had catalase activity of 22,539, 7253, 4680 and 13,867 U g^{-1} DCW, respectively. Catalase activities of other strains were lower than 5000 U g^{-1} DCW and that of strain hb16 was only about 1239 U g^{-1} DCW. Thus, strain hb4 was chosen as a candidate due to its higher catalase activity compared to other strains. Its catalase activity was more 18 times that of strain hb16.

Strain hb4 was cultured for catalase production in fermentation medium at 30 °C and 200 rpm for 36 h. The curves of growth and catalase production of strain hb4 were determined (Fig. 2). Growth of strain hb4 exhibited a typical bacterial growth curve with a logarithmic phase from 12 to 24 h. Production of catalase emerged during a rapid-growth period during fermentation. Catalase activity increased about threefold in this period (18–21 h). This period coincided with a later phase of logarithmic growth.

Catalase isozymes of strain hb4 were separated and analyzed by PAGE. A total of three isozymes were separated and could be distinguished in the gel (Fig. 3b). Only one catalase

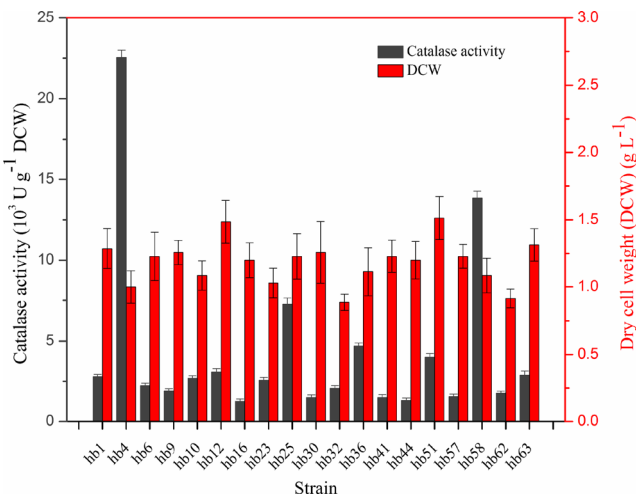

Fig. 1 Catalase activity of honey-associated bacteria cultured in nutrient broth (NB) medium for 36 h

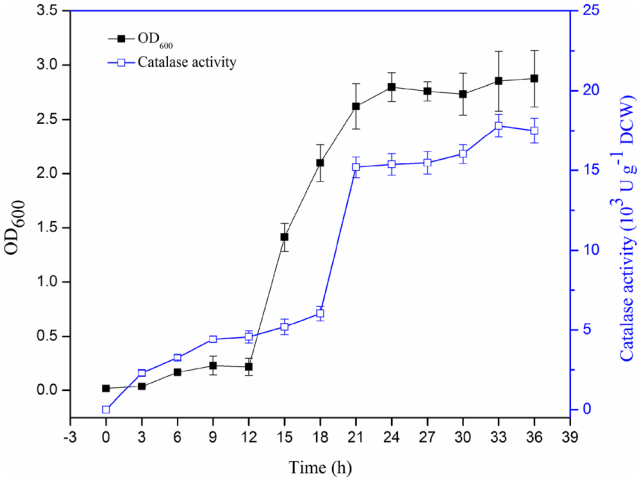

Fig. 2 Profile of growth and catalase production of strain hb4 cultured in NB medium for 36 h

was clearly visible before 12 h, while a total of three isozymes were distinctive after 18 h. The quantity of each isozyme also increased to different degrees after 18 h. It was noteworthy that the increase in isozymes emerged in the logarithmic growth phase with the rapid-growth of catalase activity.

Identification of strain hb4 by phenotypic tests, and 16S rRNA/*gyrB* gene sequences

The 16S rRNA gene of strain hb4 was sequenced and deposited with the GenBank database (KF857270). Strain hb4 showed 100 % 16S rRNA gene sequence similarity to *Bacillus aerius* MTCC 7303^T, *Bacillus aerophilus* MTCC 7304^T, *Bacillus stratosphericus* MTCC 7305^T and *Bacillus altitudinis* MTCC 7306^T. Secondly, strain hb4 showed 99 % sequence similarity to *Bacillus pumilus* ATCC 7061^T, *Bacillus safensis* FO-36b^T. Based on the similarity analysis of the 16S rRNA gene sequence, a phylogenetic tree was constructed by neighbor-joining (NJ) method using MEGA software (Fig. 4a). Based on a test of inferred phylogeny, a NJ tree was constructed successfully using the program Interior Branch Test of Phylogeny with the Kimura two parameter model. The phylogenetic tree revealed that strain hb4 shared the closest phylogenetic relationship with *Bacillus altitudinis* MTCC 7306^T. Therefore, strain hb4 could be preliminarily identified as the same genus as *Bacillus altitudinis*. In order to further classify strain hb4, phenotypic differences between strain hb4 and some type strains were compared in detail by physiological and biochemical tests (Table 2).

The phenotypic properties of the five type strains used as references were collected from different reports (Logan and Berkeley 1984; Shivaji et al. 2006; El Hadj-Ali et al. 2007; Anwar et al. 2009). All strains exhibited the same characteristics as follows: Gram-positive, rod-shaped cells, positive for catalase, forming spores, acid production from sucrose and glucose. Besides these latter properties, they all exhibited

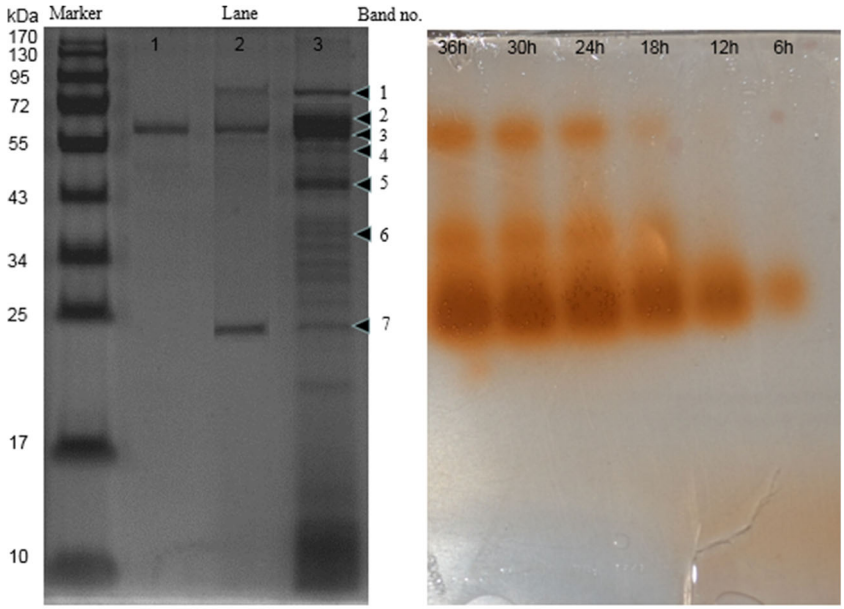

Fig. 3a,b Polyacrylamide gel electrophoresis (PAGE) analysis of proteins prepared from extracts of strain hb4 cells. **a** SDS-PAGE analysis of proteins; molecular mass standards (size in kDa) are listed on the *left*; protein bands (*black arrowheads 1–7*) were analyzed by MALDI-TOF. **b** Native PAGE analysis of catalases isozymes extracted from different fermentation times as noted above the gel

different phenotypic properties amongst themselves. For example, strain hb4, *B. pumilus* ATCC 7061^T and *B. pumilus* MTCC 1640^T were negative for amylolysis and utilized rhamnose, while *B. altitudinis* MTCC 7306^T, *B. aerophilus* MTCC 7304^T and *B. stratosphericus* MTCC 7305^T were positive. Strain hb4 exhibited different properties from

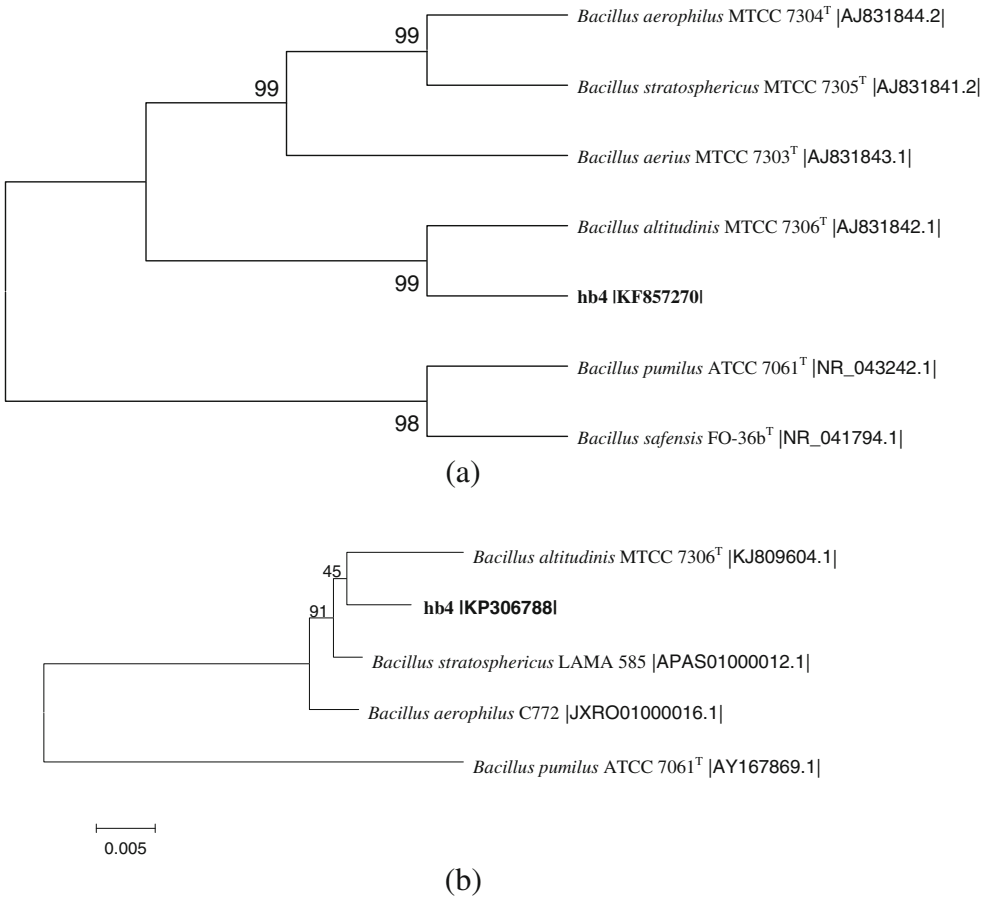

Fig. 4 **a**,**b** Neighbor-joining (NJ) trees based on sequences of the 16S rRNA (**a**) and *gyrB* (**b**) genes of strain hb4 and other *Bacillus* strains. Bootstrap values (expressed as percentages of 1000 replications) are given at nodes; sequence accession numbers are shown in parentheses

Table 2 Phenotypic characteristics[a] differentiate strain hb4, *Bacillus pumilus* ATCC 7061^T, *B. pumilus* MTCC 1640^T, *Bacillus altitudinis* MTCC 7306^T, *Bacillus aerophilus* MTCC 7304^T and *Bacillus stratosphericus* MTCC 7305^T

Phenotype	hb4	*B. pumilus* ATCC 7061^T	*B. pumilus* MTCC 1640^T	*B. stratosphericus* MTCC 7305^T	*B. aerophilus* MTCC 7304^T	*B. altitudinis* MTCC 7306^T
Gram stain	+	+	+	+	+	+
Spore	+	+	+	+	+	+
Amylolysis	-	-	-	+	+	+
V-P	+	+	+	+	+	-
M-R	+	ND	ND	-	-	ND
Catalase	+	+	+	+	+	+
Gelatin	+	ND	-	+	+	+
Citrate	-	ND	-	-	+	-
Urease	-	ND	ND	+	-	ND
Growth pH 5.0	+	ND	-	-	-	+
Maltose	+	-	ND	+	+	ND
Sucrose	+	+	+	+	+	+
Rhamnose	-	-	-	+	+	+
Glucose	+	+	+	+	+	+
Cellobiose	+	ND	-	+	-	+
D-Mannose	+	+	+	+	+	ND
Alpha lactose	+	+	+	+	+	ND
D-Sorbitol	-	ND	-	-	+	+
D-(+)-Xylose	+	ND	+	+	+	+
Inositol	-	-	-	+	-	+

[a] + Positive, − negative, *ND* no data shown

B. altitudinis MTCC 7306^T in amylolysis, V-P test and in utilizing rhamnose, D-sorbitol and inositol. Thus, chemotaxonomic analyses indicated that strain hb4 was different from the type strain of *B. altitudinis* MTCC 7306^T.

To further validate this speculation, the housekeeping gene *gyrB* of strain hb4 was also used for identification (Galloway-Pena et al. 2014). It has been reported *gyrB* is effective in precise species determination of bacteria (Collado et al. 2014; Draghi et al. 2014; Geng et al. 2014; Kpikpi et al. 2014; Lenaerts et al. 2014; Wang et al. 2014). Therefore, the *gyrB* sequence of strain hb4 was obtained by PCR and deposited with GenBank (KP306788). A fragment of 1130 bp exhibited 99 % similarity to *B. altitudinis* MTCC 7306^T, and fragment of 1133 bp showed 93 % similarity to *B. pumilus* ATCC 7061^T. A NJ tree was constructed using the Interior Branch Test of Phylogeny program with the Kimura two-parameter model based on the *gyrB* sequences (Fig. 4b). The NJ tree revealed that strain hb4 formed a branch with *B. altitudinis* MTCC 7306^T. However, the bootstrap value (given at the node in Fig. 4b) of this branch is only 45 % (less than 50 %). Thus, phylogenetic analysis of the *gyrB* gene indicated that strain hb4 differed slightly from *B. altitudinis* MTCC 7306^T. Combining the 16S rRNA and *gyrB* genes analyses and phenotypic tests, strain hb4 could be identified and designated as *Bacillus altitudinis* SYBC hb4.

Catalase gene cloning and phylogenetic analysis

Four fragments were obtained from the *B. altitudinis* SYBC hb4 genome by PCR using four pairs of catalase gene primers (*cat1*, *cat2*, *cat3* and *cat4* as shown in Table 1); these four fragments were also numbered *cat1*, *cat2*, *cat3* and *cat4*, respectively. The corresponding sequences were deposited with GenBank with accession numbers as follows: KF857271 (*cat1*), KF857272 (*cat2*), KF857273 (*cat3*) and KF857274 (*cat4*). These four sequences all exhibited more than 60 % similarity to homologous genes from some strains of *Bacillus* by the BLASTN program. They all exhibited the highest similarity (98–99 %) to the homologous catalase genes from *B. altitudinis* MTCC 7306^T and *B. pumilus* MTCC B6033. The sequences of *cat1*, *cat2*, *cat3* and *cat4* exhibited 89 %, 91 %, 86 % and 88 % sequence similarity to their homologs in *B. pumilus* SAFR-032, respectively. The sequence similarities between *B. altitudinis* SYBC hb4 catalase genes and other homologous genes are shown in Table 3. Based on similarity of catalase genes, a NJ tree (Fig. 5) was constructed and corrected with 1000 bootstrap iterations using model of Kimura two parameter by MEGA 4.0.

The phylogenetic tree revealed that the four catalase genes of *B. altitudinis* SYBC hb4 are separated into two basic clusters: the monofunctional heme catalase gene (*katE*) and Mn

Table 3 Sequence conservation (% identity) of catalase genes and proteins among *Bacillus* species

Gene/Protein	*B. subtilis*			*B. licheniformis*		*B. anthracis*
	katA/KatA	*katX*/KatX	*katN*/KatN	*katA*/KatA	*katX*/KatX	*katB*/KatB
katX/KatX	63/67	76/91	–/–	66/68	71/85	63/72
katB/KatB	69/70	64/70	–/–	65/70	64/71	70/83
katN1/KatN1	–/–[a]	–/–	76/92	–/–	–/–	–/–
katN2/KatN2	–/–	–/–	–/–	–/–	–/–	–/–

[a] –/–, no significant similarity (<50 %)

catalase gene (*katN*) clusters. The catalase genes of *cat1* and *cat2* are both members of *katE* but form two separate clusters by further steps of evolution. Similarly, *cat3* and *cat4* are both classified into two subgroups of *katN*. Generally, *Bacillus* catalase genes include *katA*, *katB*, *katX* and *katN* according to the gene expression phase (Loewen and Switala 1987; Bagyan et al. 1998). Catalase genes of *B. subtilis* and *B. licheniformis* can act as a reference to speculate on the origin of homologous *Bacillus* genes (Gioia et al. 2007). The phylogenetic tree indicated that *cat1* formed a primary cluster with *B. subtilis* and *B. licheniformis katX. Cat2* was located in the same primary cluster with *B. subtilis* and *B. licheniformis katA*. Further phylogenetic analysis indicated that *cat2* shared a closer relationship with *B. anthracis* str. Ames *katB*. However, *B. pumilus* SAFR-032 *katX2* was more closely related with *cat2* than *B. anthracis* str. Ames *katB*. KatB of *B. anthracis* str. Ames

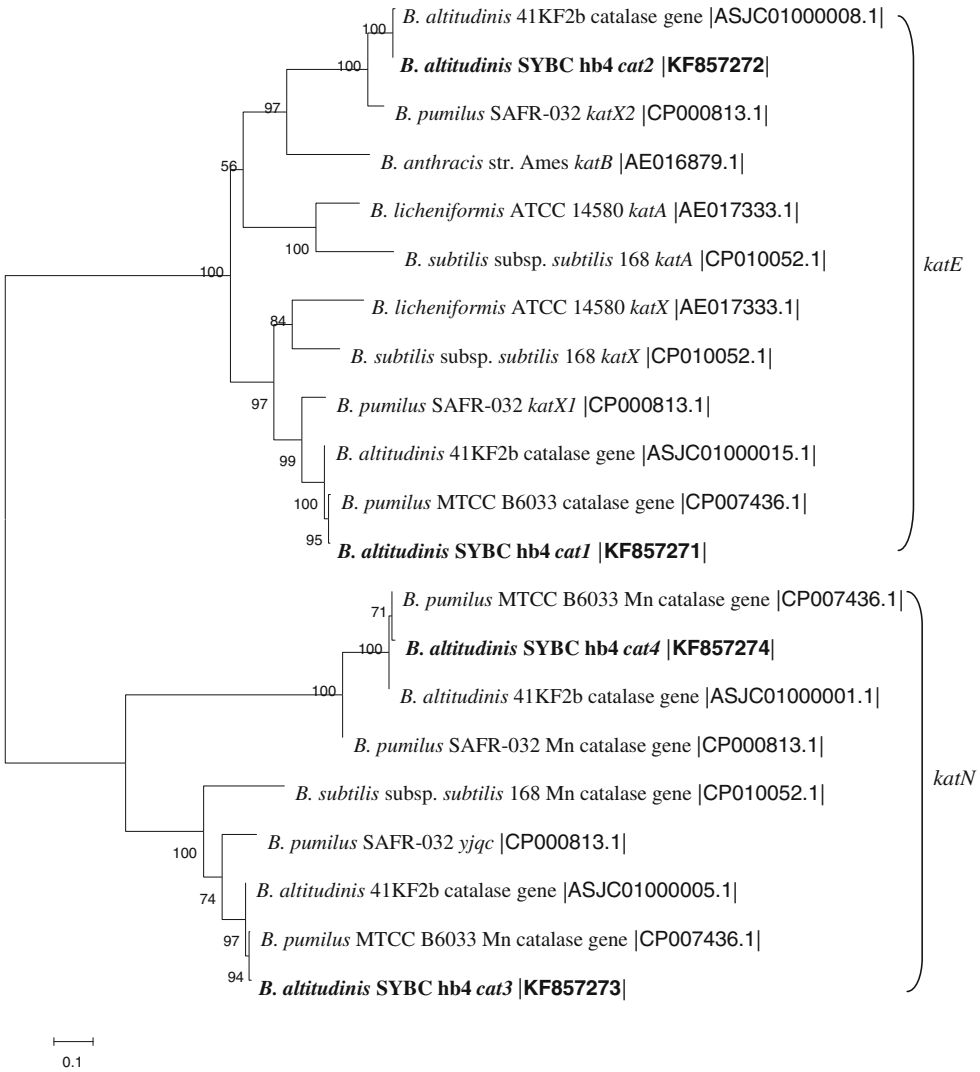

Fig. 5 Neighbor-joining tree based on homologous catalase genes of *B. altitudinis* SYBC hb4 and other *Bacillus* strains. The GenBank accession numbers of all sequences are listed in parentheses

has been validated as a vegetative catalase (Tu et al. 2012). There is doubt about authenticity of *B. pumilus* SAFR-032 katX2 as a spore catalase due to lack of experimental data. Therefore, *cat1* and *cat2* of *B. altitudinis* SYBC hb4 were designated as *katX* and *katB*, respectively. The phylogenetic tree also revealed that *cat3* and *cat4* both shared the closest relationship with *B. pumilus* MTCC B6033 Mn catalase genes. Meanwhile, *cat3* was phylogenetically more closely related to *B. subtilis* subsp. *subtilis* 168 Mn catalase gene than *cat4*. Thus, *cat3* and *cat4* were both identified as *Bacillus katN*. *B. altitudinis* SYBC hb4 *cat3* and *cat4* were designated as *katN1* and *katN2*, respectively.

In addition to the above phylogenetic analyses of catalase genes sequences, conservation analyses of their deduced amino acid sequences were also conducted using the program BLASTP (Table 3). As mentioned, *B. altitudinis* SYBC hb4 catalase genes would be considered homologs of *B. subtilis* and *B. licheniformis* catalase genes if their deduced amino acid sequences aligned with ≥ 50 % identity to the homolog of either species (Gioia et al. 2007). Sequence conservation analyses indicated that *B. altitudinis* SYBC hb4 KatX shared higher identity (91 %) to *B. subtilis* KatX. Although *B. altitudinis* SYBC hb4 KatB shares 70 % identity with KatA and KatX of *B. subtilis* and *B. licheniformis*, it has higher identity (83 %) to *B. anthracis* KatB. KatN1 of *B. altitudinis* SYBC hb4 shares 92 % identity with *B. subtilis* KatN. However, KatN2 has no significant identity to *B. subtilis* KatN. Thus, combined with phylogenetic analysis, sequence conservation analyses further validated the authenticity of identification of *B. altitudinis* SYBC hb4 catalase genes.

Identification and characterization of KatB

Crude enzyme prepared from *B. altitudinis* SYBC hb4 cell extract was separated and analyzed by SDS-PAGE (Fig. 3a). The clear bands were extracted and analyzed by MALDI-TOF/MS. Seven bands (numbered 1–7 in Fig. 3a) were identified successfully but only one was identified as a homologous catalase of *B. stratosphericus* (Table 4). A total of 11 peptide fragments (individual ions scores of six fragments> 59; shown in Table 4) were matched with the amino acid sequence of *B. stratosphericus* catalase. Combined with the deduced amino acid sequences of *B. altitudinis* SYBC hb4 catalases, the 11 fragments were all well matched with KatB.

In order to characterize *B. altitudinis* SYBC hb4 catalases, crude enzyme of catalases was purified according to the method described by Zeng et al. (2011). The purification procedures mainly included ammonium sulfate precipitation, anion exchange chromatography and gel filtration. All protein samples from gel filtration and anion exchange chromatography were analyzed by SDS-PAGE (Fig. 3a, Lanes 1 and 2). A

Table 4 Proteins identified in crude enzyme of *B. altitudinis* SYBC hb4 cells extract

Band no.[a]	Identified protein	Mass	NCBI accession no.	% Sequence coverage	Mascot score [b]	Peptide (s)
1	Endopeptidase	86,409	WP_008359605.1	2	88	R.VLGPGGSGTTENVLAGIDR.A
2	Peptide-binding protein	61,769	WP_008348494.1	5	191	K.HILGDVPIKDLGENEFNR.K
						K.VREDIAVVVQQQLK.E
3	Catalase	56,928	WP_007497088.1	27	815	K.NLTTNQGVPVGDNQNSR.T
						R.GAGAYGVFEVENSMEK.H
						R.AAFLSEEGKQTDVFVR.F
						K.FYTEEGNYDLVGNNLPIFFIR.D
						R.NLSMEEAAEIQANDFQHATR.D
						K.NLTTNQGVPVGDNQNSR.T
4	Phage portal protein	49,137	WP_003213180.1	2	76	R.LSQGEFLFTFDAR.D
5	Leucine dehydrogenase	40,216	WP_003215658.1	12	223	K.AIIAIHDTTLGPALGGTR.M
						R.MWTYENEEAAIEDALR.L
6	Frv operon protein	39,398	WP_008346014.1	21	441	K.VMQSYIETYADEVSTDR.L
						R.FQTVGGWWSQVMLAQR.V
						K.KGDITGVIGSKPPHVLSPEAR.K
						K.NLKDTQHENIAYSVATVQEEVGLR.G
7	Superoxide dismutase	22,741	WP_005606784.1	19	202	K.TPLLGLDVWEHAYYLNYQNR.R
						R.RPDYISAFWNVVNWDEVAR.R

[a] Bands of proteins were marked in Fig. 3;

[b] Ions score is $-10\times\mathrm{Log}(P)$, where P is the probability that the observed match is a random event. Individual ions scores>59 indicate identity or extensive homology ($P<0.05$). Protein scores are derived from ions scores as a non-probabilistic basis for ranking protein hits

Table 5 Summary of purification of the catalase from *B. altitudinis* SYBC hb4

Purification step	Total activity (U)	Total protein (mg)	Specific activity (U mg^{-1} protein)	Purification (fold)	Yield (%)
Crude extract	56,220	119.48	470.54	1	100
Ammonium sulfate precipitation	18,380	35.50	517.75	1.1	32.69
DEAE sepharose FF	8904	7.62	1168.56	2.48	15.84
Superdex 200 gel filtration	8095	1.74	4652.3	9.89	14.4

single band indicated that a purified catalase was obtained. The molecular mass of the subunit was about 57 kDa. The molecular mass of the purified catalase was estimated to be 228 kDa by Superdex 200 column (data not shown). Thus, we speculated that the purified catalase was a tetramer catalase consisting of four homo-subunits. This catalase was purified 9.89-fold with a yield of 14.4 % by the above procedures (Table 5), and displays a specific activity of 4652 U mg^{-1} protein. Combined with the mass weight of KatB identified by MALDI-TOF, we speculated that the purified catalase was the product of *B. altitudinis* SYBC hb4 *katB*.

The catalytic activity of KatB was determined under different pH and temperature values (Fig. 6). KatB had maximum activity at 30 °C and pH 5.0. The activity of KatB had a slight decrease at 35–45 °C and pH range 6.0–8.0. Under optimal temperature and pH, the activity of KatB decreased gradually with time. It maintained 78 % of maximal activity after 6 h. However, bovine liver catalase retained only 40 % relative activity after 45 min (Fig. 6c). The activity of KatB was enhanced with increased H_2O_2 concentration up to a maximum under 25 mM H_2O_2. The activity of KatB was inhibited by higher concentrations of H_2O_2 (>30 mM). The kinetic parameters of KatB were analyzed by Lineweaver–Burk plot. The apparent K_m and V_{max} for KatB at 30 °C and pH 5.0 were 62 mM and 33.4 mol min^{-1} mg^{-1}, respectively. These results indicated that KatB exhibited higher activity and stability in slightly acidic conditions (about pH 5.0). Thus, *B. altitudinis* SYBC hb4 KatB might have potential as an acid-stable catalase used in acidic conditions.

Discussion

In this work, a total of 127 bacterial strains was isolated from different honey samples. Strain hb4 exhibited higher production of catalases compared to other strains. Based on phenotypic properties, and phylogenetic analyses of the 16S rRNA and *gyrB* genes, strain hb4 was identified and designated as *Bacillus altitudinis* SYBC hb4, differing slightly from *B. altitudinis* MTCC 7306^T (type strain). Enzyme assay

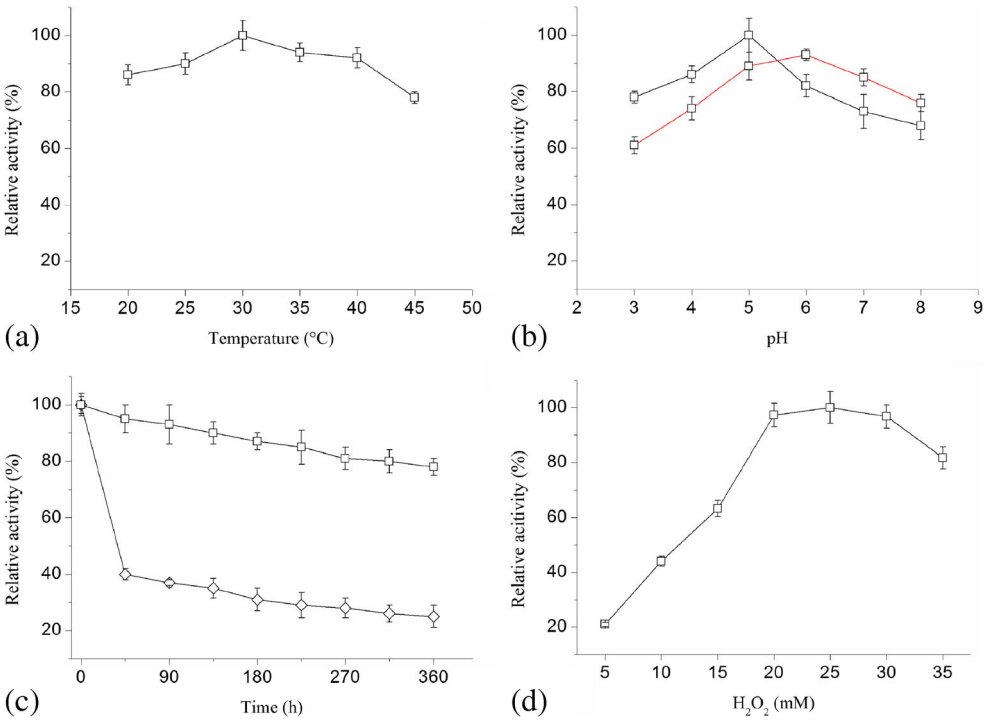

Fig. 6a–d Enzymatic properties of the catalase purified from *B. altitudinis* SYBC hb4 cell extract. **a** Effect of temperature on activity of the purified catalase, **b** *Black line* Effect of pH value on activity of the catalase, *red line* effect of pH (3.0–8.0) on catalase stability. **c** The stability of catalases at optimal temperature (30 °C) and pH 5.0; □ purified catalase, ◇ bovine liver catalase. **d** Effect of concentration of H_2O_2 on purified catalase activity under optimal conditions. Values in all figures represent means; *error bars* SD

indicated that *B. altitudinis* SYBC hb4 catalases were produced largely in the later phase of logarithmic growth (18–21 h). In this period, a total of three distinct catalase isozymes were visualized by isozyme zymography. This indicated that *B. altitudinis* SYBC hb4 produced at least three catalases. Generally, *Bacillus* catalase genes are diverse and consist of *katA*, *katB*, *katX* and *katN* (Loewen and Switala 1987; Bagyan et al. 1998) and this is consistent with the *B. altitudinis* SYBC hb4 catalase genes detected by gene cloning.

Based on phylogenetic analysis and sequence conservation, four catalase genes were cloned and designated as *katX*, *katB*, *katN1* and *katN2*, respectively. The phylogenetic tree revealed that the four catalase genes of *B. altitudinis* SYBC hb4 are classified into *katE* (*katX* and *katB*) and *katN* (*katN1* and *katN2*). Based on analysis of conserved domains, KatX and KatB are both small subunit catalases with a heme b binding pocket and comprising a tetramer. KatN1 and KatN2 both belong to the ferritin-like superfamily with a dimanganese center. However, the four catalases differ amongst themselves and were separated into four clusters by further phylogenetic analysis. Therefore, we speculated that *B. altitudinis* SYBC hb4 has four different catalases. For example, KatX and KatB could be further classified into spore catalase and vegetative catalase, respectively. According to known data, it is speculated that largely only KatB is produced by *B. altitudinis* SYBC hb4 after 36 h fermentation. Without appropriate induction, KatX, KatN1 and KatN2 are produced only slightly by *B. altitudinis* SYBC hb4. Isozyme zymography also indicated that only three catalases were produced. In the subsequent protein identification step, only one protein was identified as catalase. However, three catalase isozymes were distinctly exhibited in PAGE by isozyme zymogram. Thus, what are the other two catalases represented by reactive staining? Generally, the reactive staining method can sensitively detect trace amounts of catalases that Coomassie brilliant blue cannot. In addition, the trace catalases might be masked by some mass protein bands. As a consequence, identification of trace catalases might be disturbed by other proteins, thus explaining why only one catalase was identified in this work.

The identified catalase was classified as *B. altitudinis* SYBC hb4 KatB. Its MW was in agreement with the value predicted by the deduced amino acid sequence. The subunit MWs of KatX, KatB, KatN1 and KatN2 were computed as 60.97, 56.74, 31.09 and 33.39 kDa, respectively. The identified catalase MW was about 57 kDa according to its relative electrophoretic mobility in PAGE. Furthermore, the size of the purified catalase was similar to that of the identified catalase. Thus, we can speculate that the purified catalase corresponds to KatB of *B. altitudinis* SYBC hb4. Based on phylogenetic analysis and sequence conservation, *B. altitudinis* SYBC hb4 KatB is different from *B. subtilis* KatA. Thus, the enzymatic properties of *B. altitudinis* SYBC hb4 KatB might differ from those of *B. subtilis* KatA. the alkali-stable property of KatA has been investigated and improved by rational mutation (Cao et al. 2014). Some strains of *B. subtilis* have been explored as excellent candidates for industrial production of catalases; however, according to known data, *B. subtilis* lacks acid-stable catalases (Zeng et al. 2011; Xu et al. 2014). There is little information about the physicochemical properties of catalases produced by *B. altitudinis*. In this work, KatB of *B. altitudinis* SYBC hb4 was purified and validated. KatB displayed optimal activity under conditions of pH 5.0, 30 °C and 25 mM H_2O_2. The apparent K_m and V_{max} were calculated to be 62 mM and 33.4 mol min^{-1} mg^{-1} protein, respectively. KatB could maintain at least 60 % relative activity after 6 h under conditions of pH 5.0 and 30 °C. Its activity and stability were higher compared to catalase from bovine liver under slightly acid conditions (pH 5.0). Thus, *B. altitudinis* SYBC hb4 KatB might represent a potential acid-stable catalase use in acidic conditions.

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Conflict of interest There is no conflict of interest in this study.

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