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Purification and Molecular Cloning of Catalase from *Rhizobium radiobacter* Strain 2-1

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A heat-stable catalase from *Rhizobium radiobacter* strain 2-1 was purified. The purified catalase showed an extremely high specific activity of 764,500 units/mg and displayed stability in a broad pH range of 5–11 and thermostability up to 60°C. Its Km was 60.06 mM and its V_{max} was 10.17 mM/min. The enzyme was considered to be composed of four identical subunits with a molecular weight 56 kDa as determined by SDS-PAGE and a total molecular mass measured by gel filtration of 230 kDa. The gene encoding the catalase was sequenced by using touchdown and inverse PCR. The deduced amino acid sequence of the catalase from strain 2-1 showed 83% homology to that of the putative catalase *Sphingomonas wittichii*. The proximal active site and the proximal heme ligand were tentatively identified. The combined results of the gene sequence analysis and biochemical characterization proved that the catalase from strain 2-1 is a typical monofunctional heme catalase.

Key words: catalase, *Rhizobium radiobacter*

1. Introduction

Hydrogen peroxide is widely used for chemical treatment in various industries particularly in the textile, paper, pulp, food and dairy industries as a more environmentally-friendly alternative to chlorine that forms toxic and carcinogenic chemicals called dioxins as by-products[7,9,33]. However, the rapid formation of reactive oxygen species (ROS) from hydrogen peroxide, including hydroxyl radicals is also a threat[6,10,25]. In light of the growing concern over environmental problems, hydrogen peroxide should be decomposed before disposal.

Currently, hydrogen peroxide is either diluted with large quantities of water, which is expensive and wasteful, or treated with agents such as sodium bisulphite or hydrosulphite, which leaves salt in the water[5]. Alternatively, the application of catalases has been suggested. These enzymes play an important role in reducing the formation of the highly reactive hydroxyl radical that arises from H_2O_2 degradation via the Fenton reaction[13]. Catalases are assigned to three phylogenetically distinct groups: two groups comprise heme catalases and one group comprises nonheme catalases[8,22]. Generally, a few thermostable versions of a monofunctional catalase[32], catalase peroxidases[4,12,32,34] and Mn catalases[2,17] have been described. True catalases are usually homo tetramers, with a subunit size of approximately 60 kDa. Each subunit contains one heme group[20,21].

Since the hydrogen peroxide bleaching step occurs at elevated temperatures and pH (>60°C and pH 9), commercially available catalases that are optimally active at 20–50°C and at neutral pH. The pretreatment was recurred the temperature and pH prior to their use[23,27]. From this, thermostable catalases hold a great potential for application in the various different industries due to the increasing trend to develop environmentally friendly technologies.

The availability of catalase that can function at high temperature and pH would be attractive for the above mentioned applications. However, many of the reported catalases exhibit low thermal stability, several are rapidly inactivated in the presence of hydrogen peroxide, and most show low activity and stability at elevated temperature and pH, making them unsuitable for industrial applications[30].

Nakayama et al. isolated strain 2-1 that can produce high-activity catalase from industrial waste. From phylogenetic analysis, strain 2-1 was identified as *Rhizobium radiobacter*, formally *Agrobacterium tumefaciens*[35,36] (Nakayama et al., unpublished data). In this study, we describe the purification of catalase from *Rhizobium radiobacter* strain 2-1. The catalase showed extremely high activity and exceptional stability at elevated temperatures and pHs compared with many other reported catalases. Furthermore, we have determined its N-terminal amino acid sequence and cloned the catalase encoding gene. The alignment result of cloned gene showed no homology with those of other

known catalases.

2. Materials and Methods

2.1. Bacterial strain cultivation

The *Rhizobium radiobacter* strain 2-1 that was isolated as an excellent catalase producer and described previously, was used throughout this study. The medium composition for catalase production (PYS medium) consists of peptone, 10 g; yeast extract, 5 g; and NaCl, 5 g in 1 *l* of deionized water. Strain 2-1 was cultivated aerobically up to the early stationary growth phase at 30°C with reciprocal shaking at 200 rpm. The cells were harvested by centrifugation at 8,000×*g* for 10 min at 4°C. The cells (4 g wet weight) were suspended in 4 ml of 50 mM potassium phosphate buffer (pH 7.0).

2.2. Catalase extraction

The cells (4 g wet weight) were suspended in 4 ml of 50 mM potassium phosphate buffer (pH 7.0). Ethanol was added at a final concentration of 1% as a catalase stabilizer. Then, the cells were disrupted trice using a French Pressure Cell (SIM Aminco, NY, USA) at a pressure of 12,500 psi. The treated cell suspension was centrifuged with reciprocal shaking at 25,000 rpm for 60 min to remove cell debris. The crude enzyme solution was incubated overnight at 50°C and then chilled at 4°C overnight. The resulting precipitate (probably composed of phospholipids and denatured proteins) was removed by centrifugation at 15,000 rpm for 10 min and the supernatant was used for column chromatography.

2.3. Ion-exchange chromatography

The crude extract was applied to a RESOURCE Q column (6.0 ml bed volume, GE Healthcare UK Ltd., England) equilibrated with 20 mM potassium phosphate buffer (pH 7.0) at a flow rate of 5.0 ml/min. After washing the column with the same buffer, the enzyme was eluted by linearly increasing the ionic strength of NaCl over the range of 0–0.5 M in the same buffer. Catalase activity was detected at concentrations of 0.2–0.3 M NaCl. The active fractions were collected and concentrated by ammonium sulfate precipitation.

2.4. Gel permeation chromatography

A superose-12 (GE Healthcare UK Ltd., England) column (1.6×50 cm) was equilibrated with 20 mM potassium phosphate buffer (pH 7.0) containing 0.2 M NaCl at a flow rate of 0.6 ml/min. The concentrated solution showing catalase activity prepared in the previous step was then applied to the column. Fractions showing catalase activity were pooled as purified catalase.

2.5. Activity assays

Catalase activity was measured spectrophotometrically by monitoring the decrease in absorbance at 240 nm caused by the disappearance of hydrogen peroxide[1]. The ε value at 240 nm for hydrogen peroxide was assumed to be 43.6 $M^{-1}cm^{-1}$ [14]. The standard reaction mixture for the assay contained 15 mM hydrogen peroxide, 3 µl of a catalase-containing solution and 50 mM phosphate buffer (pH 7.0), in a total volume of 2 ml. The reaction was run for 1 min and the initial linear rate was used to estimate the activity using a V-550i spectrophotometer (JASCO International Co., Ltd. Japan) equipped with a pen recorder. The amount of enzyme that decomposed 1 µmol of hydrogen peroxide per min was defined as 1 U of activity. Protein concentration was determined using a protein assay kit purchased from Bio-Rad laboratories with bovine serum albumin as a standard.

2.6. Sequence analysis of N-terminal amino acids

Protein SDS-PAGE was performed as described by Laemmli[19]. Proteins were electroblotted from SDS-polyacrylamide gels onto a polyvinylidene difluoride (PVDF) membrane (Clear Blot Membrane-P, ATTO Co., Ltd., Japan). The digested band containing catalase was excised and used for the amino acid sequence analysis. The N-terminal amino acid sequence analysis was carried out by Toray Research Center Inc., Japan. The N-terminal amino acid sequence obtained was compared with those closely related bacteria by homology search of the NCBI database.

2.7. Cloning of catalase gene

The molecular cloning of the catalase gene of strain 2-1 was performed by the PCR technique. The forward PCR primer CatF: (5'-GAYATGAATAARAARCARGG-3'); (Y=C+T; R=A+G) was constructed on the basis of the N-terminal amino acid sequence of the purified catalase derived from strain 2-1. The reverse primers, CatR1: (5'-RAANACNGGNGTRTTRTTNCCNAC-3'); (N=A+T+G+C), CatR2: (5'-TGNGGCCANACYTTNGTNARRTCRAANGGTT-3') were derived from the conserved region of the catalase of strain 2-1 and other catalases whose sequences were aligned using the tblastn program of the DDBJ/GenBank/EMBL database. The total chromosomal DNA was extracted from strain 2-1 using ISOPLANT II kit (Nippon Gene Co., Ltd., Japan) and used as a template. Touchdown PCR using Ex *Taq* polymerase (Takara Bio Co., Japan) was performed using an *i*-cycler (Bio-Rad Co., USA) with the following protocol: initial denaturation at 94°C for 2 min, followed by 19 cycles of denaturation at 95°C for 20 s, annealing at 60°C for 20 s, extension at 72°C for 1 min. The annealing temperature was decreased by 0.5°C at every subsequent set of cycles. This was followed by 10 cycles of 20 s at 95°C, 20 s at 50°C and 40 s at 72°C, with a final extension of 4 min at 72°C. After PCR, the total reaction mixture (20 µl) was separated by electrophoresis in a 2.0% agarose gel and fragments of 0.4 kbp (CatF-CatR1) and 0.9 kbp (CatF-CatR2) were recovered and purified using the Geneclean III kit (Qbiogene, Inc. USA).

Table 1. Purification of catalase from *Rhizobium radiobacter* strain 2-1.

Step	Volume (ml)	Total Protein (mg)	Total Activity (U)	Sp. activity (U/mg of protein)	Yield (%)	Purification (fold)
Crude Extract	4.5	85.5	4816350	56331	100	1
Protein Denaturation	3.0	14.5	4128300	284710	85	5
Ion Exchange	3.0	4.5	1376100	305800	28	6
Gel Filtration	3.5	2.8	2140600	764500	12	14

2.8. Determination of flanking nucleotide sequences of catalase using inverse PCR

Inverse PCR is a convenient and versatile method of cloning unknown sequences upstream or downstream of known sequences without the constructing of genomic libraries[11,26,29]. The total chromosomal DNA of strain 2-1 was digested with *Sal* I and *Pst* I and self-ligated using T4 DNA ligase (Takara bio Co.). Inverse PCR was carried out with the forward primer invF (5'-GCCCACGGTCAGAGAGTTGCGGTCGCTTCA-3') and the reverse primer invR1 (5'-CGTGGCGAGCATCCGGCCTGGACGATGTCG-3'). The PCR conditions were as follows: 94°C for 3 min, 98°C for 10 s, 70°C for 5 min 30 s and 70°C for 10 min for 30 cycles. The second inverse PCR was performed to obtain the downstream side sequence of the same gene. The forward primer (invF) and a newly constructed reverse primer invR2 (5'-ACTGGTGCGCGAGGTGTTCAATGATGACGAG-3') were used to perform inverse PCR as described above except that the number of cycles was 25.

2.9. DNA sequencing and data analysis

DNA sequencing was carried out using an ABI Prism 310 DNA sequencer (PE Biosystems, Foster City, USA). The nucleotide and amino acid sequences were analyzed using the GENETYX-MAC program ver. 8 (Software Development Co., Ltd., Tokyo, Japan), FASTA and BLAST on the DDBJ/GenBank/EMBL nucleotide sequence databases. Selected amino acid sequences were aligned using the Clustal X program[28].

3. Results

3.1. Catalase purification

A simple, three-step purification procedure consisting of protein denaturation, ion- exchange chromatography, and gel permeation chromatography was developed to obtain purified catalase from *Rhizobium radiobacter* strain 2-1. The results of each purification step are given in Table 1. The catalase was purified 14-fold with a yield of 12% and a specific activity of 764,500 U/mg. SDS-PAGE analysis of the purified sample confirmed to be a single band (Fig. 1). The molecular weight of the purified catalase was 56 kDa as determined by SDS-PAGE. The molecular weight of the native catalase was also estimated to be about 230 kDa by gel filtration using a Superose 12HR10/30 column (data not shown). These observations indicate that the native catalase exists as a tetramer.

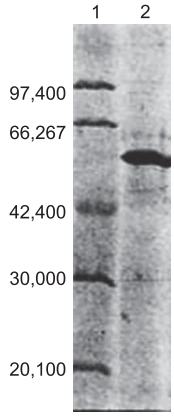

Fig. 1. SDS-PAGE of purified catalase.
Lane 1, Molecular weight marker; lane 2, purified catalase.

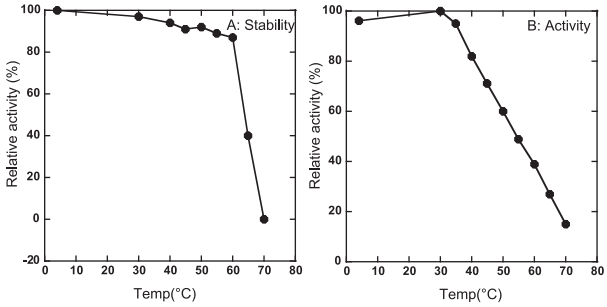

Fig. 2. Effect of temperature on stability (A) and activity (B) of catalase purified from strain 2-1.
For stability assay, the enzyme was incubated for 30 min at the given temperatures prior to the initiation of the reaction. After incubation, catalase activities were measured at 30°C, pH 7. The activity is expressed as relative activity, taking the highest activity observed as being 100%.

3.2. Optimal conditions for stability/activity of catalase

The catalase was stable within 30 min of incubation at 60°C, but was 40% inactivated at 65°C (Fig. 2A). The optimum temperature for the catalase was 30°C (Fig. 2B). The pH for the optimum stability and activity of purified catalase was also determined at 30°C. The catalase stability remained almost the same at the pH range from 5 to 11 (Fig. 3A), and the optimal pH range was also broad at 5 to 10 (Fig. 3B). The Km and V_{max} of the purified catalase under optimum conditions were 60.06 mM and 10.17 mM/min, respectively.

3.3. Cloning of catalase gene by PCR

The N-terminal amino acid sequence, TDMNKKQG-GTGSTTGTGAPA, revealed from purified catalase of strain 2-1 was compared with those of the other catalase genes by similarity search using the tblastn program on the DDBJ/GenBank/EMBL database. It was revealed that the N-terminal amino acid sequence showed 68% identity to that of *Nitrobacter hamburgensis* strain X14 catalase. Then, catalases showing high similarity to *N. hamburgensis* X14 catalase were selected using the blastx program. Among these, catalases from *N. hamburgensis* X14 and closely related to *R. radiobacter* were selected and the amino acid sequences were aligned. Consequently, two regions where the sequences are highly conserved were identified (Table 2).

Touchdown PCR was performed using the forward primer CatF and the reverse primers CatR1and CatR2, to amplify the part of catalase gene from the strain 2-1 chromosomal DNA. Consequently, PCR products of approximately 400 bp (CatF-CatR1) and 900 bp (CatF-CatR2) were obtained. The nucleotide sequence of the CatF-CatR completely matched with the first half of the nucleotide sequence of CatF-CatR2. The size of this fragment roughly matched with those of the corresponding regions of catalases used for the alignment.

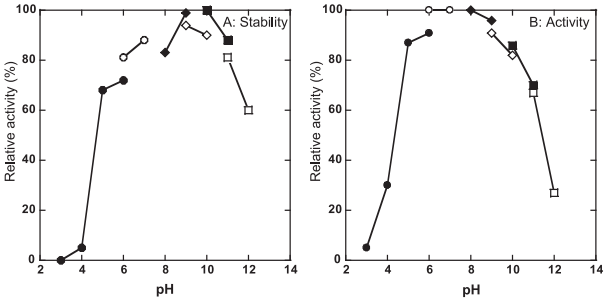

Fig. 3. Effect of pH on stability (A) and activity (B) of catalase purified from strain 2-1.
The buffers (50 mM) used were sodium citrate (pH 4–6), sodium phosphate (pH 6–7), Tris HCl (pH 8–9), Glycine-NaOH (pH 9–10), Na_2CO_3-$NaHCO_3$ (pH 10–11) and Na_2PO_4-NaOH (pH 11–12). For stability assay, the enzyme was incubated in each buffer at 30°C for 30 min. After incubation, catalase activities were measured at 30°C, pH 7. For activity assay, the enzyme activity was measured at 30°C in each of the above buffers. The activity is expressed as relative activity, taking the highest activity observed as being 100%

To clone the entire length of the catalase gene, inverse PCR was carried out on the basis of the partial nucleotide sequence of the catalase mentioned above. An approximately 800 bp PCR product was amplified from the *Sal* I digested and circulated chromosomal DNA of strain 2-1. Sequence analysis suggested that the PCR product contained 80 bp of the upstream and 626 bp downstream regions of the putative catalase gene. The deduced N-terminal amino acid sequence completely matched with that of the purified catalase from strain 2-1. However, because the stop codon was not found in the downstream side, inverse PCR was performed again to determine the entire length of the catalase gene. Using the reverse primer for inverse PCR (invR2) revealed an approximately 3.5 kbp fragment that was amplified from the *Pst* I digested and circulated chromosomal DNA. Finally, a 351 bp downstream sequence including a putative stop codon was determined. The nucleotide sequence of the cloned catalse gene, designated as *katH*, have been submitted to GenBank and received the accession number AB370115.

3.4. Nucleotide sequence and deduced amino acid sequence

Figure 4 shows the complete nucleotide sequence of *katH* of strain 2-1. The resultant sequence contained one open reading frame (ORF) consisting of 1,854 nucleotides, and the N-terminal amino acid sequence (20 amino acids) matched completely to that of the purifed catalase from strain 2-1. The stop codon TAA at the position 1555–1557 was followed by the stem-loop structure (5'-ACGCCGGC-CCTCACAAGGGGCCGGCGT-3'), which is a putative transcription termination point. The complete amino acid sequence of the catalase derived from strain 2-1 is also shown in Fig. 4. This catalase (KatH) consists of 491 amino acids with a molecular mass of 55059.39 Da. This result was in good agreement with the molecular mass (56,000 Da) of the purified catalase determined by SDS-PAGE (Fig. 1). The deduced amino acid sequence was searched against the GenBank database using the tblastx program[3]. It was found that *R. radiobacter* KatH showed more than 70% identity to catalases from *Sphingomonas wittichii* RW1, *Nitrobacter hamburgensis, Thermobifida fusca* and *Micrococcus luteus* (Table 3). In addition, the proximal *active site* was tenta-

Table 2. Alignment of amino acid sequences of catalases derived from strain 2-1 and closely related bacteria.

Species/Strain [Accession number]	Conserved region	
Strain 2-1	(194) VGNNTPVF	(343) NPFDLTKVWPH
Nitrobacter hamburgensis X14[ABE61714]	(131) VGNNTPVF	(280) NPFDLTKVWPH
Sinorhizobium meliloti [NP_384870]	(126) VGNNTPVF	(275) NPFDLTKVWPH
Sinorhizobium meliloti [AAC44649]	(194) VGNNTPVF	(343) NPFDLTKVWPH
Rhizobium sp. [AAA99823]	(123) VGNNTPVF	(272) NPFDLTKVWPH
Streptomyces coelicolor A3(2)[CAC16511]	(131) VGNNTPVF	(280) NPFDLTKVWPH

Two highly conserved regions are shown. The number in the parentheses indicates the amino acids position.

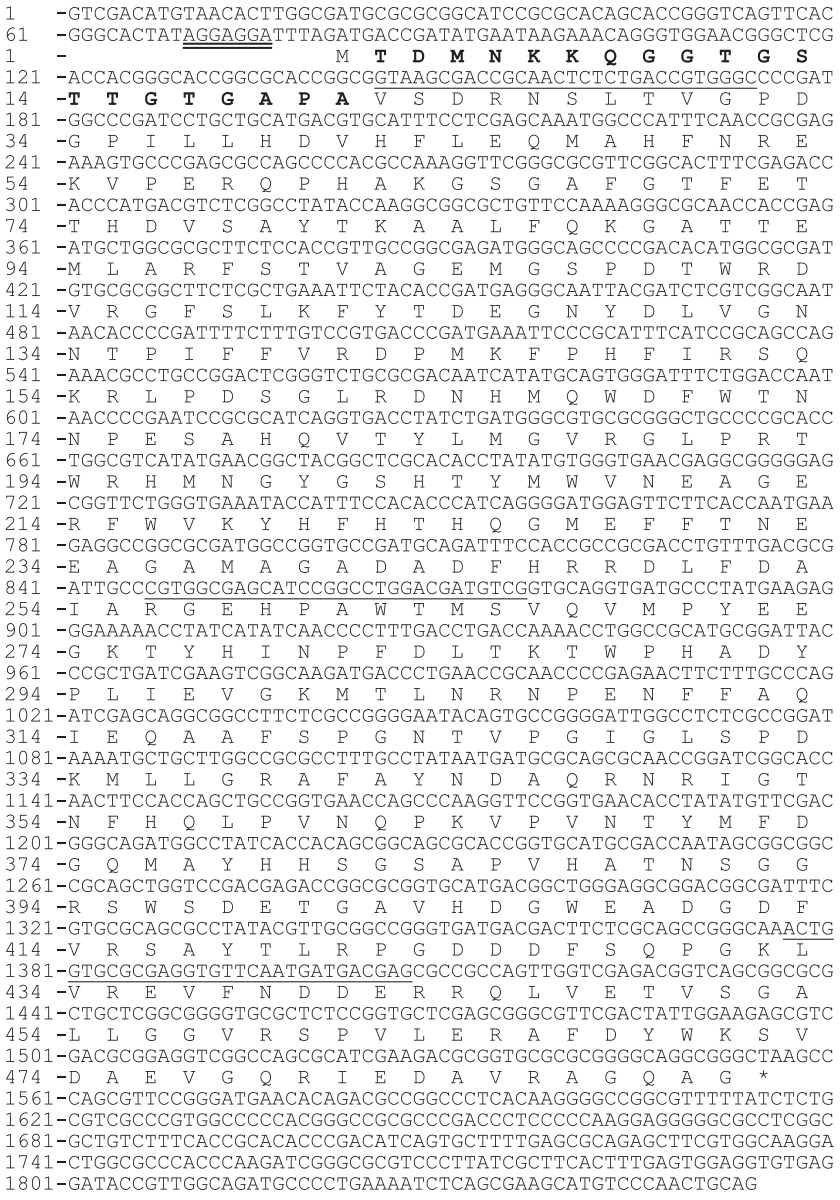

Fig. 4. Complete nucleotide sequence of catalase gene from strain 2-1.
The double underline indicates the putative ribosome binding site located 7 bp upstream of the start codon. The stem loop (5'-ACGCCG GCCCTCACAAGGGGCCGGCGT-3') located downstream of the stop codon (TAA) is the putative transcription termination point. The underlined parts indicate the inverse PCR primers. Bold letters indicate matching parts of the purified catalase amino acid sequence and the N-terminal amino acid sequence.

Table 3. Amino acid homology analysis of catalase from strain 2-1.

Catalase gene	Homology
Sphingomonas wittichii RW1 [SwitDRAFT_2065]	83%
Nitrobacter hamburgensis [Nham_0804]	79%
Thermobifida fusca (strain YX) [Tfu_1649]	74%
Micrococcus luteus (*Micrococcus lysodeikticus*) [katA]	70%

tively identified as residue 50–66 (apparent consensus FNREKVPERQPHAKGSG) and the proximal heme ligand was tentatively identified as residue 339–347 (apparent consensus RAFA YNDAQ).

4. Discussion

The purification of catalase was performed using a high-yielding catalase producing bacteria, *Rhizobium radiobacter* strain 2-1. Although the amino acid sequence of KatH showed more than 70% homology with the four putative monofunctional heme catalases (Table 3), it showed no more than 40% of the identities with other known *R. radiobacter (Agrobacterium tumefaciens)* strains (data not shown). Consequently, the present enzyme is a novel *Rhizobium* catalase, which can be classified as a monofunctional heme catalase.

The molecular mass of a subunit of the catalase from strain 2-1 was estimated to be 56 kDa. The native molecular mass of the enzyme was estimated to be 230 kDa by gel permeation chromatography, revealing that the catalase consists of four identical subunits. The subunit and native molecular sizes for this enzyme are verily similar to those reported for other tetrameric catalases (i.e., *Bacillus* sp. with 70.5 and 282 kDa, *Rhodobacter capsulatus* with 59 and 236 kDa, *T. brockianus* with 42.5 kDa and 178 kDa, and *V. rumoiensis* with 57.3 and 230 kDa, subunit and native molecular sizes, respectively)[31,37]. The Km of the catalase from strain 2-1 is 60.06 mM. This value is the same as those of the reported thermostable catalases such as from *T. aurantiacus, T. brockianus,* and *B. subtilis* (catalase-1 and catalase-2), whose Km values are 48, 35.5, 40.1 and 70 mM, respectively[16,37]. These observations strongly suggest that this catalase is a typical catalase.

However, the specific activity of purified catalase from strain 2-1 was 143-fold higher than that of typical *T. brockianus* catalase, about 4-fold higher than that of *M.luteus* catalase, and about 2-fold higher than that of *V. rumoiensis* catalase. These catalases are known for exhibiting high catalase specific activity[16,31,37].

In addition, the purified catalase from strain 2-1 showed high temperature stability ranging from 4 to 60°C (Fig. 2A). The activity also remained at about 40% at 65°C for 15 min, but the catalase was completely inactivated at 70°C. Although the optimum reaction temperature of this catalase was 30°C (Fig. 2B) and the activity decreased gradually at higher temperature, about 40% of the activity still remained at 60°C. The purified catalase exhibited a broad pH stability range (pH 5–11) and activity range (pH 5–10) with maximum activity at pH 6 (Fig. 3). There was no apparent substrate inhibition or inactivation of this purified catalase at hydrogen peroxide concentrations up to 50 mM (data not shown). These results suggest that this catalase is suitable for industrial use[27].

From the commercial perspective, the purified catalase has many beneficial properties such as high specific activity, simple purification, high temperature stability and stability in a wide pH range which makes this novel enzyme suitable for commercial exploitation and for removing hydrogen peroxide from industrial wastewater. Further studies are required to clarify the role of this catalase from the industrial perspective.

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