Research Paper

Production, characterization, cloning and sequence analysis of a monofunctional catalase from *Serratia marcescens* SYBC08

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A monofunctional catalase from *Serratia marcescens* SYBC08 produced by liquid state fermentation in 7 liter fermenter was isolated and purified by ammonium sulfate precipitation (ASP), ion exchange chromatography (IEC), and gel filtration (GF) and characterized. Its sequence was analyzed by LC-MS/MS technique and gene cloning. The highest catalase production (20,289 U · ml^{-1}) was achieved after incubation for 40 h. The purified catalase had an estimated molecular mass of 230 kDa, consisting of four identical subunits of 58 kDa. High specific activity of the catalase (199,584 U · mg^{-1} protein) was 3.44 times higher than that of *Halomonas* sp. Sk1 catalase (57,900 U · mg^{-1} protein). The enzyme without peroxidase activity was found to be an atypical electronic spectrum of monofunctional catalase. The apparent K_m and V_{max} were 78 mM and 188, 212 per μM H_2O_2 μM $heme^{-1}$ s^{-1}, respectivly. The enzyme displayed a broad pH activity range (pH 5.0–11.0), with optimal pH range of 7.0–9.0: It was most active at 20 °C and had 78% activity at 0 °C. Its thermo stability was slightly higher compared to that of commercial catalase from bovine liver. LC–MS/MS analysis confirmed that the deduced amino acid sequence of cloning gene was the catalase sequence from *Serratia marcescens* SYBC08. The sequence was compared with that of 23 related catalases. Although most of active site residues, NADPH-binding residues, proximal residues of the heme, distal residues of the heme and residues interacting with a water molecule in the enzyme were well conserved in 23 related catalases, weakly conserved residues were found. Its sequence was closely related with that of catalases from pathogenic bacterium in the family Enterobacteriaceae. This result imply that the enzyme with high specific activity plays a significant role in preventing those microorganisms of the family Enterobacteriaceae against hydrogen peroxide resulted in cellular damage. Calalase yield by *Serratia marcescens* SYBC08 has potential industrial application in scavenging hydrogen peroxide.

Keywords: Monofunctional catalase / Gene coning and sequencing / ESI-Q-TOF MS/MS / *Serratia marcescens*

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Introduction

Aerobic organisms use molecular oxygen (O_2) for respiration or oxidation of nutrients to obtain their energy. Reactive oxygen species (ROS) including superoxide anion radical (O_2), hydrogen peroxide (H_2O_2), and the highly reactive hydroxyl radicals (·OH) are generally generated by the leakage of single electrons from cellular respiratory chain. The biological targets for these highly ROS are DNA, RNA, proteins, and lipids. Much of the damage is caused by hydroxyl radicals generated from H_2O_2 [1]. Catalase is one of the central components of the detoxification pathways that prevent the formation of highly reactive hydroxyl radical by catalyzing the decomposition of H_2O_2 into water and dioxygen by

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two-electron transfer [2], and exist in aerobic, facultative aerobic and anaerobic microorganisms [3–5], it is widely used in several industrial fields such as textiles, pulp and paper. Commercially available catalase is prepared from bovine livers or microorganisms, an approach limited by low yield [6].

Based on enzymological properties, catalases can be classified into one of their three types, heme-containing monofunctional catalases, heme-containing bifunctional catalase-peroxidases, and non-heme-containing or Mn-containing catalases [7]. Monofunctional catalases have a two stage mechanism for the degradation of H_2O_2 in which one hydrogen peroxide molecule oxidizes the heme to an oxyferryl species in catalytic sites and hydrogen peroxide molecule is utilized as a reductant of compound I to regenerate the resting-state enzyme, water and oxygen [8]. Some amino acid residues of monofunctional catalase such as in NADPH binding sites and catalytic sites play a very important role in the reaction [8, 9], in this way, amino acid sequence analysis has a better understanding of its catalytic behavior in the process of decomposing H_2O_2.

In the present study, a catalase of *Serratia marcescens* SYBC08 from sludge with hydrogen peroxide was purified and characterized as a monofunctional enzyme. Its amino acid sequence was analyzed by LC-MS/MS technology and gene cloning.

Materials and methods

Strains and culture conditions

A strain with highest catalase production among 104 catalase-producing microorganisms was isolated from sludge with hydrogen peroxide in bleaching workshop of textile factory. The strain was identified and designated as *Serratia marcescens* SYBC08 by 16S rDNA sequence (Genbank Accession no. GU188473). It was subsequently conserved in China General Microbiological Culture Collection Center (Preserved no. CGMCC 3449). Prior to use, the strain was recovered from 10% glycerol stocks stored at −70 °C. For seed preparation, the microorganism was inoculated into 50 ml seed medium (glucose 20 $g \cdot l^{-1}$, peptone 10 $g \cdot l^{-1}$, beef 5 $g \cdot l^{-1}$ extract (NaCl 5 $g \cdot l^{-1}$, pH 7.2) in 250 ml flasks and cultivated at 30 °C on a rotary shaker at 200 rpm for 12 h. Seed with 4% size of inoculation ($V \cdot V^{-1}$) was inoculated into the optimized fermentation medium (corn steep liquor powder 33.8 $g \cdot l^{-1}$, citric acid 30 $g \cdot l^{-1}$, initial pH 5.91). Batch fermentation was carried out in a 7 l fermentor with a working volume of 5 l. The aeration rate was 1.5 $V \cdot V^{-1} \cdot min^{-1}$, and agitation speed was 400 rpm. The temperature and pH were controlled at 32.8 °C and 7.0, respectively.

Preparation of crude enzyme and determination

The broth was centrifuged at 4 °C and 10,800 × *g* for 15 min. The precipitation was dried at 105 °C to constant weight followed by weighting with electrical level to determine the biomass or was disrupt by supersonic instrument at 0 °C for 20 min to prepare crude enzyme extract. The cell debris was removed by centrifugation at 4 °C and 18,000 × *g* for 15 min, and the supernatants were pooled as crude enzyme extract.

Catalase activity was measured spectrophotometrically by monitoring the decrease in absorbance at 240 nm caused by the decomposition of hydrogen peroxide [10]. The ε of H_2O_2 at 240 nm was 43.6 mM^{-1} cm^{-1}. The reaction mixture contained suitable amount of enzyme solution, 30 mM H_2O_2, and 50 mM Na_2HPO_4–NaH_2PO_4 buffer (pH 7.0) in a total volume of 4 ml. The linear range of the reaction (30 s) was used to calculate the rate of the reaction, and one unit of catalase activity was defined as the amount of enzyme that required to transform 1 μmol of hydrogen peroxide to water and oxygen per min [11]. Catalase activity was determined for three times for each sample. The catalase activity was calculated and analyzed by SPSS 11.5 software.

Peroxidase activity was measured spectrophotometrically by monitoring the increasing in absorbance at 470 nm. The reaction mixture contained suitable amount of enzyme solution, 50 mM Na_2HPO_4-citrate buffer (pH 6.0), 10 mM H_2O_2, and 10 mM guaiacol in a total volum of 4 ml. One unit of peroxidase was expressed as the enzyme amount required for producing 1 μmol guaiacol oxidants [12].

The protein concentration was determined by the method according to Bradford [13] with bovine serum albumin as the standard. Residual citric acid was detected according to the method described by Cen *et al.* [14].

Catalase purification

The pooled crude enzyme extract was firstly precipitated by using 40% ($w \cdot v^{-1}$) ammonium sulfate saturation. The pellets were removed, and the supernatants with catalase activity were collected by centrifugation at 4 °C and 10,800 × *g* for 20 min. After the precipitation by using 60% ($w \cdot v^{-1}$) ammonium sulfate saturation, the precipitates were collected by centrifugation at 4 °C and 12,000 × *g* for 20 min. The precipitates were fully dissolved in a small amount of 50 mM Na_2HPO_4-NaH_2PO_4 buffer (pH 8.0) followed by centrifugation (4 °C and 17,300 × *g* for 20 min) to discard the undissol-

ved fractions. The supernatants were dialyzed against the same buffer for 12 h and then subjected to a DEAE (1.0 cm × 10.0 cm) column which had been equilibrated with the same buffer. The adsorbed enzyme was eluted with a linear gradient of NaCl from 0 to 0.7 M in 50 mM Na_2HPO_4–NaH_2PO_4 buffer (pH 8.0) at the flow rate of 1 ml × min^{-1}. The fraction with catalase activity was pooled and 2 ml of the active fraction was loaded on a Sephacryl Tm S-200 column (16 mm × 60 cm) which had been equilibrated with the same buffer. The enzyme was eluted with the same buffer at the flow rate of 1 ml × min^{-1}, and the eluted catalase fractions were collected. The solution in each purified step was collected for assaying catalase activity and protein content.

The molecular masses of the catalase subunits and holoenzyme were determined by 12% (w × v^{-1}) SDS-PAGE according to the method of Laemmli [15] and GF, respectively. The gels were silver staining according to the Ref. [16].

Spectrophotometric analysis

The absorption spectrum of the purified catalase was recorded at each 2 nm at room temperature between 280 and 700 nm using double beam UV-Vis spectrophotometer.

Effect of pH and temperature on the activity and stability of the purified catalase

Effect of catalytic pH value on catalase activity was determined by incubating the purified enzyme in 50 mM Na_2HPO_4-citric acid buffer (pH 4.0 ~ 6.0), 50 mM NaH_2PO_4–Na_2HPO_4 buffer (pH 7.0 ~ 8.0), and 50 mM Na_2CO_3–$NaHCO_3$ buffer (pH 9.0 ~ 11.0) at 30 °C, and the highest catalase activity was defined as 100%. The effect of the pH range from 5.0 to 11.0 on catalase stability was investigated by incubating the enzyme at 30 °C for 180 min, and its initial activity was expressed as 100%. The effect of temperature on catalase activity was measured at the temperature from 0 to 70 °C at pH 7.0, and the highest activity was regarded as 100%. For thermal stability determination, the enzyme was incubated at 60 °C, 65 °C and 70 °C at pH 7.0 followed by periodical measurement at 30 °C, and initial activities of the sample at corresponding temperatures were calculated as 100%.

Kinetic parameters (V_{max} and K_m)

The effect of H_2O_2 concentration (7.5, 10, 12.5, 15, 20, 25, 30 mM) on catalase activity was evaluated in 50 mM NaH_2PO_4–Na_2HPO_4 buffer (pH 7.0) at 20 °C. The kinetic parameters (Michaelis-Menten constant, Km, and maximal reaction velocity, V_{max}) were estimated by linear regression from double-reciprocal plots according to Lineweaver and Burk [17].

Amino acid sequence analysis using ESI-Q-TOF MS/MS

The purified enzyme partially digested with trypsin (Sigma-Aldrich, Germany). Mass spectrometry analysis was carried out in electrospray ionization quadrupole time-of-flight mass spectrometr (ESI-Q-TOF-MS/MS). MS/MS data were investigated using MASCOT searching tool (Matrix Science Ltd., London, UK)

DNA preparation and gene cloning

DNA was extracted according to the method described by Tao *et al*. [18]. Primers for PCR amplification were designed according to the DNA sequence of *serratia proteamaculans* 568 catalase (Genbank Accession no. CP000826.1). It included upstream primer (ACCGGAATTCATGAGCAAGAAAGGACTG) and downstream primer (ACCGGCGGCCGCTTATTTCAGACCTAA CGCC). The reaction system included genomic DNA, the PCR reaction buffer, and two units of Taq polymerase were mixed and performed PCR amplification in the condition which was an initially denatured step at 95 °C for 4 min, followed by 35 cycles of a three-stages program with 1 min at 94 °C, 1 min at 52 °C for renaturation, then 1.5 min at 72 °C, and a final elongation step runed for 6 min at 72 °C. The PCR products were then recovered with Agarose Gel DNA Purification Kit Ver. 2.0 (TaKaRa). The purified products were ligated into pET 28 vectors and transformed into *E. coli* DH5α. The transformants were selected on Luria-Bertani (LB) broth containing 100 μg · ml^{-1} ampicillin. The positive clone was screened by H_2O_2 bubbling test. Plasmid DNA from positive colonies was extracted from *E. coli* using a Plasmid DNA Extraction Kit (Takar) for sequence analysis using an automated DNA sequencer ABI3700.

Amino acid sequence analysis

The deduced amino acid sequence of *Serratia marcescens* SYBC08 was blasted in NCBI database (http://www.ncbi.nlm.nih.gov/Database/). Twenty four amino acid sequences of related catalases from *Serratia marcescens* SYBC08 (accession no.ADI55329.1), *Yersinia enterocolitica* subsp. enterocolitica 8081 (accession no. YP_001005695.1), *Vibrio fischeri* MJ11 (accession no. YP_002157580), *Syntrophobacter fumaroxidans* MPOB (accession no. YP_845843.1), *Serratia proteamaculans* 568 (accession no. YP_001479504.1), *Saccharomonospora viridis* DSM 43017 (accession no. YP_003133951.1), *Rhodococcus jostii RHA1* (accession no. YP_705771.1), *Ralstonia*

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eutropha H16 (accession no. YP_727552.1), *Pseudovibrio* sp. JE062 (accession no. ZP_05083841.1), *Pseudomonas aeruginosa* pao1 (accession no. NP_252926.1), *Providencia rustigianii* DSM 4541 (accession no.ZP_05973780.2), *Proteus Mirabilis* Pr (accession no. 2CAH_A), *Polaromonas naphthalenivorans* CJ2 (accession no. YP_982885.1), *Photorhabdus luminescens* subsp. laumondii tto1 (accession no. NP_930300.1), *Pelobacter propionicus* DSM 2379 (accession no. YP_901599.1), *Nitrosomonas* sp. al212 (accession no. ZP_05316133.1), *Moritella* sp. PE36 (accession no. ZP_01899777.1), *Desulfovibrio vulgaris* str. 'Miyazaki F'(accession no. YP_002435660), *Cupriavidus metallidurans* CH34 (accession no. YP_587727.1), *Colwellia psychrerythraea* 34H (accession no. YP_269157.1), *Arsenophonus nasoniae* (accession no. CBA76514.1), *Aromatoleum aromaticum* EBn1(accession no. YP_158186.1), *Aliivibrio salmonicida* LFI1238 (accession no. YP_002264567) and Bovine liver (accession no. NP_001030463.1) were chosen for alignment by DNAMAN Version.v5.2.2. The phylogenetic relationships of the 24 sequences were generated by using CLUSTALX version 1.8 and the software packages MEGA version 4.1. Unrooted phylogenetic trees were constructed by using the neighbour joining [19]. Minimum evolution and maximum parsimony methods was carried out according to the reference [20], and they were evaluated by bootstrap resampling (1000 replications).

Results

Catalase production in 7 l fermenter

The fermentation time course for catalase production by *Serratia marcescens* SYBC08 in a 7 l fermenter was presented in Fig. 1, which reveals the relationship between the biomass, specific activity, and catlase production. The maximum catalase production (20,289 U · ml^{-1}) and specific activity (11,863 U · mg^{-1} of protein) was achieved at 40 h after incubation, while biomass constantly increased at all the time course. Specific activity and catalase production were significantly associated (*r* 0.97). Biomass was closely associated with catalase production (*r* 0.86).

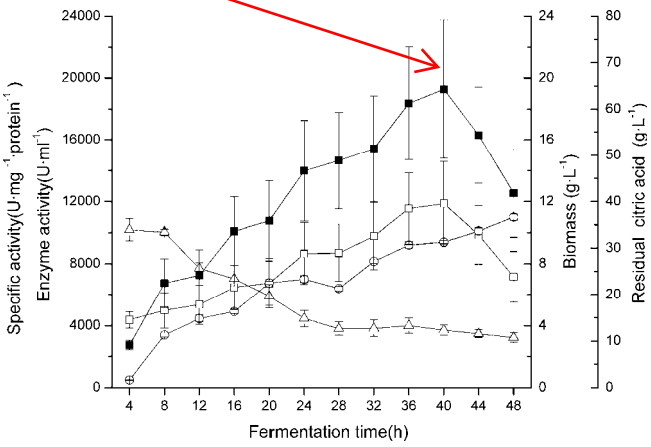

Figure 1. Time course of production of catalase from *Serratia marcescens* SYBC08 under optimized medium in 7 l fermenter. The aeration rate was 1.5 V · V^{-1} · min^{-1}, agitation speed was 400 rpm, and pH was 7.0. Values given are the means of at least triplicate experiments, and error bars represent the SD. Catalase activity (■), Specific activity (□), Biomass (○), Residual citric acid (△).

Catalase purification

Serratia marcescens SYBC08 catalase was purified by ASP, IEC and GF. The purification procedure was summarized in Table 1.

Catalase from *Serratia marcescens* SYBC08 was purified 1.6-fold after ASP. During IEC, five protein peaks were appeared and the fifth peak contained catalase activity (data not shown). The catalase was purified 5.4-fold after this process. The protein solution from GF was separated and appeared six peaks, and only the sixth peaks contained catalase activity (data not shown). The enzyme was purified 13.8-fold with a recovery of 22% after this procedure. It displays high specific activity of 99,584 U · mg^{-1} protein, 3.44 time higher than that *Halomonas* sp. Sk1 catalase (57,900U · mg^{-1} protein) [21].

Samples from each procedure were analyzed by SDS-PAGE, and the result is present in Fig. 2. A single band from GF was revealed by silver staining and suggested that the purified catalase was obtained, and the molecular mass of the subunit was 58 kDa. Molecular mass of the purified catalase was estimate to be 230 kDa by GF (data not shown). Thus, we proposed that the purified catalase was tetramer consisted of 4 homosubunits.

Table 1. Summary of the purification of catalase from *Serratia marcescens* SYBC 08.

Step	Total activity (U)	Total protein (mg)	Specific activity (U · mg^{-1} protein)	Yield (%)	Purification (fold)
Crude extract	5,790,186	399	14,487	100	1.0
ASP	2,569,373	112	22,863	44	1.6
IEC	1,742,347	22.1	78,683	30	5.4
GF	1,325,786	6.6	199,585	22	13.8

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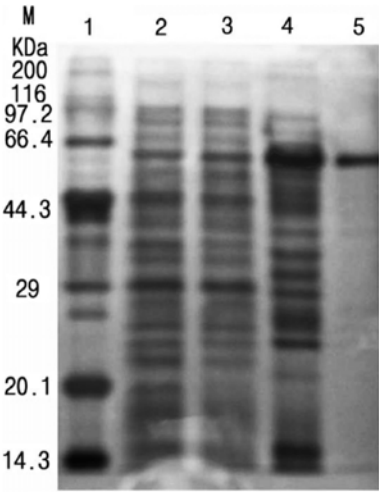


Figure 2. Electrophoretic analysis of catalase from *Serratia marcescens* SYBC08 by SDS-PAGE with silver staining. Lane 1, Marker. Lane 2, crude extract; Lane 3, ASP; Lane 4, IEC; Lane 5, GF.

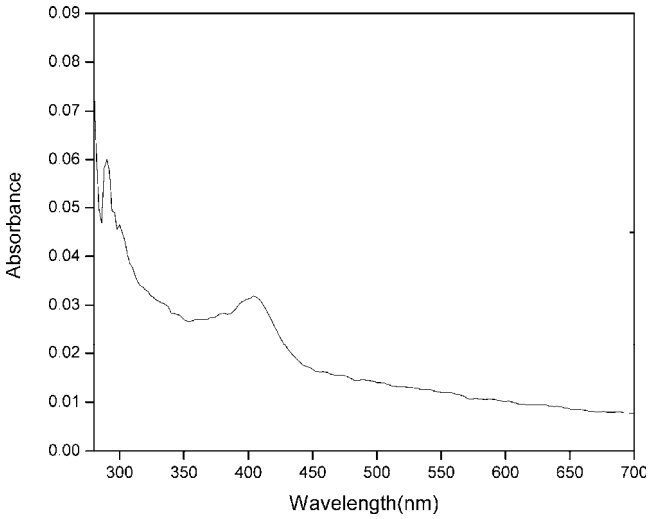
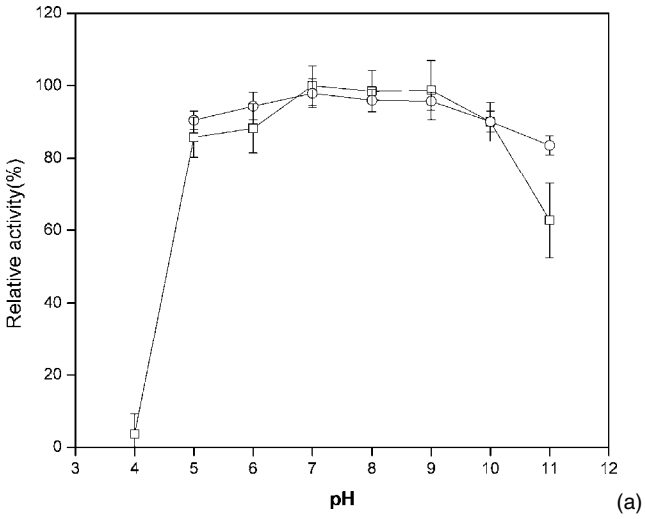
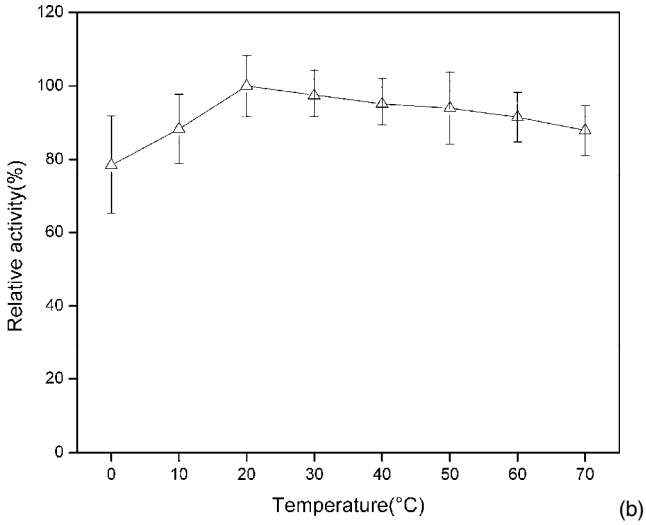


Figure 3. Spectrum analysis of the purified catalase from *Serratia marcescens* SYBC08. The spectra of the enzyme were recorded against a blank of identical buffer.





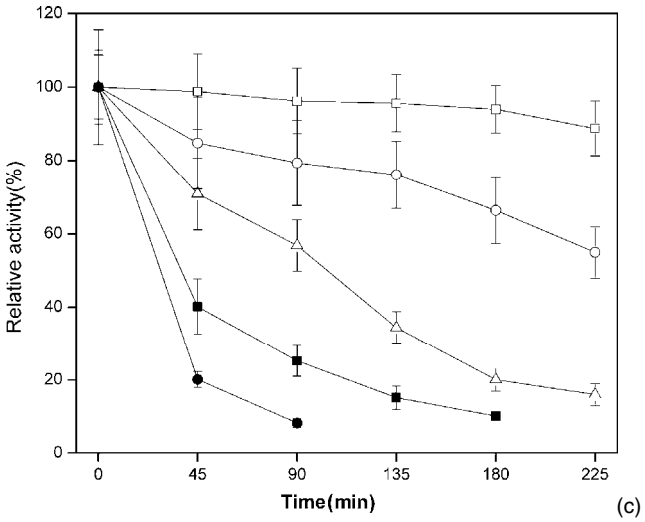


Figure 4. (a) Effect of pH on catalase activity from *Serratia marcescens* SYBC08. Values given are the means of at least triplicate experiments, and error bars represent the SD. pH catalytic activity (□); pH stabilition (○). (b) Effect of temperture on catalase catalytic activity from *Serratia marcescens* SYBC08. Values given are the means of at least triplicate experiments, and error bars represent the SD. (c) The thermal stability of catalase at various temperatures. Values given are the means of at least triplicate experiments, and error bars represent the SD. *Serratia marcescens* SYBC08, 60 °C (□); 65 °C (○); 70 °C (△); bovine liver, 60 °C (■); 65 °C (●).

Spectroscopic analysis

Spectroscopic analysis of the purified catalase was performed, and the result was displayed in Fig. 3. The two maxima at 405 nm (Soret peak) and 280 nm (protein maximum) was obviously appeared, and the Rz value (A405/A280) of 0.42 ± 0.041 was calculated.

Effect of pH and temperature on catalase activity and stability

The catalytic activity of the purified catalase under different pH values is presented in Fig. 4a, and had a wide pH range of 5.0–11.0. The enzyme was also highly stable in a broad pH range of 5.0–11.0 (Fig. 4a). Although the enzyme had a slight dependence of temperature, it still showed optimum temperature of 20 °C, and it maintained 78% of the maximal activity at 0 °C (Fig. 4b). The temperature stability was determined by incubating the purified enzyme at 60 °C, 65 °C and 70 °C at pH 7.0, respectively (Fig. 4c). At 60 °C, the enzyme from *Serratia marcescens* SYBC08 was stable for 240 min, while bovine liver catalase only retained 40% activity for 45 min. At 65 °C, the enzyme from *Serratia marcescens* SYBC08 retained more than 55% of its activity by incubating the enzyme at pH 7.0 after 225 min, while bovine liver catalase only retained 20% activity for 45 min. At 70 °C, the enzyme could retain 57% of its initial activity after incubating at pH 7.0 for 90 min.

Kinetic analysis

The kinetic parameters of the purified catalase were analyzed by Lineweaver–Burk plot (Fig. 5). The K_m and V_{max} for the enzyme at 20 °C was 78 mM and 188,212 per μM H_2O_2 μM heme^{-1} s^{-1}, respectively.

MS peptide sequence

The peptides mass fingerprint from LC-MS/MS were used as a query against the NCBI Protein database (MASCOT search), and the result is represent in Table 2. Four peptide sequences was identical with the sequences of the two catalases from *Yersinia enterocolitica* subsp. enterocolitica 8081 (gi|123441711) and *Serratia proteamaculans* 568 (gi|157371515), but two peptide sequences did not completely matched that of the two catalases, respectively.

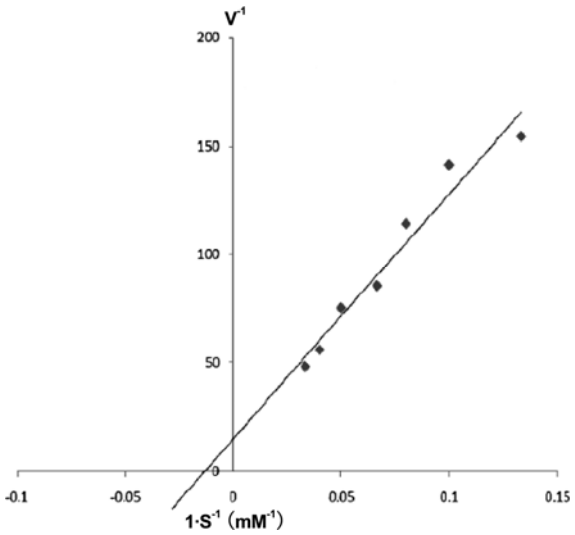


Figure 5. Lineweave-Burk plot of the catalase from *Serratia marcescens* SYBC08.

Gene cloning and sequence analysis

A encoding gene was cloned by using two PCR primers which was designed according to highly homologized gene sequence from *Serratia proteamaculans* 568 catalase under LC-MS/MS analysis, and it was deposited in the GenBank under the accession number HM 068611. The deduced 479 amino acid sequence according to an open reading frame of 1437bp completely matched mass spectrometric sequence in Table 3. Comparative analysis of those amino acid squences revealed it had high homology with the sequences of other catalases from *Serratia proteamaculans* 568 (94% amino acid sequence identity), *Yersinia enterocolitica* subsp. *enterocolitica* 8081 (91%), and other sources (the range of 53%–85%). Multiple alignments of 24 catalases were performed (data not shown). The amino acid residues of the active site are very important in preservation of enzyme functions and much research revealed it was

Table 2. Identification of tryptic peptides of catalase from *Serratia marcescens* SYBC 08.

Observed ion (m/z)	Expected molecular mass	Calculated molecular mass	Delta	Sequence	Matched organism
494,5961	1480,7665	1480,7786	–0.0122	LAHFDREVIPER	1,2
741,3950	1480,7754	1480,7786	–0.0032	LAHFDREVIPER	1,2
761,4196	1520,8246	1520,8351	–0.0105	DPLKFPDLNHVVK	1,2
644,2604	1286,5062	1286,5051	0.0011	EDDDYYSQPR	1,2
841,9160	1681,8174	1681,8060	0.0114	GSGAYGTFTVTHDITR	1
784,4251	1566,8356	1480,7786	–0.0032	IAGELSQVPEQIQR	2

1 or 2 present *Yersinia enterocolitica* subsp. enterocolitica 8081 (gi|123441711) or *Serratia proteamaculans* 568 (gi|157371515).

Table 3. Weakly conservative amino acid residuals from important functional sites.

Organism	M53	S93	H341	H284	K216	F194
Serratia marcescens SYBC08	M	S	H	H	R	F
Proteus mirabilis PR	M	S	H	H	R	F
Providencia rustigianii DSM 4541	M	S	**Q**	H	R	F
Photorhabdus luminescens subsp. *laumondii* TTO1	M	S	**Q**	H	R	F
Arsenophonus nasoniae	M	S	H	H	R	F
Serratia proteamaculans 568	M	S	H	H	R	F
Yersinia enterocolitica subsp. *enterocolitica* 8081	M	S	**A**	H	R	F
Pseudomonas aeruginosa PAO1	M	S	H	H	**K**	F
Aliivibrio salmonicida LFI1238	M	S	**Q**	H	V	F
Vibrio fischeri MJ11	M	**T**	**Q**	H	**K**	F
Moritella sp. PE36	M	**T**	**Q**	H	**K**	F
Colwellia psychrerythraea 34H	M	**T**	**Q**	H	**E**	F
Nitrosomonas sp. AL212	M	**T**	**Q**	H	**K**	F
Rhodococcus jostii RHA1	M	S	**Q**	**K**	R	F
Saccharomonospora viridis DSM 43017	M	S	**Q**	**K**	R	F
Aromatoleum aromaticum EBN1	M	S	**Q**	H	**E**	F
Pelobacter propionicus DSM 2379	M	S	**Q**	**K**	R	F
Syntrophobacter fumaroxidans MPOB	M	S	**Q**	**K**	R	F
Desulfovibrio vulgaris str. 'Miyazaki F'	M	S	H	H	R	F
Cupriavidus metallidurans CH34	M	S	**A**	**K**	**V**	F
Pseudovibrio sp. JE062	M	S	**A**	**K**	**E**	F
Polaromonas naphthalenivorans CJ2	M	S	**Q**	H	**V**	F
Ralstonia eutropha H16	**V**	S	**Q**	H	**K**	F
Bovine liver H16	**V**	S	H	H	**K**	**Y**

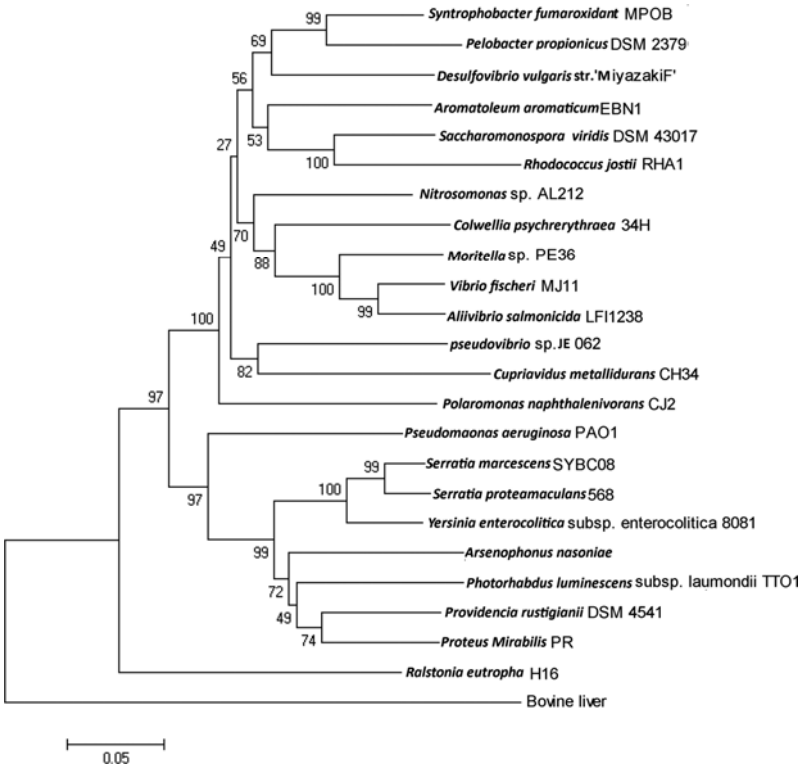


Figure 6. The phylogenetic relationship of the catalase from *Serratia marcescens* SYBC08 with other 23 related catalase sequences. The dendrogram was constructed from a matrix of pairwise genetic distances by the neighbor-joining method using the MEGA 4.1 software. Numbers above branches indicate a bootstrap values (1000 replicates). The scale bar represents five per substitutions 1000 amino acid positions.

rather conserved in catalases [22, 23]. According to the amino acid residues of bovine liver catalase site and alignment analysis of amino acid sequence, the active sites of the catalase from *Serratia marcescens* SYBC08 were consisted of H54, N127, and S93. Its sites of proximal side of heme were composed of P301, R333, Y337, M329, and H341, and sites of the distal side of the heme contained M53, N127, F132, and F140. The Function of catalase-bound NADPH in bovine and human catalase was that both prevents and reverses the accumulation of compound II, an inactive form of catalase that is generated slowly when catalaseis exposed to hydrogen peroxide [24]. Amino acid residues involved in NADPH binding sites of the catalase were H173, S180, R182, and H284. In the bovine catalase, a water molecule has been considered as possibly involved in a redox mechanism of NADPH [25]. The amino acid residues involved in such procedure were K216, Y194, and H214. A phylogenetic relation of 24 catalases was presented in Fig 6. From this figure, it was found that the catalase of *Serratia marcescens* SYBC08 had closest relationship with *Serratia proteamaculans* 568 and *yersinia enterocolitica* subsp. enterocolitica 8081, and it was distincted from the bovine liver catalases. Those catalases from *arsenophonus nasoniae*, *photorhabdus luminescens* subsp. laumondii tto1, *proteus mirabilis* pr and *providencia rustigianii* dsm 4541 belonging to the family Enterobacteriaceae were located on a small branch.

Discussion

The highest catalase yield (20,289 $U \cdot ml^{-1}$) was achieved in 7 l fermenter after incubation of 40 h. Many literatures showed catalase productions by microorganisms did not exceed 5,000 $Um \cdot ml^{-1}$ [6, 26–29]. Nakayama *et al.* [11] reported that *Micrococcus luteus* strain showed rather high catalase production of 34,601 $U \cdot ml^{-1}$. Although, in our study, the yield of catalase from *Serratia marcescens* SYBC08 was slightly lower compared to the report of Nakayama *et al.* [11], it had a great rising space by adding some suitable inducers such as H_2O_2. Therefore, the enzyme yield had a good attraction in application.

The 13.8-fold purification achieved in this study was lower than most of reports. Literature survey revealed that main ranges of purification fold was 54.1-fold for a catalase from *Vibrio rumoiensis* S-1^T [30] to 1,538-fold for a catalase from *Methanosarcina barkeri* [5]. Since one of the goals of our study was to evaluate its industrial applications, low fold purification obtained from the above procedure meant high ratio of catalase production and helped to reduce its purification cost.

The protein had a molecular mass of 230 kDa and a subunit size of approximately 58 kDa. Thus, we proposed that the purified monofunctional catalase was tetramer consisted of 4 homosubunits. The subunit number and native enzyme sizes for this monofunctional enzyme were similar to those of bacteria (i.e., *Vibrio rumoiens*is S-1^T with 57.3 kDa and 230 kDa [30], Halophilic bacterium *Halobacterium halobium* with 68 and 240 kDa [31], *Deinococcus radiodurans* with 65 kDa and 240 kDa subunit and native molecular mass [32], *Vibrio salmonicida* with 57 kDa and 235 kDa [33], respectively).

The Rz value of the purified catalase from *Serratia marcescens* SYBC08 (0.042 ± 0.041) was lower than that of monofunctional catalase which usually exhibit ratios of approximately 1. Thus, the observed spectrum would be considered as atypical electronic spectrum of monofunctional catalase. Similar atypical spectra were found in catalases from other bacteria such as *Methanosarcina barkeri* (0.48) [5] and *Rhodobacter sphaeroides* ATH 2.4.1(0.513) [34]. *Shima et al.* and Terzenbach *et al.* explained the phenomenon, which is caused by partially loss of the heme in this purified procedure [5, 33].

The purified enzyme of *Serratia marcescens* SYBC08 showed maximum catalase activity in the pH range from 7.0 to 9.0. The broad pH optimum range is a common feature of monofunctional catalases, but catalase-peroxidases have narrow pH optimum range [12, 30, and 31]. Our catalase from *Serratia marcescens* SYBC08 was found to be stable in the broad pH range from pH 5.0 to 11.0. This result was similar to the report of the monofunctional catalase of Yumoto *et al.* [30]. It displayed high relative activity at wide temperature range from 0 to 70 °C. The temperature dependence of catalase activity was poor. The phenomenon was also observed in other monofunctional catalases [30, 34]. The heat stability of the catalase from *Serratia marcescens* SYBC08 was higher than that of commercial bovine liver catalase. From an industrial application of view, those property of pH and temperature could meet the demands of waste water treatment under wide pH or temperature conditions.

Generally, monofunctional catalses have high K_m values of about over 50 mM, while catalase-peroxidases have low K_m values of 1–20 mM [5, 34, 35]. Catalase from *Serratia marcescens* SYBC08 exhibited a K_m of 78 mM which is very similar to other monofunctional enzyme, but its K_m values was much lower than that of other *Serratia marcescens* catalase (228) [36]. This indi-

cated that the catalase is more efficient with regard to the scavenging of hydrogen peroxide than the *Serratia marcescen* catalase in literature [36].

Rapid advances in protein analytical technologies, fuelled by the addition of MS and sequence databases, have made it possible for protein chemists to identify new proteins and designed primers for gene coining [37, 38]. Two peptide sequences did not completely matched catalase sequence of *Yersinia enterocolitica* subsp. enterocolitica 8081 (gi|123441711) or *Serratia proteamaculans* 568 (gi|157371515). Therefore, the catalase was regarded as a new protein. 479 amino acid residues encoded by the open reading frame matched the LC-MS/MS sequences. This confirmed that the enzyme gene was obtained. The above study represents a excellent application of LC–MS/MS technology.

Catalase primarily responsible for the metabolism of hydrogen peroxide, is an essential antioxidant enzyme that is present throughout phylogeny, from bacteria to animal [39]. Amino acid residues of *Serratia marcescens* SYBC08 catalase in the active sites (H54 and N127), NADPH binding sites (H173, S180, and R182), proximal sites of heme (P301, R333, Y337, and M329), distal sites of the heme (N127, F132, and F140) and sites interacting with a water molecule (K216 and Y194) were well conserved in 23 catalases. Met changed to Val in bovine liver and *ralstonia eutropha* h16 (Table 3). The Met in *Proteus mirabilis* PR could produce some steric hindrance impairing the accessibility of large substrates or inhibitors to the iron of the active site, this result of Met replacing with Val caused significantly greater sensitivity to aminotriazole of a specific inhibitor of catalases than *P. mirabilis* PR [40]. Some other replacements of the residues from *Serratia marcescens* SYBC08 catalase (Table 3) might further supported some degree of specificity in their catalysis behaviors. The catalase of high specific activity was closely related with the enzyme from pathogenic bacterium in the family Enterobacteriaceae which developed the ability to survive in host against the presence of H_2O_2. This supported the growth environment of *Serratia marcescens* SYBC08.

As a summary, in the study, a high catalase production was obtained by *Serratia marcescens* SYBC08. The purified catalase was characterized as a monofunctional enzyme. LC–MS/MS technology confirmed that the cloning gene was the encoding gene of the monofunctional enzyme. Amino acid sequences analyses suggest the enzyme from *Serratia marcescens* SYBC08 has highly conserved catalysis behaviors in the microorganisms from the family Enterobacteriaceae.

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