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ORIGINAL ARTICLE

Optimization of catalase production and purification and characterization of a novel cold-adapted Cat-2 from mesophilic bacterium *Serratia marcescens* SYBC-01

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Abstract Maximum catalase production by mesophilic bacterium *Serratia marcescens* SYBC-01 was obtained by an optimization of culture medium and conditions. A novel cold-adapted catalase from the strain was purified and characterized. The Cat-2 without peroxidase activity was a homodimer with a molecular mass of 154 kDa, consisting of two identical subunits of about 70 kDa. Its apparent K_m and V_{max} value were 29.7 mM and 80,925 U/mg of protein, respectively. The Cat-2 exhibited maximal activity at pH 7.0, being relatively stable in alkaline conditions. The enzyme was most active at approximately 20°C and had 73.8% activity at 0°C. After incubation at 60°C for 60 min, the enzyme still maintained 75% of its initial activity. The Cat-2 displayed relatively higher thermostability compared to that of other cold-adapted and some mesophilic catalases.

Keywords Cold-adapted · Catalase · Mesophilic · *Serratia marcescens*

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Introduction

Catalases (H_2O_2: H_2O_2 oxidoreductase; EC1.11.1.6) are widely spread in aerobic (facultative or not) bacteria such as *Escherichia coli* and *Bacillus subtilis* (Rochat et al. 2005). Catalases are 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 oxygen by two-electron transfer (Shin et al. 2008). Based on their enzymological properties, bacterial catalases have been classified into three types: heme-containing monofunctional catalases, heme-containing bifunctional catalase-peroxidases, and non-heme-containing catalases (Chelikani et al. 2004). Multiple catalases have been found in almost all bacterial species, including *Escherichia coli* (Claiborne and Fridovich 1979) and *Bacillus subtilis* (Loewen and Switala 1987). There have been several reports of unique catalases from halophiles (Brown-Peterson and Salin 1993, 1995), thermophiles (Kagawa et al. 1999), and psychrophiles (Yumoto et al. 2000; Lorentzen et al. 2006).

Hydrogen peroxide (H_2O_2) is a powerful oxidant, and is used as a bleaching or microbiocidal agent in the paper, food, textile, and semiconductor industries. However, due to its toxicity to environment and human health, hydrogen peroxide needs to be eliminated after industrial processes. Thus, catalases can be utilized in these industrial sections. Generally, cold-adapted enzymes display high catalytic activity at low temperatures associated with reduced thermal stability at relatively higher temperatures (Georlette et al. 2001). Due to saving energy consumption, cold-adapted catalases have been valuably applied in low-temperature wastewater treatment (Margesin and Schinner 1999), and catalases have also been applied in high-

temperature conditions. In this way, if the stability of cold-adapted catalases can be enhanced without deteriorating their catalytic competence at low temperatures, these enzymes will be proven to have a potential application in a wide industrial field.

In our previous work, a high catalase-producing strain was identified as *Serratia marcescens* SYBC-01 (Qian et al. 2008). Although *Serratia marcescens* is a ubiquitous Gram-negative bacterium which is capable of causing disease in diverse organisms, including humans (Richards et al. 2000), coral (Patterson et al. 2002), insects (Adamo 2004), and plants (Pair et al. 2004), it has also been applied in fermentation for the production of keratin hydrolyzing enzyme (Khardenavis et al. 2009), protease (Romero et al. 2001), phospholipase (Fu et al. 2008), and chitinase (Duzhak et al. 2002).

In this study, we characterized a unique catalase (Cat-2) from *Serratia marcescens* SYBC-01 exhibiting cold-adaptation and relatively high thermostability, and optimized the cultural medium and conditions for catalase production.

Materials and methods

Strains and preparation of inoculum

The mesophilic *Serratia marcescens* SYBC-01 was isolated from Wuxi City in China where the environmental temperature is mainly maintained at 20–30°C. Prior to use, the strain was recovered from 10% (v/v) glycerol stocks stored at –70°C. The strain was grown on Luria-Bertani medium (yeast extract, 5 g/l; NaCl, 10 g/l; protein peptone, 10 g/l) at 30°C for 12 h and then were aseptically transferred to a 250-ml flask containing 50 ml seed medium (protein peptone, 2 g/l; yeast extract, 1 g/l; NH_4SO_4, 6 g/l; $MgSO_4 \cdot 7H_2O$, 0.5 g/l; K_2HPO_4, 10 g/l; NaCl, 0.5 g/l) incubated at 30°C with shaking at 180 rpm for 12 h. Seed broth was aseptically added to fermentation medium with inoculm size of 4% (v/v) for catalase production.

Optimization of culture medium and conditions for catalase production

The fermentation medium (glycerol, 10.0 g/l; protein peptone, 10.0 g/l) was used as basal medium and the initial pH of the medium was adjusted to 7.2 before sterilization. Fermentations were carried out at 30°C with shaking at 180 rpm for 24 h. For selection of the best carbon source for catalase production, glycerol was substituted with equal total carbon molar ratio in dextrin, glucose, fructose, soluble starch, cane sugar, or lactose. Gradient concentrations of the best carbon sources (2.5, 5, 7.5, 10, 12.5, 15, and 17.5 g/l, final concentration) were tested in order to determine their effect on simultaneous catalase production. At the optimum concentration of the best carbon source, the influences of different nitrogen sources in simultaneous catalase production were evaluated by replacing peptone with yeast extract, yeast, peptone, $NaNO_3$, Urea NH_4Cl, or $(NH_4)_2SO_4$ with equal total nitrogen molar ratio. The optimum concentration of the best nitrogen source was achieved by changing concentration (2.5, 5, 7.5, 10, 12.5, 15, and 17.5 g/l, final concentration). Subsequently, the other five factors affecting simultaneous catalase production, including KH_2PO_4 concentration, initial pH, loading liquid volume, inoculum size and temperature, were investigated. Finally, catalase production was determined in different incubation periods (2, 4, 6, 8, 10, 12, 14, 16, 18, and 20 h).

Preparation of crude enzyme and determination

Fermentation broth was centrifuged at 4°C and 10,800 *g* for 15 min. The precipitation was divided into two parts, one part was dried at 105°C to constant weight followed by weighing with electrical balance, and the other part was disrupted by supersonic instrument at 0°C for 20 min. The cell debris from the disrupted part 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 (Beers and Sizer

Table 1 Effect of carbon and nitrogen sources on catalase production by *Serratia marcescens* SYBC-01

Carbon source	Catalase (U/ml)	Nitrogen source	Catalase (U/ml)
Starch	621.8±68.5	Yeast extract	965.8±145.2
Glycerin	469.5±54.6	Yeast extract and peptone	950.0±165.6
Cane sugar	463.2±70.7	Peptone	624.2±83.5
Dextrin	450.5±85.5	NH_4SO_4	3.2±0.6
Fructose	444.2±72.5	NH_4Cl	3.0±0.8
Lactose	412.4±75.2	Urea	1.7±0.2
Glucose	412.4±79.6	$NaNO_3$	0.3

Data are mean values from three triplicate experiments

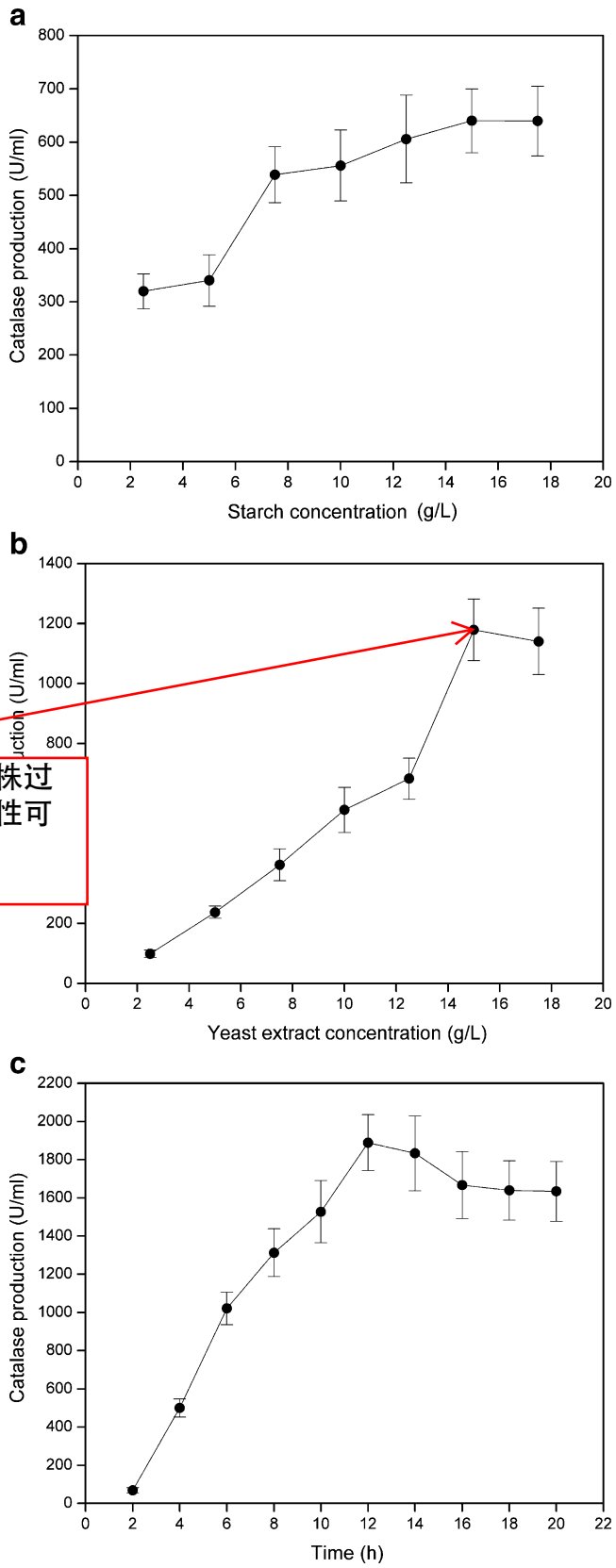

Fig. 1 Effects of **a** starch concentrations and **b** yeast extract concentrations on catalase production by *Serratia marcescens* SYBC-01; **c** time course of *Serratia marcescens* SYBC-01 under the optimized condition in flask cultures on a rotary shaker at 200 rpm. Values given are the means of three triplicate experiments, *error bars* SD

1952). The ε for H_2O_2 at 240 nm was 43.6 /M /cm. The reaction mixture in a total volume of 4 ml was composed of 100 µl enzyme solution, 50 mM K_2HPO_4-KH_2PO_4 (pH 7.0), 15 mM H_2O_2. One unit of catalase activity was defined as the amount of enzyme required to transform 1 µmol of hydrogen peroxide to water and oxygen per min.

Peroxidase activity was measured spectrophotometrically by monitoring the increasing in absorbance at 470 nm at 30°C. The reaction mixture in a total volume of 4 ml contain 100 µl enzyme solution, 50 mM Na_2HPO_4-citrate buffer (pH 6.0), 10 mM H_2O_2, 10 mM guaiacol. One unit of peroxidase was expressed as the enzyme amount required forming 1 µmol guaiacol oxidants.

The protein concentration was determined by the Bradford method (Bradford 1976).

Cat-2 purification

The pooled crude enzyme extract was firstly fr ammonia sulfate precipitation at 40% saturati the catalase active fractions were collected b tion at 4°C and 10,800 *g* for 20 min. A se precipitation was performed by adding ammonium sulfate to 60% saturation and incubating for 12 h at 4°C. The final precipitates were collected by centrifugation (4°C and 17,300 *g* for 20 min) and then fully dissolved in a small amount of 50 mM phosphate buffer (pH 7.0). The supernatants were dialyzed and concentrated with PEG-20000.

分离出的菌株过氧化氢酶活性可以达到1200

The concentrated solution was loaded on a Sephacryl Tm S-200 column (16 mm×60 cm) which had been equilibrated with 50 mM potassium phosphate buffer (pH 7.0). The enzyme was eluted with the same buffer at the flow rate of 1 ml/min. The fractions with catalase activity were pooled and concentrated with PEG-20000, and then subjected to a Mono Q column (10 mm×100 mm) which had been equilibrated with the same buffer. The adsorbed enzyme was eluted with a linear gradient of NaCl (0 to 0.7 M). The eluted catalase fractions were combined and dialyzed against the same buffer at 4°C for 12 h and used as the purified catalase sample.

Isoenzyme and molecular weight

SDS-PAGE was performed for determining the molecular masses of the catalase subunits according to the method of Laemmli (1970), using 5% (w/v) stacking polyacrylamide gel and 7.5% (w/v) separating gel, and run at 120 V.5% (w/v) stacking polyacrylamide gel and 12% (w/v) separat-

Table 2 Purification of Cat-2 from *Serratia marcescens* SYBC-01

Step	Total activity (U)	Total protein (mg)	Sp activity (U/mg)	Yield (%)	Purified folds
Crude extract	584,609	784	745.7	100	1
Ammonia sulfate precipitation	411,776	385	1,070.0	70.4	1.4
Sephacryl Tm S-200	241,786	36.7	6,591.8	41.4	8.8
Mono Q	187,411	4.0	46,619.7	32.1	62.5

ing gel was applied in nature PAGE. The gels were stained by Commasie brilliant blue or catalase active staining according to the method of Woodbury et al. (1971). Native molecular weight was detected by gel filtration.

Effects of pH and temperature on catalase activity

Effect of pH on catalytic activity was determined by incubating the purified enzyme in 50 mM K_2HPO_4-citric acid buffer (pH 3.0–8.0) and 50 mM glycine-NaOH buffer (pH 9.0–10.0) at 30°C, and the highest catalase activity was defined as 100% of the activity. The effect of the pH range of 3.0–10.0 on catalase stability was assayed by incubating the enzyme at 30°C for 24 h, and the highest residual activity is expressed as 100% of the activity. 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 defined as 100% of the activity. For thermal stability determination, the enzyme was incubated at different temperatures for 60 min followed by measuring activity in 50 mM phosphate buffer (pH 7.0) at 30°C, and the highest initial activity was defined as 100% of the activity.

Kinetic parameters (V_{max} and K_m)

The effect of H_2O_2 concentrations from 2.5 to 30 mM on catalase activity was evaluated in 50 mM phosphate buffer (pH 7.0) at 30°C. The kinetic parameters were estimated by linear regression from double-reciprocal plots according to Lineweaver and Burk (1934).

Results

The optimum fermentation conditions

The influence of carbon source on catalase production by *Serratia marcescens* SYBC-01 is shown in Table 1. The best catalase production occurred with soluble starch as the carbon source. Caridis et al (1991) detected catalase production when microorganism grew in submerged fermentation condition using various carbon sources including starch. Their result showed that starch was not the suitable carbon for catalase production. Catalase production increased at the concentration range of soluble starch from 2 to 15 g/l, and then decreased above 15 g/l (Fig. 1a).

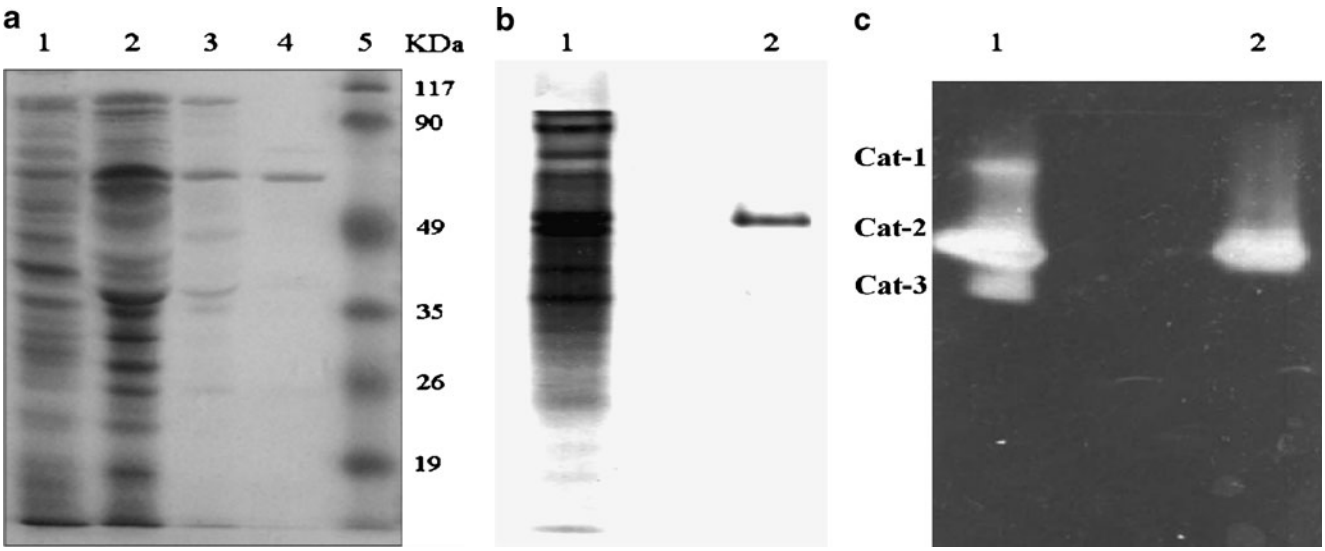

Fig. 2 **a** Electrophoretic analysis of catalase from *Serratia marcescens* SYBC-01 by SDS-PAGE; *lane 1* enzyme extractl *lane 2* $(NH_4)_2SO_4$ precipitationl *lane 3* gel filtration, *lane 4* ion exchange, *lane 5* marker. **b** Electrophoretic analysis of catalase from *Serratia marcescens* by native PAGE and Commasie brilliant blue; *lane 1* enzyme extract, *lane 2* purified Cat-2. **c** Electrophoretic analysis of catalase isozyme from *Serratia marcescens* by native PAGE and catalase active staining; *lane 1* enzyme extract, *lane 2* purified Cat-2

The catalase production was also impacted by nitrogen. Catalase production by the strain growing in the media with inorganic nitrogen sources was much lower than the media with organic nitrogen. A similar result had been found in the study of Caridis et al. (1991). Yeast extract was the best nitrogen source for catalase production (Table 1). Optimum concentration of yeast extract for catalase production was 15 g/l (Fig. 1b).

Subsequently, the optimum conditions of other factors for catalase production were obtained (data not shown). The results showed that the optimal fermentation medium was as follows: soluble starch, 12.5 g/l; yeast extract, 15 g/l; KH_2PO_4, 0.5 g/l. The suitable fermentation conditions were as follows: initial pH 8.0, temperature 28°C, medium volume 60 ml/250 ml, inoculum volume 10% (v/v). In such optimal nutrition and environmental conditions, the highest catalase production (1,888.9 U/ml) was achieved after 12 h of incubation (Fig. 1c). Although many microorganisms including *Saccharomyces cerevisiae* CFR-201 (Venkateshwaran et al. 1999), *Penicillium variable* (P16) (Petruccioli et al. 1995), *Penicilli minioluteum* 40 (Kurakov et al. 2001) and *Alternaria alternata* (Caridis et al. 1991) have been reported as sources of catalase, their catalase yields usually did not exceed 1,500 U/ml. The fermentation time of 12 h was much shorter than other reports, such as 7 days in *Alternaria alternata* (Caridis et al. 1991), 72 h in *Penicillium variabile* (P16) (Petruccioli et al. 1995), and 72 h in *Saccharomyces cerevisiae* (Venkateshwaran et al. 1999). A short fermentation time and relatively high catalase production can make *Serratia marcescens* SYBC-01 as a potential catalase producer.

Purification of Cat-2 from *Serratia marcescens* SYBC-01

The Cat-2 of *Serratia marcescens* SYBC-01 was purified by ammonia sulfate precipitation, gel filtration and ion exchange. The results are summarized in Table 2.

During gel filtration, three protein peaks appeared, and the second peak contained catalase activity (data not shown). After the above procedure, the Cat-2 was purified 8.8-fold. The catalase active fraction was further purified by ion exchange and separated into five fractions, and the fraction of the fourth peak contained catalase activity (data not shown). The enzyme was thus purified 62.5-fold in a 32% yield.

Samples from each procedure were analyzed by SDS–PAGE (Fig. 2a). A single band from ion exchange was revealed by native PAGE and SDS-PAGE (Fig. 2a-c). It indicated that the Cat-2 without peroxidase activity was purified to apparent electrophoretic homogeneity and was a main isoenzyme among three isoenzymes. The subunit size of about 70 KDa was estimated by SDS-PAGE, and about 145 KDa of the molecular mass was revealed using gel filtration. We concluded that the purified Cat-2 appeared to be a homodimer composed of two identical subunits. Most

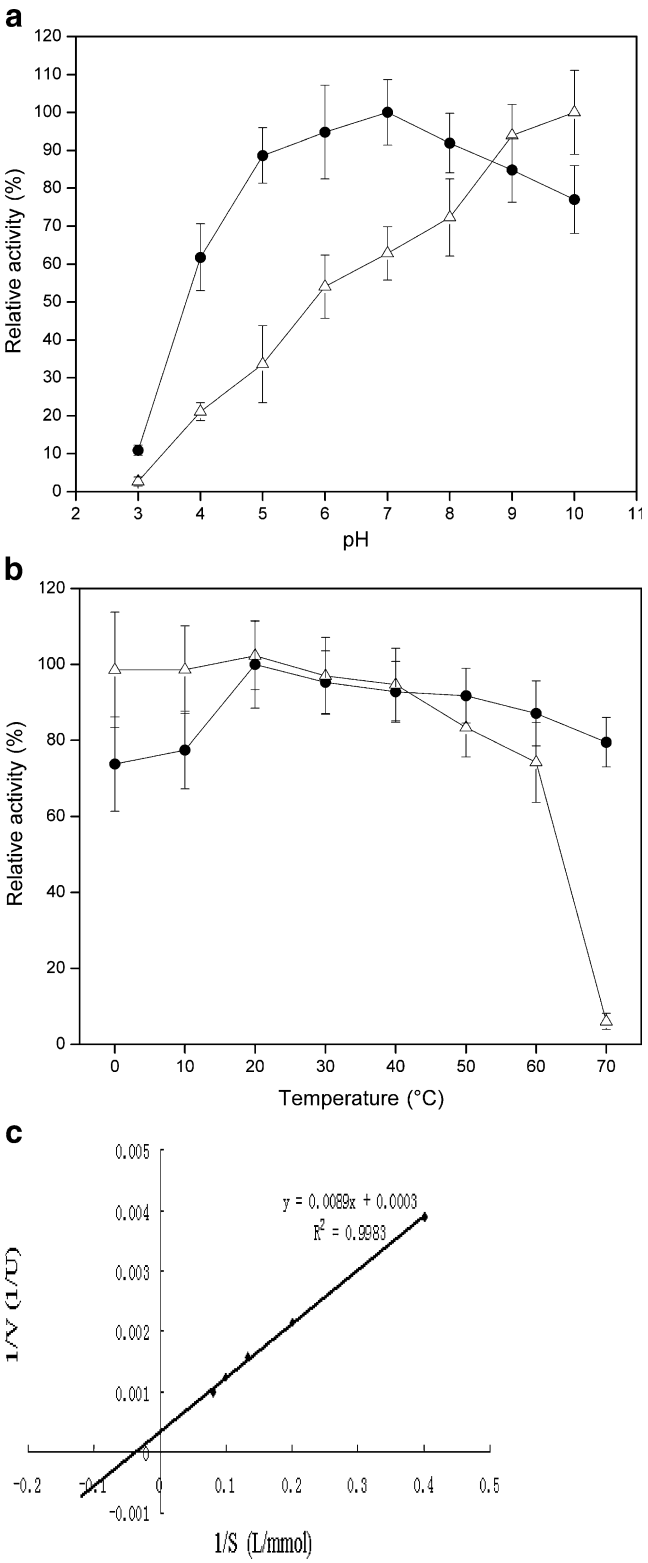

Fig. 3 **a** Effect of pH on catalase activity (•) and stability by incubating the Cat-2 in buffer (pH 3.0-10.0) for 24 h (Δ). **b** Effects of temperture on catalase activity (•) and temperature stability by incubating at 0, 10, 20, 30, 40, 50, 60 and 70°C for 60 min (Δ). Values given are the means of three triplicate experiments, *error bars* SD. **c** Lineweaver–Burk plot of Cat-2. The enzyme activity was measured in 50 mM phosphate buffer (pH 7.0) and at 30°C

of the monofunctional catalases are homo-tetramers, 200–340 kDa in size (Zámocký and Koller 1999). As far as we know, the homodimer from monofunctional catalase has only been reported in *Bacillus subtilis* (Zhang et al. 2005), *Klebsiella pneumoniae* (Goldberg and Hochman 1989) and *Rhodobacter sphaeroides* ATH 2.4.1 (Terzenbach and Blaut 1998).

Effect of pH and temperature on the Cat-2 activity

The purified Cat-2 showed the optimal pH at 7.0 and a broad catalytic pH range from 3.0 to 10.0 (Fig. 3a). This is common feature of monofunctional catalases, but catalase-peroxidases have narrow catalytic pH range (Brown-Peterson and Salin 1993, 1995; Yumoto et al. 2000). Its activity retained over 60% after incubating the Cat-2 at 30°C in the buffer (pH 7.0–10.0) for 24 h. The enzyme had high stability at pH (7.0–10.0) and highest stability at pH 10.0 (Fig. 3a). It is similar to the report that monofunctional catalase *TvC-II* in yeast *Trigonopsis variabilis* displays the highest half-life times ($t_{1/2}$) at pH of 10 (Monti et al. 2003).

The effect of temperature on Cat-2 stability and activity is represented in Fig. 3b. The optimum temperature was approximately 20°C, and about 73.8% the activity was observed at 0°C. The purified Cat-2 was stable at the temperature range from 0°C to 40°C and maintained 75% of its initial activity at 60°C when it was incubated for 60 min.

Kinetic analysis

The kinetic parameters for Cat-2 were analyzed by Lineweaver–Burk plot (Fig. 3c). The K_m and V_{max} at 30°C were 29.7 mM and 80,925 U/mg of protein, respectively. The K_m of *Serratia marcescens* SYBC-01 was the lowest of all the catalases (Table 3) and was only 1/12 compared to that of the same species in the literature (Switala and Loewen 2002). Low K_m indicates the catalase have a higher affinity for decomposition H_2O_2.

Discussion

The cold-adapted enzyme as enzymes in which the optimum temperature is about 30°C and high activity is retained at 0°C (Margesin and Schinner 1999). The catalase from *Bacillus* sp. N2a can be categorized as a cold-adapted enzyme only mainly based on its low optimal temperature of 25°C (Wang et al. 2008). The mesophilic *Serratia marcescens* SYBC-01 was isolated from Wuxi City of China where the environmental temperature was usually at 20–30°C. The optimum temperature of Cat-2 from mesophilic *Serratia marcescens* SYBC-01 was approximately 20°C, and it displayed high relative activity (73.8%) at 0°C. Therefore, our results indicate that the Cat-2 was a kind of cold adaptation enzyme.

Cold-adapted catalase has been firstly characterized in facultative psychrophilic bacterium *Vibrio rumoiensis* S-1^T. The thermostability of *Vibrio rumoiensis* S-1^T catalase is significantly lower than that of mesophilic catalase from *Micrococcus luteus* and bovine liver at moderate temperature (Yumoto et al. 2000). The second cold-adapted catalase from *Vibrio salmonicida* displays higher thermoinstability compared to mesophilic *Proteus mirabilis* catalase (Lorentzen et al. 2006). The third cold-adapted catalase from *Bacillus* sp. N2a has a low optimum temperature of 25°C, and displays lower thermostability as compared with other small-subunit catalase homologues from mesophile-like bacteria (Wang et al. 2008). Based on these studies, cold-adapted catalases generally display higher thermoinstability at moderately high temperatures compared to mesophilic counterparts. However, temperature stability experiments of the cold-adapted Cat-2 revealed that it

Table 3 Comparison partial property of monofunctional catalases from microorganisms

Microorganisms	Conditions and residual activity (%)	Optimum temperature (°C)	K_m (mM)	References
Serratia marcescens SYBC-01	60°C, 60 min, 75%	20	29.7	Present work
Proteus mirabilis	60°C, 50 min, 50%	20-50	127	Lorentzen et al. 2006
Klebsiella pneumoniae	60°C, 45 min, 20%	ND	ND	Goldberg and Hochman 1989
Deinococcus radiodurans	60°C, 30 min, 30%	30	ND	Kobayashi et al. 2006
Bacillus sp. N2a	60°C, 15 min, 12%	25	41.5	Wang et al. 2008
Comamonas terrigena N3H	60°C, 15 min, 0%	ND	ND	Zámocký et al. 2004
Vibrio rumoiensis S-1^T	60°C, 15 min, 0%	40	ND	Yumoto et al. 2000
Vibrio salmonicida LFI1238	60°C, 5 min, 50%	0-10	103.6	Lorentzen et al. 2006
Streptomyces coelicolor ATCC 10147	60°C, 5 min, 0%	ND	ND	Kim et al. 1994

ND Not determined.

remained 75% of its initial activity after incubation at 60°C for 60 min, suggested that it had higher thermostability than other three cold-adapted catalases and some mesophilic catalases (Table 3).

In conclusion, we reported the growth conditions for producing catalase by a mesophilic bacterium *Serratia marcescens* SYBC-01. The main isoenzyme (Cat-2) from the strain was characterized as a novel cold-adapted catalase with relatively high thermostability.

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