A catalase-peroxidase from a newly isolated thermoalkaliphilic Bacillus sp. with potential for the treatment of textile bleaching effluents

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Introduction

1. Why is this important?

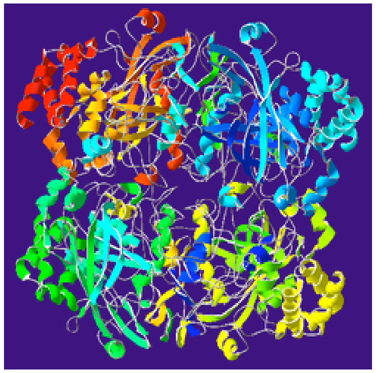
- Application of catalase to eliminate hydrogen peroxide (H_2O_2) in textile bleaching effluents
- The enormous water consumption(100 L/kg of fabric) of the textile processing industry could be reduced
- Only very small amount needed, compare to the use of chemicals

2. Thermoalkalistable enzymes

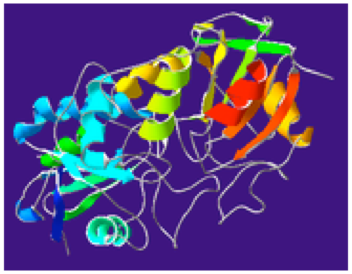
- Temperature above 50°C and high pH value (above 9)
- New Bacillus sp. (Bacillus SF)

3. Catalase

- $2H_2O_2 \rightarrow 2H_2O + O_2$
- Protect aerobic cells from the toxic effect of hydrogen peroxide
- Composed of 4 identical subunits
- Each of the subunits binds one protoheme IX group



4. Peroxidase



- Heme-containing enzyme
- Use H_2O_2 as the electron acceptor to catalyse a number of oxidative reactions

5. Catalase-peroxidase (CP)

- Bifunctional enzyme
- A new class of bacterial enzyme
- Ancestral form of catalase or peroxidase in evolutionary flow
- Little sequence homology with typical heme-containing monofunctional catalase
- High homology with fungal cytochrome c peroxidase and plant ascorbate peroxidase
- A part of the class I of the superfamily of plant, fungal and bacterial peroxidases
- Overwhelming catalase activity with *k*cat/*K*m values comparable with monofunctional catalase
- Reduced by dithionite
- Not inhibited by the catalase-specific inhibitor, 3-amino-1,2,4-triazole, but inactivated by hydrogen peroxide
- Narrow pH range

6. Treatment of bleaching

- Hydrogen peroxide and caustic soda are commonly used for the bleaching of a range of textile materials in order to improve fabric whiteness.
- Washing process after bleaching is a step that consumes large amounts of water, since any residual hydrogen peroxide has to be removed to avoid problems in subsequent dyeing processes.

7. Purpose of this paper

- Describe the purification of a thermoalkalistable catalase-peroxidase from this Bacillus sp.
- Compare the properties of this enzyme to those of other known catalase-peroxidases

Results

1.Thermoalkaliphilic Bacillus SF

- Isolated from textile finishing effluent
- Gram positive
- Catalase positive
- Non-spore forming
- Rod-shaped
- Optimum pH 9.5 (7.5~10.1)
- Optimum temperature around 60°C (upper limit at 65°C)
- Thermophilic Bacillus sp.
- Alkalophilic Bacillus sp.
- Member of the genus Bacillus

2. Purification of catalase-peroxidase

- Fig 1A: one isoform of an enzyme with hydroperoxidase activity
- Fig 1B: 82 kDa by SDS-PAGE, 165±10 kDa by Superdex 200 HR 10/30 column
- The enzyme is composed of two subunits of identical size
- Table 1: Catalase activities of 48 U/mg, 70.3 fold purified protein, and 11.3 % of recovery of catalase activity
- PI is at pH 6.0 by IEF

Figure 1A,B. Native and sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis of the purified catalse-peroxidase (CP) from *Bacillus* SF

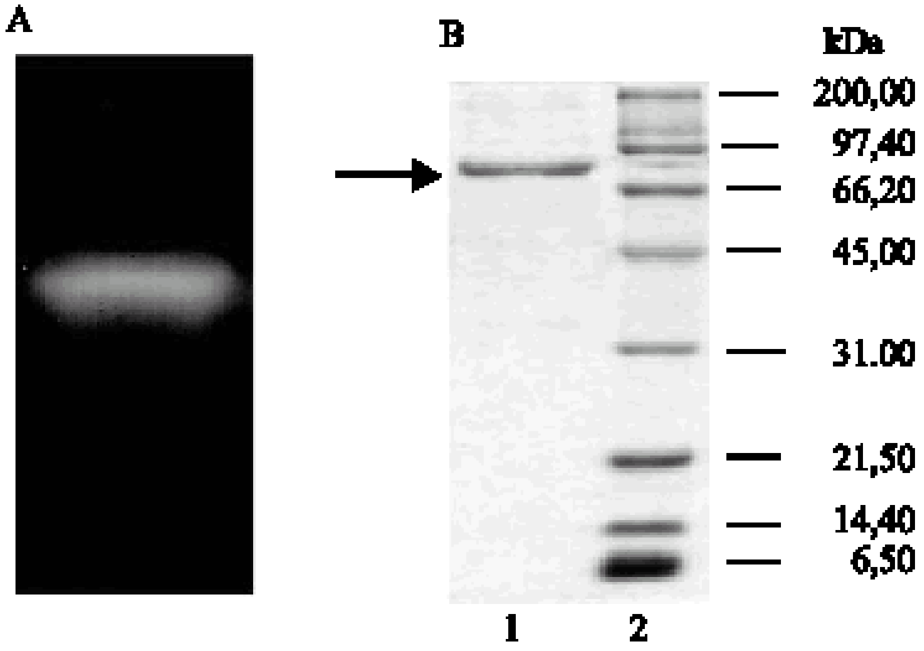


Fig. 1A,B. Native and sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis of the purified catalase-peroxidase (CP) from *Bacillus* SF. **A** Native 10% polyacrylamide gel stained for catalase activity according to Harris and Hopkinson (1976). **B** Denaturing SDS 12% polyacrylamide gel stained for protein with Coomassie brilliant blue. *Lane 1*, purified CP; *lane 2*, Pharmacia molecular mass standard. *Arrow* indicates application of the catalase

Table 1. Purification of catalse peroxidase from the thermoalkaliphilic *Bacillus* SF

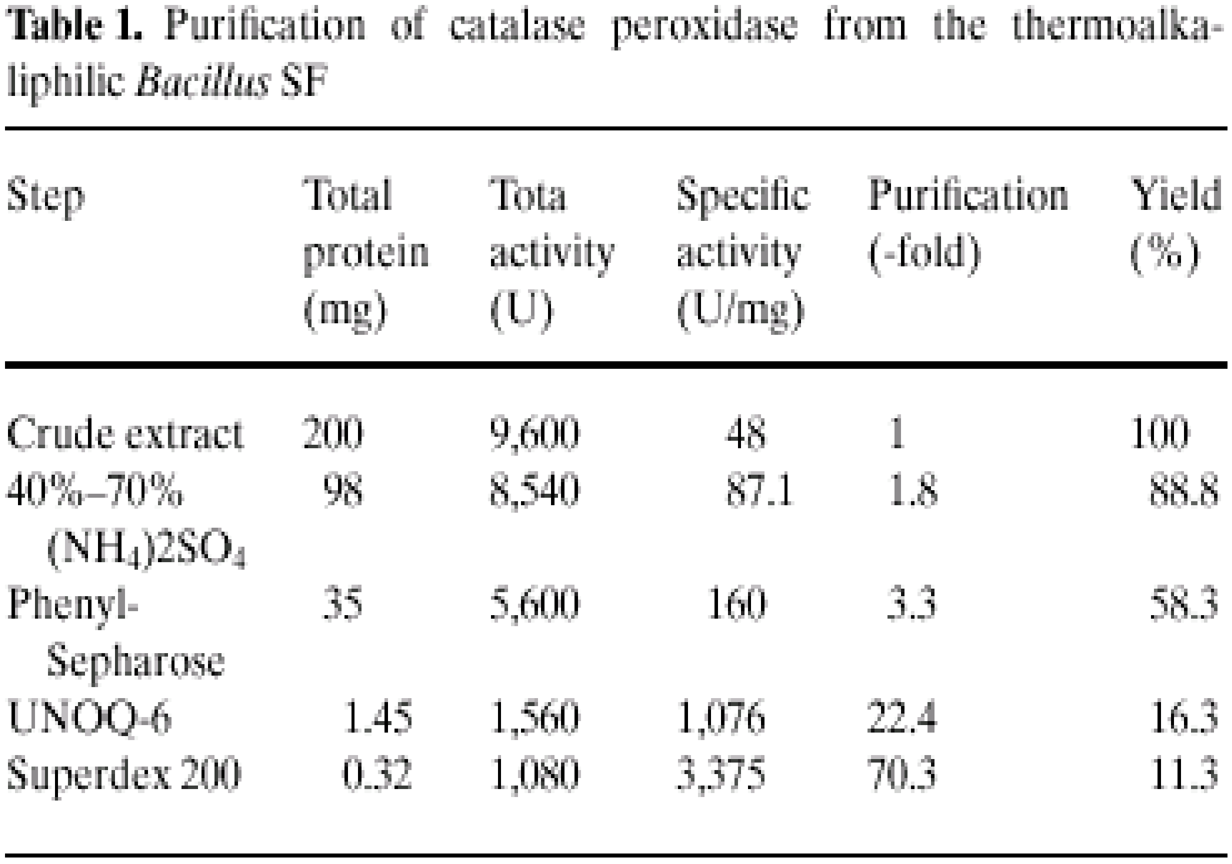


Table 1. Purification of catalase peroxidase from the thermoalkaliphilic *Bacillus* SF

Step	Total protein (mg)	Tota activity (U)	Specific activity (U/mg)	Purification (-fold)	Yield (%)
Crude extract	200	9,600	48	1	100
40%–70% $(NH_4)2SO_4$	98	8,540	87.1	1.8	88.8
Phenyl-Sepharose	35	5,600	160	3.3	58.3
UNOQ-6	1.45	1,560	1,076	22.4	16.3
Superdex 200	0.32	1,080	3,375	70.3	11.3

Results cont.

3.Physical & Chemical characterization of the CP from Bacillus SF

- Optimum temp for catalase activity at pH 9.5 is 55°C,

  10% decreased at 60°C, 50% decreased at 70°
- Fig. 2:

  active pH 6.0 ~10.0

  Sharp activity maximum: pH 7.0 (25°C)

  Activity rapidly decreased until it reached 25% at pH 8.0, remained almost constant until pH 9.0, and only 4% at pH 10.0
- Table 2:

  Stable at pH 10 and 25°C, showing half-lives of at least 4 days (104h)

  At 25°C, higher stabilities at pH 8.0 (260h) & pH 9.0 (240h) than pH 7.0 (190h)

  At 60°C, longest half lives at pH 8.0 & pH 9.0 and its half-life decreased to only 5 h at pH 10.0

Figure 2. Effect of pH on the catalse activity of the purified CP from *Bacillus* SF

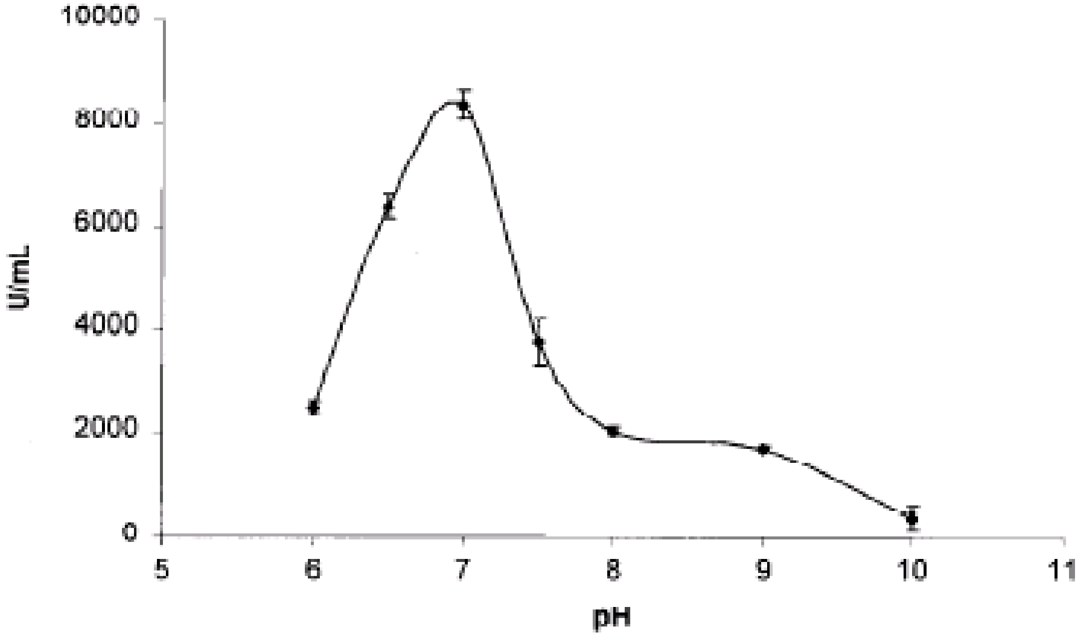


Fig. 2. Effect of pH on the catalase activity of the purified CP from *Bacillus* SF. Catalytic activity was assayed at 25°C as described in Materials and methods

Table 2. Stability of a catalase peroxidase from *Bacillus* SF

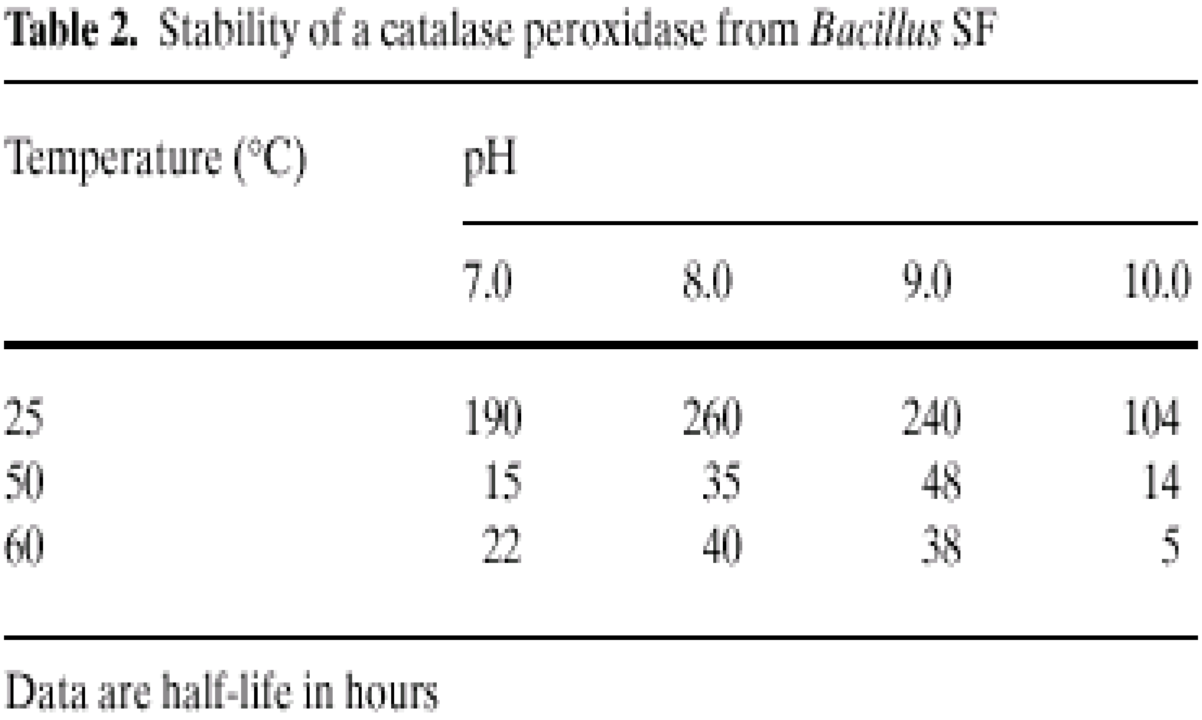


Table 2. Stability of a catalase peroxidase from *Bacillus* SF

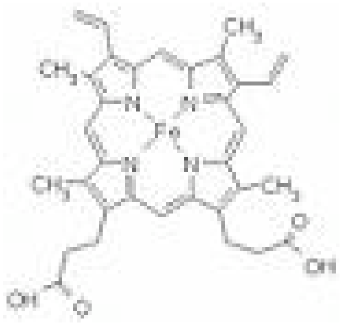
Temperature (°C)	pH			
	7.0	8.0	9.0	10.0
25	190	260	240	104
50	15	35	48	14
60	22	40	38	5

Data are half-life in hours

Results cont.

4. Catalytic and spectral properties

- Strong absorbance at 405 nm and peak at 623 nm
- OD_{405}/OD_{280} ratio of the enzyme preparations is 0.36, consistent with one heme/homodimer
- Fig 3:
  Adding of cyanide, peak absorbance shifted 405 -> 417 nm
  A high to low spin transition of the iron center of
  the heme
  Catalase-peroxidase was reduced by dithionite



- Table 3:
  Catalytic activity of the purified enzyme: 3375 U/mg protein
  *K*m: 2.6 ± 0.1mM H_2O_2
  1/*k*cat: 11475 s^{-1}
  *k*cat/ *K*m = 4.41x10^6
- The purified enzyme was irreversibly inhibited by high hydrogen peroxide conc.(15mM of H_2O_2)
- Monofunctional catalase, 3-amino-1,2,4-triazole does not inhibit CP from Bacillus SF

Figure 3. Absorption spectrum of purified CP from *Bacillus* SF

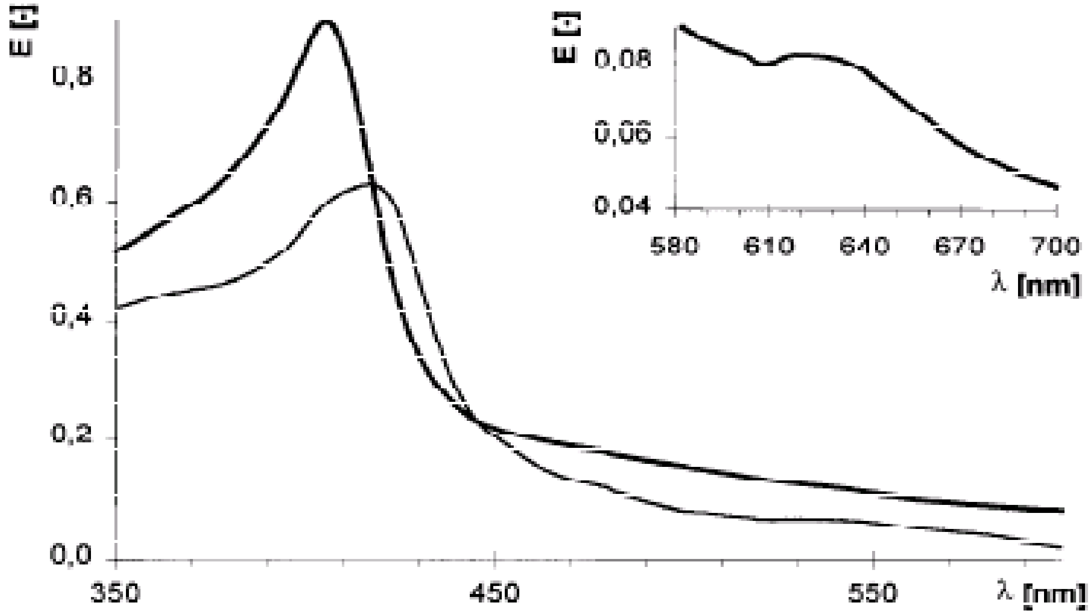


Fig. 3. Absorption spectrum of purified CP from *Bacillus* SF. *Thick line*, 9 µM native enzyme; *thin line*, the cyano-adduct obtained by the addition of 25 mM KCN to the native enzyme. *Inset* is an expanded scale of the visible portion of the spectrum. Experimental conditions were as described in Materials and methods

Table 3. Kinetic parameters of various catalse-peroxidases

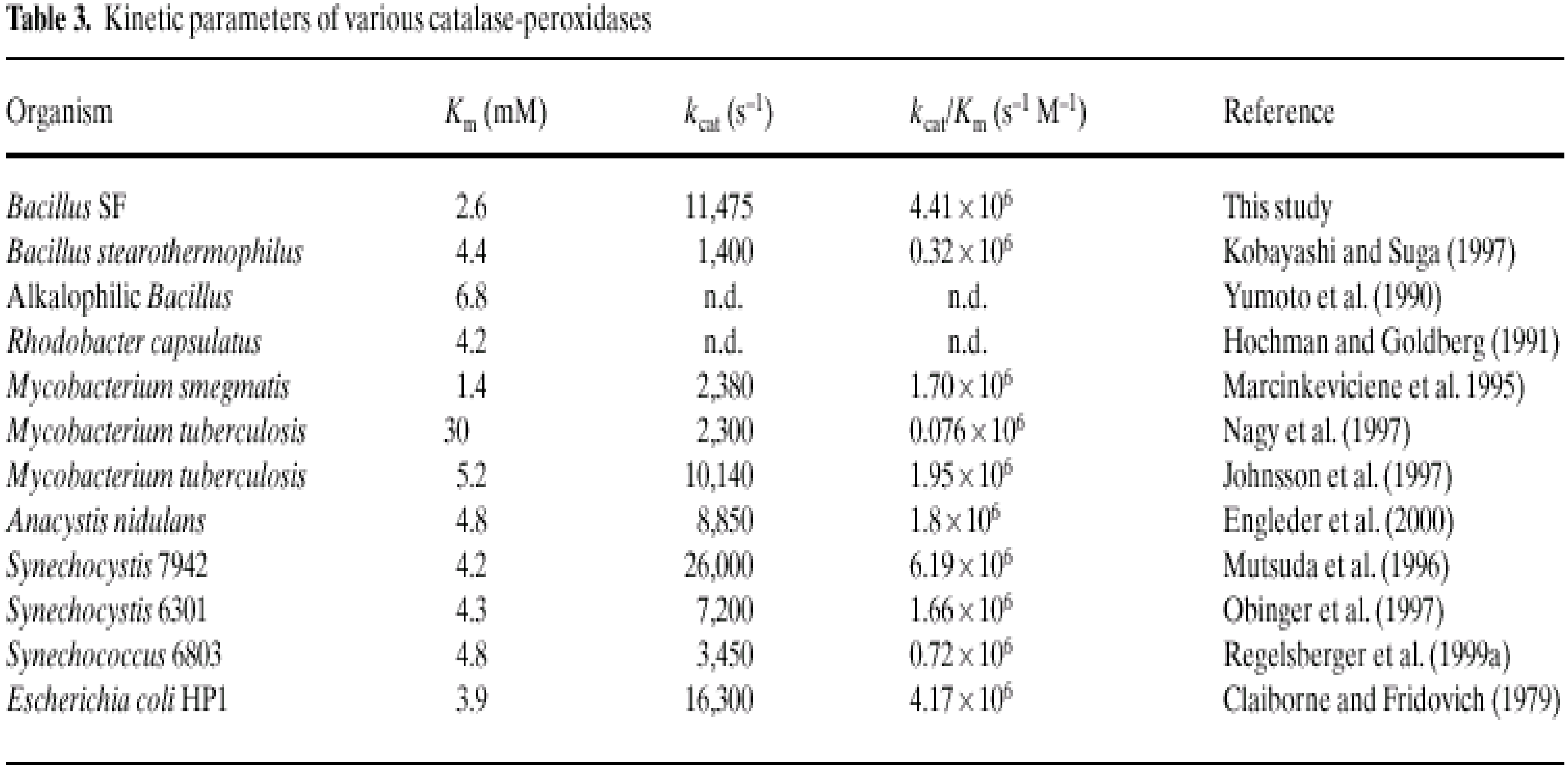


Table 3. Kinetic parameters of various catalase-peroxidases

Organism	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m (s^{-1} M^{-1})	Reference
Bacillus SF	2.6	11,475	4.41×10^6	This study
Bacillus stearothermophilus	4.4	1,400	0.32×10^6	Kobayashi and Suga (1997)
Alkalophilic *Bacillus*	6.8	n.d.	n.d.	Yumoto et al. (1990)
Rhodobacter capsulatus	4.2	n.d.	n.d.	Hochman and Goldberg (1991)
Mycobacterium smegmatis	1.4	2,380	1.70×10^6	Marcinkeviciene et al. 1995)
Mycobacterium tuberculosis	30	2,300	0.076×10^6	Nagy et al. (1997)
Mycobacterium tuberculosis	5.2	10,140	1.95×10^6	Johnsson et al. (1997)
Anacystis nidulans	4.8	8,850	1.8×10^6	Engleder et al. (2000)
Synechocystis 7942	4.2	26,000	6.19×10^6	Mutsuda et al. (1996)
Synechocystis 6301	4.3	7,200	1.66×10^6	Obinger et al. (1997)
Synechococcus 6803	4.8	3,450	0.72×10^6	Regelsberger et al. (1999a)
Escherichia coli HP1	3.9	16,300	4.17×10^6	Claiborne and Fridovich (1979)

Results cont.

- Addition of cyanide and azide to monofunctional catalase inhibits the enzyme activity
- For azide, the inhibition was competitive with a *K*I(EI) of 15 _M for dissociation of the enzyme-inhibitor complex
- For cyanide, the inhibition was competitive with a 1.5 _M
- Comparing function of guaiacol & *o*–dianisidine as electron donors for Bacillus SF catalas-peroxidase after compound I formation with peroxoacetic acid: *o*–dianisidine was the better substrate

Discussion

pH	6.5	11	10	10
Temp (°C)	58	60	60	RT
CP Half life	30min	60min	300min	104h
origin	Synechococcus ppc 7942 & Mycobacterium tuberculosis	Alkalophilic Bacillus sp	Bacillus SF	Bacillus SF

- CP from B. stearothermophilus : increased k_{cat} with a concomitant decrease of K_m after heating
- No second active form could be found on thermal activation for the CP from Bacillus SF

Discussion cont.

* General characteristics of CP from Bacillus SF

- share a number of structural and spectroscopic properties with other bacterial CP
- Dimer of 82 kDa subunits, containing 1 ferric (Fe^{3+}) protoporphyrin IX prosthetic group
- Exhibit a high-spin ferric heme optical spectrum
- In presence of CN^-, high-spin to low-spin conversion
- Homodimer/homotetrameric structure for hydroperoxidase
- Identical subunits of CP are 78~85 kDa, demonstrated for E. coli, streptomyces sp. IMSNU-1, Mycobactrium smegmatis, Mycobacterium tuberculosis, Rhodobacter capsulatus, and B. stearothermophilus
- Double length of the bacterial peroxidases for gene duplication

Organism	Bacillus SF	Streptomyces sp.	M.Smegmatis	E.Coli HPI	R. Capsulatus	Synechocystis sp.
OD_{405}/OD_{280} per subunit of CP	0.36	0.54	0.56	0.52	0.3	0.35~0.46

Discussion cont.

- Competitive inhibition by cyanide & azide, and the lack of inhibition by the catalase inhibitor, 3-amino-1,2,4-triazole
- Inhibition by cyanide, the heme iron atom of the CP is the site of both peroxide and cyanide binding, as is the case for other peroxidase
- $K_{I(EI)}$ of 1.5 _M for inhibition of the CP from Bacillus SF by cyanide was significantly lower than the $K_{I(EI)}$ of 19.7 _M
- Dithionite reduction distinguishes the CP from typical catalases, which are not reduced by this agent, whereas typical peroxidases are rapidly reduced by dithionite.
- In addition to its catalase activity, the enzyme functions as a peroxidase : oxidizing guaiacol and *o*-dianisidine
- No activity on guaiacol was previously found for CP from Synechocystis sp. and Klebsiella pneumoniae
- *o*-dianisidine seemed to be a substrate for most CP

Conclusion

- Physical characteristics indicates that this enzyme belongs to the developing class of bacterial enzymes that catalyze both catalase- and peroxidase-like reaction
- The low heme content of catalase-peroxidases is contradiction to sequence alignment models proposing one heme binding/monomer
- OD_{405}/OD_{280} ratio seems to be one of the characteristics of this type of hydroperosidase

- In future: More information about the structure-function relationship by 3-D