Recombinant Sox enzymes from *Paracoccus pantotrophus* degrade hydrogen sulfide, a major component of oral malodor

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Topic of the manuscript:

For the first time we report our findings on the H_2S-degrading activity of a mixture of seven recombinant Sox enzymes from *Paracoccus pantotrophus* with respect to oral malodor prevention. Oral malodor is a common problem, but effective methods to

17 control it have not been established. Major malodorous components are volatile sulfur
18 compounds including H_2S. We show the possibility of applying bacterial Sox system
19 to oral malodor prevention.
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21 **Running headline:** *P. pantotrophus* rSox enzymes degrade H_2S
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25

Abstract

Hydrogen sulfide (H_2S) is emitted from industrial activities, and several chemotrophs possessing Sox enzymes are used for the removal of H_2S. In dental field, oral malodor is a common problem and major malodorous components are volatile sulfur compounds (VSCs), including H_2S and methyl mercaptan. *Paracoccus pantotrophus* is an aerobic, neutrophilic facultatively autotrophic bacterium that has sulfur-oxidizing (Sox) enzymes to use sulfur compounds as an energy source. In this study, we cloned Sox enzymes of *P. pantotrophus* GB17 and evaluated their VSC-degrading activity with respect to oral malodor prevention. Six genes, *soxX, soxY, soxZ, soxA*, *soxB*, and *soxCD*, were amplified from *P. pantotrophus* GB17. Each fragment was cloned into a vector for the expression of 6×His-tagged fusion proteins in *Escherichia coli*. Recombinant Sox (rSox) proteins were purified from whole-cell extracts of *E. coli* using nickel affinity chromatography. The enzyme mixture was investigated for the degradation of VSCs using gas chromatography. Each of the rSox enzymes was purified to apparent homogeneity, as confirmed by SDS-PAGE. The rSox enzyme mixture degraded H_2S in dose- and time-dependent manners. All rSox enzymes were necessary to degrade

H_2S. The H_2S-degrading activity of rSox enzymes was stable at 25-80℃, and the optimum pH was 7.0. The amount of H_2S produced by periodontopathic bacteria or oral bacteria collected from human subjects decreased after incubation with rSox enzymes. These results suggest that the combination of rSox enzymes from *P. pantotrophus* GB17 could be useful for the prevention of oral malodor.

Key words: hydrogen sulfide, oral bacteria, oral malodor, *Paracoccus pantotrophus*, sulfur-oxidizing enzyme

Introduction

Hydrogen sulfide (H_2S) is emitted from industrial activities, especially from biogas and protein-rich industrial wastes. H_2S has high toxicity and is very corrosive to internal combustion engines. Currently, many commercial chemical technologies are used for H_2S removal in industry (41). However, these chemical H_2S removal processes are expensive due to high chemical requirements, energy, and disposal costs, and they tend to have short-term effects. Biological treatment methods for H_2S removal are desirable, and several studies have investigated this (10, 32). The oxidation of H_2S is mediated by various aerobic lithotrophic and anaerobic phototrophic bacteria (35).

In dental fields, oral malodor, the presence of unpleasant or foul-smelling breath, is a common problem throughout the world and can cause significant social or psychological effects on those who are suffering from it (6, 15, 21). Oral malodor is a mixture of malodorous components, including volatile sulfur compounds (VSCs), such as H_2S, methyl mercaptan (CH_3SH), and dimethyl sulfide (6, 15). VSCs are produced in saliva, gingival crevices, on the tongue surface, and in other areas via the putrefactive activities of microorganisms on sulfur-containing amino acids, such as cysteine and

67 methionine (3, 6). H_2S and CH_3SH are primarily responsible for oral malodor and
68 comprise approximately 90% of the VSC content in mouth air (43).

69 VSCs also have adverse effects on oral tissues. It has been shown that H_2S
70 damages gingival epithelial cells (42), increases the permeability of oral mucosa *in vitro*
71 (24), and causes apoptosis in human gingival fibroblasts (2). H_2S was predominantly
72 detected in the pockets (27) associated with periodontal bacteria, including
73 *Fusobacterium nucleatum*, one of the most active oral bacteria to produce H_2S from
74 L-cysteine (26). Many reports have also demonstrated that the presence of CH_3SH is
75 involved in the induction or progression of periodontal disease. Exposure to CH_3SH
76 inhibits cell migration in periodontal ligament cells (18), epithelial cell growth and
77 proliferation (38). *Porphyromonas gingivalis*, a black-pigmented anaerobic bacterium
78 and a major pathogen in adult periodontitis, produces large amounts of CH_3SH in
79 human serum (26) from L-methionine.

80 There have been several attempts to prevent and reduce oral malodor (6, 40, 45).
81 Mechanical prophylaxis using a toothbrush and a tongue scraper is a basic method to
82 remove bacterial cells and substrates from the oral cavity, but the organisms still grow

and accumulate. Mouth rinses containing antimicrobial agents, such as chlorhexidine and cetylpiridinium chloride, have been used to reduce bacterial numbers, leading to a reduction in oral malodor (9, 47). Additionally, zinc ions have been used to reduce VSCs through their oxidizing effects on the thiol groups in VSC precursors (1, 16). However, the effects of specifically formulated mouth rinses for treating oral malodor are, in general, unclear (40). Therefore, new methods to control VSCs in the oral cavity should be established for the prevention of oral malodor.

The purpose of this study was to examine the possibility of applying sulfur-oxidizing (Sox) enzyme system to control oral malodor. We chose the sulfur-oxidizing bacterium, *Paracoccus pantotrophus*, which is an aerobic, Gram-negative, neutrophilic facultatively autotrophic bacterium that grows using thiosulfate or molecular hydrogen as an energy source and heterotrophically using a large variety of carbon sources (13). The Sox enzyme system in *P. pantotrophus* has been studied so far. The *sox* gene region of *P. pantotrophus* comprises 12 open reading frames and seven genes, soxXYZABCD, encode proteins essential for sulfur oxidation in vitro (34). The Sox proteins of *P. pantotrophus* are located in the

99 periplasm (12), and four proteins, SoxXA, SoxYZ, SoxB, and SoxCD, are required for
100 H_2S-, sulfur-, thiosulfate-, and sulfite-dependent horse cytochrome *c* reduction (34).
101 We report the cloning of *sox* genes from *P. pantotrophus* GB17 – *soxX, soxY, soxZ,*
102 *soxA, soxB*, and *soxCD* – and the characteristics of recombinant Sox (rSox) enzymes.
103 We investigated rSox enzymatic degradation of VSCs produced by periodontopathic
104 bacteria and oral bacteria.
105
106 **Materials and methods**
107 *Bacterial strains and culture conditions*
108 All strains were cultivated at 37°C. *P. pantotrophus* GB17 (NBRC 102493) was
109 obtained from NBRC (Kisarazu, Japan) and used throughout this study. Seed cultures
110 were grown aerobically in brain heart infusion (BHI; Becton, Dickinson and Company,
111 Sparks, MD, USA) broth supplemented with 4 mM magnesium sulfate. *Escherichia*
112 *coli* XL II was grown aerobically in Luria Bertani (LB; Difco Laboratories, Detroit, MI,
113 USA) medium. *P. gingivalis* W83 was grown anaerobically in GAM broth (Nissui
114 Medical Co., Tokyo, Japan) supplemented with hemin (5 μg mL^{-1}) and menadione (1 μg

115 mL^{-1}). *F. nucleatum* ATCC10953 was grown anaerobically in BHI broth

116 supplemented with 5 mg mL^{-1} yeast extract and 0.3 mg mL^{-1} cysteine-HCl. Ampicillin

117 (100 μg mL^{-1}) was added when appropriate.

118

119 *DNA manipulation*

120 Standard DNA recombinant procedures, such as DNA isolation, restriction

121 endonuclease digestion, ligation, transformation of competent *E. coli* cells, and agarose

122 gel electrophoresis, were carried out as described by Sambrook *et al*. (37).

123 Chromosomal DNA was isolated from *P. pantotrophus* GB17 cells using the Dr.

124 GenTLE (from yeast) High Recovery DNA extraction kit (Takara Bio Inc., Shiga,

125 Japan).

126

127 *DNA amplification*

128 To improve the fidelity of the PCR assay for *soxX, soxY, soxZ, soxA*, *soxB*, and *soxCD*

129 genes, we used Tks Gflex DNA polymerase (Takara Bio Inc.). The reaction mixture

130 (50 μL total) contained 25 μL of 2× Gflex PCR buffer (containing 2 mM of Mg^{2+} and

131 400 μM dNTPs; Takara Bio Inc.), 1 μL of Tks Gflex DNA polymerase (Takara Bio
132 Inc.), 0.01 nM each primer, 1 μL DNA template, and the volume was adjusted with
133 nuclease-free water (Roche Diagnostics, Indianapolis, IN). The reaction was carried
134 out for 30 cycles under the following conditions: initial denaturation at 94°C for 1 min,
135 denaturation at 98°C for 10 s, annealing at 48°C for 15 s, and extension at 68°C for 2.5
136 min.
137
138 *Cloning of sox genes from P. pantotrophus GB17*
139 Six *sox* genes (*soxX, soxY, soxZ, soxA*, *soxB*, and *soxCD*) were amplified by PCR from
140 the 5′-terminus of the *sox* gene (13 kbp): *soxX* fragment (474 bp), *soxY* fragment (423
141 bp), *soxZ* fragment (330 bp), *soxA* fragment (873 bp), *soxB* fragment (1695 bp), and
142 *soxCD* fragment (2431 bp). These short double-stranded DNAs were compatible with
143 a *Bam*HI site on the 5′-end and a *Hind*III site on the 3′-end. The primers were
144 designed to create *Bam*HI and *Hind*III restriction sites (underlined) within the PCR
145 product (Table 1). The pQE30 vector (Qiagen, Tokyo, Japan) was used for
146 construction of histidine-tagged recombinant proteins. Each DNA fragment,

147 containing a *sox* gene, was digested with *Bam*HI and *Hind*III and ligated into the pQE30
148 vector for the expression of 6×His-tagged fusion proteins. To obtain the Sox products
149 of *P. pantotrophus* GB17, *E. coli* XL II competent cells were transformed with each
150 resulting plasmid (*soxX, soxY, soxZ, soxA, soxB*, and *soxCD*). Positive colonies were
151 selected and re-plated on tryptic soy (TS; Becton, Dickinson and Company) agar
152 containing ampicillin (100 μg mL^{-1}). The nucleotide sequences of the inserted
153 fragments were confirmed by PCR to verify that the fragments were correct and did not
154 contain nucleotide substitutions or deletions. BLAST nucleotide sequence analysis
155 was also performed for DNA sequence identification.
156
157 *Preparation of recombinant Sox proteins*
158 The transformants were grown in LB medium with ampicillin (100 μg mL^{-1}) at 37°C
159 until the optical density at 550 nm (OD_{550}) reached 0.5.
160 Isopropyl-β-D(-)-thiogalactopyranoside was added to the culture at a final concentration
161 of 1 mM, and the cultures were grown for an additional 4 h. The cells were harvested
162 by centrifugation (5,000 × *g*, 15 min, 4°C) and lysed using an ultrasonic sonifier

163 equipped with a microtip (Model W-220-F; Heat Systems Ultrasonics Inc., Plainview,
164 NY, USA) on ice. The cell extract was obtained by centrifugation (10,000 × *g*, 15
165 min, 4°C) and subjected to Ni-NTA resin (Qiagen) affinity column chromatography.
166 The purification procedures followed the manufacturer's instructions, and the purity of
167 the recombinant Sox proteins was analyzed by SDS-PAGE. Eluted proteins were
168 refolded by sequential dialysis against a urea-decreasing phosphate buffer at 4°C (48).
169 The amounts of proteins were determined using the Lowry method with bovine serum
170 albumin as the standard (19).
171
172 *SDS-PAGE and Western blotting*
173 SDS-PAGE was performed using 15% polyacrylamide gels according to the method of
174 Laemmli (17). After electrophoresis, the gel was stained with Coomassie brilliant blue
175 R-250. The low molecular weight electrophoresis calibration kit (Amersham
176 Pharmacia Biotech, Uppsala, Sweden) was used for molecular mass markers. For
177 Western blotting, the proteins subjected to SDS-PAGE were transferred
178 electrophoretically to a nitrocellulose membrane according to the method of Burnette

(8). After blocking with 2% skimmed milk in Tris-buffered saline (20 mM Tris, 150 mM NaCl, pH 7.2) containing 0.1% Triton X-100 (TBS-T), the membrane was reacted with HRP-conjugated mouse anti-6×His monoclonal antibody (Wako Pure Chemical Industries Ltd., Osaka, Japan). The membrane was washed three times with TBS-T, and fluorescence detection was performed using a chemiluminescence detection system (Clarity western ECL blotting, Bio-Rad Laboratories, Inc., Hercules, CA).

Enzyme assay

We investigated the ability of a rSox enzymes mixture reconstituted from rSoxX, rSoxY, rSoxZ, rSoxA, rSoxB, and rSoxCD to degrade H_2S generated from NaHS. The assay mixture (1 mL) for the determination of H_2S-degrading activity contained various amounts of *P. pantotrophus* GB17 cells or rSox enzymes and 20 nmol of sodium hydrogen sulfide (NaHS) to generate H_2S in 10 mM phosphate buffer (pH 7.0). All the reactions were performed in sterile 15-mL polypropylene tubes sealed with a silicone plug. The NaHS was added to start the reaction. After incubation for 2 h at 37°C, a sample (2.5 mL) of the vapor above the assay mixture in the tube was removed

using a gas-tight syringe and analyzed by gas chromatography (model GC-2014; Shimadzu Corp., Kyoto, Japan) using a glass column packed with 25% β, β'-oxydipropionitrile on a 60-80-mesh Chromosorb W AW-DMCS-ST device (Shimadzu Corp.) fitted with a flame photometric detector at 70°C. The concentration of VSCs was determined using standard H_2S and CH_3SH gas prepared with a Permeater PD-1B (Gastec, Ayase, Japan).

The assay mixture (1 mL) used to determine the rSox enzyme activity to degrade VSCs produced by periodontopathic bacteria were prepared following the method of Yoshimura *et al*. (49). Briefly, bacterial strains were grown at 37°C until an OD_{550} of about 0.6 was reached. The cells were harvested and washed three times with a buffered salt solution (40 mM potassium phosphate buffer, 50 mM NaCl, pH 7.7). The cells were suspended in the buffered salt solution to an OD_{550} of 0.3 for *F. nucleatum* ATCC10953 and OD_{550} of 1.0 for *P. gingivalis* W83. To determine H_2S, a reaction mixture was prepared consisting of 100 μL of *F. nucleatum* ATCC10953 or *P. gingivalis* W83 cell suspension and 0.5 nmol each of rSoxX, rSoxY, rSoxZ, rSoxA, rSoxB, and rSoxCD, and buffered salt solution was added to a tube and sealed with a

silicone plug. The reaction was initiated by adding 30 μL of 33 mM L-cysteine. For the determination of CH_3SH, 30 μL of 33 mM L-methionine were added instead of L-cysteine. The assay mixtures were kept at 37°C and after a 2-h incubation, the reactions were stopped by adding 500 μL of 3 M phosphoric acid. Then, 10 min later, a sample (2.5 mL) of the vapor above the assay mixture was analyzed by gas chromatography, as described above.

The assay mixture used to determine the degrading activity of H_2S produced by oral bacteria was prepared as described by Tonzetich and Johnson (44) with modifications. Paraffin-stimulated whole saliva was collected from three healthy and non-smoking participants (28-58 years of age), which was approved by the ethics committee of Kagoshima University (authorization number 572). The bacterial sediment was collected by centrifugation (10,000 × *g*, 15 min, 4°C) and washed three times with buffered salt solution. The bacterial sediment was disrupted by ultrasonication on ice and suspended in the buffered salt solution to an OD_{550} of 1.0 to measure the extracellular H_2S produced from L-cysteine by oral bacteria. The reaction mixture (3 mL) consisted of 2 mL bacterial suspension and 0.5 nmol each of rSoxX,

rSoxY, rSoxZ, rSoxA, rSoxB, and rSoxCD in buffered salt solution. The subsequent procedures were as described above.

To evaluate heat stability, rSox enzymes were mixed and heated at 25 to 100°C for 30 min and subjected to the H_2S-degrading assay. To examine the effect of pH on rSox enzyme activity, 10 mM phosphate buffer with pH 4 to 9 was prepared.

Statistical analysis

Data were averaged for three independent experiments. Statistical analysis was performed using Student's *t*-test or one-way analysis of variance (ANOVA) followed by Dunnett's test. *P*-values < 0.05 were considered to indicate statistical significance.

Results

Purification of recombinant Sox proteins

Figure 1A shows purified recombinant proteins. rSoxX migrated at 23 kDa; rSoxY at 18 kDa; rSoxZ at 21, 22, and 43 kDa; rSoxA at 43 kDa; and rSoxB at 68 kDa (Fig. 1A). In Western blot analysis, the anti-6×His antibody reacted with the rSoxX, rSoxY,

243 rSoxZ, rSoxA, and rSoxB proteins (Fig. 1B). rSoxCD was separated into two bands
244 (51 and 54 kDa) by SDS-PAGE, and the 54-kDa band reacted with the anti-6×His
245 antibody. Because the 6×His was attached to rSoxC, we suggest that the 51-kDa band
246 was rSoxD and the 54-kDa band rSoxC. rSoxZ showed three bands and the antibody
247 to 6×His reacted with all of these components, so we suggest that the 43-kDa band was
248 a dimer, and the 21-kDa band lacked some portion of rSoxZ (the 22-kDa band).
249
250 P. pantotrophus *GB17 activity to degrade H_2S*
251 Whole cells of *P. pantotrophus* GB17 were examined for degradation of H_2S generated
252 from NaHS. *P. pantotrophus* GB17 cells showed H_2S-degrading activity, and the H_2S
253 concentration decreased in a cell number-dependent manner (Fig. 2). Approximately
254 50% of H_2S was degraded when a bacterial suspension containing 1.0×10^7 cells was
255 used.
256
257 *rSox enzyme activity to degrade H_2S*

A combination of the six rSox proteins (rSoxX, rSoxY, rSoxZ, rSoxA, rSoxB, and rSoxCD) was investigated for its ability to degrade H_2S generated from NaHS. After incubation, the rSox enzymes mixture showed degradation of H_2S in a dose-dependent manner (Fig. 3A). The concentration of H_2S decreased significantly and reached a stable level after 0.125 nmol of proteins were used. In time-course experiments, the ratio of H_2S concentration (sample/control) decreased to approximately 45% at 2-h incubation and was stable thereafter (Fig. 3B). In the thermal stability study, the amount of H_2S in the assay mixture increased gradually as the temperature was raised (Fig. 4A). However, the amount of H_2S was still significantly lower than that in the control even after heating at 80°C. After heating at 100°C, there was no difference in the amount of H_2S compared with the control. Regarding the effect of pH on rSox enzyme activity, the percentage of H_2S concentration (sample/control) was lowest at pH 7.0, and higher values were observed at other pH values (Fig. 4B).

Effect of each component of the rSox enzyme mixture on H_2S degradation

Table 2 shows the effects of the components of the rSox enzyme mixtures on degradation of H_2S. In this experiment, the rSoxX and rSoxA or rSoxY and rSoxZ pairs were omitted from the assay, because those pairs of enzymes work together in the Sox system (12, 34). When a mixture of rSox enzymes reconstituted from all rSox enzymes was used, the H_2S level decreased significantly compared with the control (no rSox enzymes). In the other cases, omitting certain rSox enzymes from the assay, H_2S levels were not significantly different from the control, and the H_2S ratios relative to control levels were approximately 70%. Bovine serum albumin, which was used as a non-sulfur-oxidizing control, showed no reducing effect of H_2S.

rSox enzyme activity to degrade VSCs produced by periodontopathic bacteria and oral bacteria

We first determined the ability of rSox enzymes to degrade H_2S produced by *F. nucleatum* ATCC10953 and *P. gingivalis* W83. The rSox enzyme mixture significantly degraded the H_2S produced by the two bacterial strains (Table 3). We also determined CH_3SH degradation by the rSox enzyme mixture and found that it

289 could decrease the CH_3SH level, but that the difference was not significant compared
290 with the control (no enzymes). Next, oral bacteria were collected from human saliva
291 and used to produce H_2S. The rSox enzyme mixture significantly degraded H_2S
292 produced from the oral bacteria from all subjects, although the degree differed among
293 the subjects (Table 4).
294

295 **Discussion**

296 In this study, we firstly demonstrated the degradation of H_2S by the rSox enzymes from
297 *P. pantotrophus* GB17 in view of oral malodor prevention. We produced rSox
298 enzymes to examine their VSC-degrading activity. We first attempted to construct
299 rSoxXA, rSoxYZ, rSoxB, and rSoxCD proteins, but only rSoxB and rSoxCD proteins
300 were obtained successfully. Thus, we next constructed each enzyme as rSoxX, rSoxY,
301 rSoxZ, and rSoxA. We demonstrated that the combination of rSoxX, rSoxY, rSoxZ,
302 rSoxA, rSoxB, and rSoxCD proteins degraded H_2S, and the activity was almost constant
303 after reaching the maximum effect (Fig. 3A). Regarding the use of recombinant Sox
304 enzymes, rSoxXA expressed in *E. coli* was found to be functional in the reconstituted

enzyme system to activate H_2S-dependent cytochrome *c* reduction (35). This report is the first to describe a Sox system composed of each recombinant Sox protein for degrading H_2S.

High cytochrome *c*-reducing activity by the Sox system was observed using H_2S as the substrate (34), and to achieve optimal activity, SoxXA, SoxYZ, SoxB, and SoxCD were required (12, 34). In the present study, we demonstrated that all of the enzymes – rSoxX, rSoxY, rSoxZ, rSoxA, rSoxB, and rSoxCD – were necessary for H_2S degradation (Table 2). The central protein of the Sox enzyme system is SoxYZ, to which the sulfur substrate is covalently linked, oxidized, and finally released as sulfate by three other Sox proteins (11). SoxXA binds the sulfur substrate and covalently links it to the thiol of the single cysteine 110 residue of the SoxY subunit. SoxY forms a complex with SoxZ. The outer sulfur atom is oxidized by sulfur dehydrogenase SoxCD, yielding SoxY-cysteine-S-sulfate. Finally, SoxY-cysteine-S-sulfate is hydrolyzed by the dimanganese SoxB protein to yield sulfate and to regenerate SoxY for a new reaction cycle of SoxYZ (13, 36). When SoxB or SoxYZ was omitted from the assay mixture, approximately 12 or 9%, respectively, residual H_2S-dependent

activities were observed compared with the mixture of SoxXA, SoxYZ, SoxB, and SoxCD (34). SoxCD was also required to achieve the maximum rate of H_2S-dependent cytochrome *c* reduction, showing a residual rate of 19% when SoxCD was omitted, compared with the mixture of all Sox enzymes. The results obtained in this study are consistent with those previous results.

In *P. pantotrophus* GB17, each *sox* gene encodes a protein with a different molecular mass: SoxX, 16,421 Da; SoxY, 13,830 Da; SoxZ, 11,850 Da; SoxA, 31,884 Da; SoxB, 61,897 Da; SoxC, 43,442 Da; and SoxD, 37,637 Da (12). In this study, the estimated molecular masses of rSox enzymes by SDS-PAGE were larger than those predicted by the nucleotide sequences (Fig. 1A). All rSox proteins, except rSoxD, had a 6×His tag, but the differences observed were larger than the size of 6×His (approximately 1.2 kDa). Previous reports (12, 29) also showed that the purified Sox proteins had different molecular masses from predicted ones (SoxX, 16 kDa; SoxY, 12 kDa; SoxZ, 16 kDa; SoxA, 29 kDa; SoxB, 59 kDa; SoxC, 47 kDa; and SoxD, 50 kDa) under denaturing SDS-PAGE. Friedrich *et al*. (12) reported that the molecular mass of SoxY determined by SDS-PAGE differed from that predicted by the nucleotide

337 sequence due to an unknown reason. Furthermore, Quentmeier *et al*. (29) also found
338 that the SoxC and SoxD proteins had different molecular masses determined by
339 SDS-PAGE versus those deduced from the nucleotide sequence of the mature protein.
340 Mobility on SDS-PAGE gels may be affected by amino acid composition,
341 glycosylation, or phosphorylation of the protein. In this study, the amino acid
342 composition and predicted pI value were obtained using the Compute pI/Mw tool in
343 ExPASy. None of the rSox enzymes seemed to have a unique amino acid
344 composition, and the pI value was between 4.5 and 6.6. These characteristics do not
345 seem to affect the mobility of the proteins on SDS-PAGE gels. BLAST nucleotide
346 sequence analysis showed the identities of all of the *sox* genes of *P. pantotrophus* GB17
347 used in this study, so the reason for the differences in mass observed remains unclear.
348 The optimal pH for the H_2S-degrading activity of rSox enzymes was 7.0, and the
349 activity was stable at 25-80°C. *P. pantotrophus* was isolated as *Thiosphaera*
350 *pantotropha* originally (33), was reclassified as *Paracoccus denitrificans* (20), and then
351 renamed (31). *T. pantotropha* GB17 obtains energy through the Sox system and can
352 grow at pH 6.5-10.5, with an optimum pH of 8.0, and at a temperature of 15-42°C, with

an optimum at 37°C (33). Another report showed optimal growth conditions of *P. denitrificans* at pH 7.5-8.0 and 30-37°C (11). These findings suggest that the Sox system functions under these pH and temperature conditions, which seem reasonable. We also found that whole cells from *P. pantotrophus* GB17 could degrade H_2S even after heating at 100°C (data not shown). Recently, thermophilic sulfur-oxidizing bacteria have been isolated from geothermal fields (25, 39). Although it is not clear whether these bacteria contain the Sox system, Sox enzymes might have thermophilic properties. Indeed, Ghosh *et al*. (14) suggested that the Sox system originated in ancient thermophilic bacteria and evolved through extensive horizontal gene transfer. Further studies are necessary to clarify the mechanism of the thermal stability of the rSox enzymes. In possible clinical applications, heat stability of rSox enzymes would be advantageous, because the intra-oral temperature changes after ingestion of various cold/hot meals and beverages (4, 22).

In this study, we demonstrated that rSox enzymes significantly degraded H_2S produced by periodontopathic bacteria. The reducing effect of rSox enzymes in reaction mixtures with *P. gingivalis* or *F. nucleatum* was smaller than that obtained in

369 reaction mixtures with NaHS. We examined the effect of rSox enzymes on the growth
370 of these bacterial cells and found no significant effects (data not shown). rSox
371 enzymes also showed potential to decrease the CH_3SH produced by *P. gingivalis*. The
372 Sox system, reconstituted from SoxXA, SoxYZ, SoxB, and SoxCD, mediates
373 thiosulfate-, sulfite-, sulfur-, and H_2S-dependent cytochrome *c* reduction(12, 34), but no
374 reports have demonstrated the CH_3SH-dependent reduction. The availability of
375 CH_3SH as a substrate for Sox system needs to be clarified in further studies. We also
376 demonstrated that H_2S produced by oral bacteria collected from human saliva decreased
377 after incubation with rSox enzymes. These results indicate the potential of rSox
378 enzymes to reduce the H_2S produced in the oral cavity.
379 Many randomized controlled trials have reported reducing effects of mouth
380 rinses containing chlorhexidine, cetylpyridinium chloride, or metal ions on oral
381 malodor, while several studies have reported no significant effects (7, 30, 40).
382 Systematic reviews have reported that available data are insufficient to determine
383 precision (5) and concluded that the strength of the recommendation to use these
384 ingredients for oral malodor reduction is graded to be ‘weak’ (40). It is unclear

whether the Sox enzyme-reducing effect on H_2S is superior to existing components or not. Chlorhexidine and cetylpyridinium chloride act to suppress the bacterial growth and they may be accompanied by several adverse effects on oral tissues. The Sox system seems to be advantageous in this point because the target of it is not oral bacteria or oral tissues but H_2S produced by oral bacteria. Furthermore, many studies have reported that H_2S participates in physiological regulation in human body (28, 46, 50). The Sox system has potential to be an effective agent for controlling body systems as H_2S regulator.

For the final goal of clinical application of this enzyme system, more data are necessary. Toxicity of Sox enzyme system should be examined and stability of rSox enzymes against proteases present in saliva is also required. Maximum effect of the rSox enzymes mixture appeared 2 h after reaction started (Fig. 3B). In this study, we examined the rSox enzymes activity using assay mixtures in the tube sealed with a silicon plug. Since many components including type and amount of sulfur source, pH of the oral fluids, salivary flow rate, and oxygen levels in saliva and dental plaque affect VSC formation in mouth (23), it is unclear how long it will take to obtain the maximum

effect of the rSox enzymes mixture in the oral cavity. We expect the reducing effect of the rSox enzymes to last for long time as possible. The effect lasted for 4 h in the present assay, but it is also unclear how long the effect will last in the oral environment. Further studies are necessary to clarify these points before clinical application.

In conclusion, we found that the combination of seven rSox enzymes from *P. pantotrophus* GB17 has H_2S-reducing activity. The results suggest that the rSox system could be useful for the prevention of oral malodor.

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Conflict of Interest

No conflict of interest declared.

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558

Table 1. Oligonucleotide primers used in this study

Fragment	Primer	Sequence (5′ to 3′)[a]
rSoxX	rSoxX-Forward	AGGGATCCATGAGCAGCCATCTATGG
	rSoxX-Reverse	CTAAGCTTGTCGAGCCTGTAGAGATC
rSoxY	rSoxY-Forward	AGGGATCCATGATCCTTTCAAGACGC
	rSoxY-Reverse	GCAAGCTTAATCTCCTGTTACTGGAC
rSoxZ	rSoxZ-Forward	AGGGATCCATGGCAGATGATGCAAAG
	rSoxZ-Reverse	TGAAGCTTGATGTTGCGGCGCTTAGG
rSoxA	rSoxA-Forward	GAGGATCCATGCCGCGCTTTACCAAG
	rSoxA-Reverse	GGAAGCTTGAAGCATTGCCCTTTCGA
rSoxB	rSoxB-Forward	ACGGATCCATGATTACCCGACGTGAG
	rSoxB-Reverse	CAAAGCTTAACGCTCCTTCGTGATTG
rSoxCD	rSoxCD-Forward	GTGGATCCATGAAAGACGAGCTCACC
	rSoxCD-Reverse	AGAAGCTTCTGTCTCATGCGTCACTT

[a] Nucleotides underlined in each primer sequence show the position of the restriction endonuclease site incorporated to facilitate cloning.

Table 2. Effect of each rSox enzyme on degradation of H_2S generated from NaHS

Sox enzymes added to the assay							H_2S (ng/ml)[a]	Ratio to
X	A	Y	Z	B	CD	BSA[b]		control (%)
-	-	-	-	-	-	-	16.9 ± 3.8	
+	+	+	+	+	+	-	6.4 ± 1.1*	37.9
-	-	+	+	+	+	-	11.9 ± 2.5	70.4
+	+	-	-	+	+	-	11.5 ± 2.1	68.0
+	+	+	+	-	+	-	12.0 ± 4.1	71.0
+	+	+	+	+	-	-	12.0 ± 1.5	71.0
-	-	-	-	-	-	+	17.2 ± 4.7	101.8

To determine the role of each rSox component, an enzyme mixture was prepared as shown in the table. Prepared rSox enzyme mixture (0.125 nmol each) was reacted with 20 nmol of NaHS for 2 h at 37°C.

[a] Values are the means ± SDs of three independent experiments.

[b] BSA, bovine serum albumin (0.125 nmol) was used as a non-sulfur-oxidizing control.

*$P < 0.05$ compared with the control (without rSox enzymes or BSA), as determined by ANOVA followed by Dunnett's test.

559

Table 3. rSox enzyme activity to degrade VSCs produced by periodontopathic bacteria

Bacterial strain	H_2S (ng/ml)[a]		CH_3SH (ng/ml)[a]	
	rSox enzymes		rSox enzymes	
	(-)	(+)	(-)	(+)
F. nucleatum ATCC10953	26.7 ± 2.2	12.1 ± 1.9*	ND	ND
P. gingivalis W83	19.6 ± 4.5	9.1 ± 3.5*	22.0 ± 2.9	15.9 ± 1.8

[a] Values are the means ± SDs of three independent experiments.

*$P < 0.05$ compared with the control (without rSox enzymes), as determined by Student's *t*-test.

ND, not determined.

560

Table 4. rSox enzyme activity to degrade H_2S produced by oral bacteria

Subject	H_2S (ng/ml)[a]		Ratio (%)
	Control	rSox enzymes	(rSox/Control)
A	23.8 ± 3.8	13.3 ± 4.7*	55.9
B	22.3 ± 6.3	6.5 ± 0.7*	29.1
C	8.1 ± 1.1	2.7 ± 0.2*	33.3

[a] Values are the means ± SDs of three independent experiments.

*$P < 0.05$ compared with the control (without rSox enzymes), as determined by Student's *t*-test.

561

562 **Figure legends**

563

564 **Fig. 1** SDS-PAGE (A) and Western blotting (B) analyses of purified rSox proteins.

565 (A) rSox proteins were suspended in SDS-PAGE reducing buffer (1% SDS, 1%

566 2-mercaptoethanol) and heated at 100°C for 3 min. Samples were subjected to

567 SDS-PAGE (15% polyacrylamide), and the gel was stained with Coomassie brilliant

568 blue R-250. Lanes: 1, molecular mass markers; 2, rSoxX (3 μg); 3, rSoxY (3 μg); 4,

569 rSoxZ (3 μg); 5, rSoxA (3 μg); 6, rSoxB (3 μg); 7, rSoxCD (6 μg). (B) rSox proteins

570 on the gel were transferred electrophoretically to a nitrocellulose membrane, and the

571 membrane was reacted with an antibody against 6×His. Lanes: 1, rSoxX (1.5 μg); 2,

572 rSoxY (1.5 μg); 3, rSoxZ (1.5 μg); 4, rSoxA (1.5 μg); 5, rSoxB (1.5 μg); 6, rSoxCD (3

573 μg).

574

575 **Fig. 2** *P. pantotrophus* GB17 activity to degrade H_2S generated from NaHS.

576 Various amounts of cell suspension of *P. pantotrophus* GB17 were incubated with 20

577 nmol NaHS for 2 h at 37°C. Values are the means ± SDs of three independent

578 experiments. *$P < 0.05$ compared with the control (no *P. pantotrophus* GB17 cells),

579 as determined by ANOVA followed by Dunnett's test.

580

581 **Fig. 3** rSox enzyme activity to degrade H_2S generated from NaHS. (A) Various

582 amounts of rSox enzyme mixture were reacted with 20 nmol NaHS for 2 h at 37°C.

583 Values are the means ± SDs of three independent experiments. *$P < 0.05$ compared

584 with the control (no rSox enzymes), as determined by ANOVA followed by Dunnett's

585 test. (B) rSox enzyme mixture (0.125 nmol each enzyme) was reacted with 20 nmol

586 NaHS for 1 to 4 h at 37°C. The assay was carried out with (sample) or without

587 (control) the rSox enzyme mixture. Values are the means ± SDs of three independent

588 experiments.

589

590 **Fig. 4** Heat stability of rSox enzymes (A) and effect of pH on the H_2S-degrading

591 activity of rSox enzymes (B). (A) After rSox enzyme mixture (0.125 nmol each

592 enzyme) was treated at 25 to 100°C for 30 min, the samples were reacted with 20 nmol

593 NaHS for 2 h at 37°C. Open bar, control (no rSox enzymes); solid bars, rSox

enzymes. Values are the means ± SDs of three independent experiments. *$P < 0.05$ compared with the control, as determined by ANOVA followed by Dunnett's test. (B) The assay was carried out with (sample) or without (control) rSox enzyme mixture (0.125 nmol each enzyme) in 10 mM phosphate buffer (pH 4 to 9) containing 20 nmol NaHS for 2 h at 37°C. Values are the means ± SDs of three independent experiments.

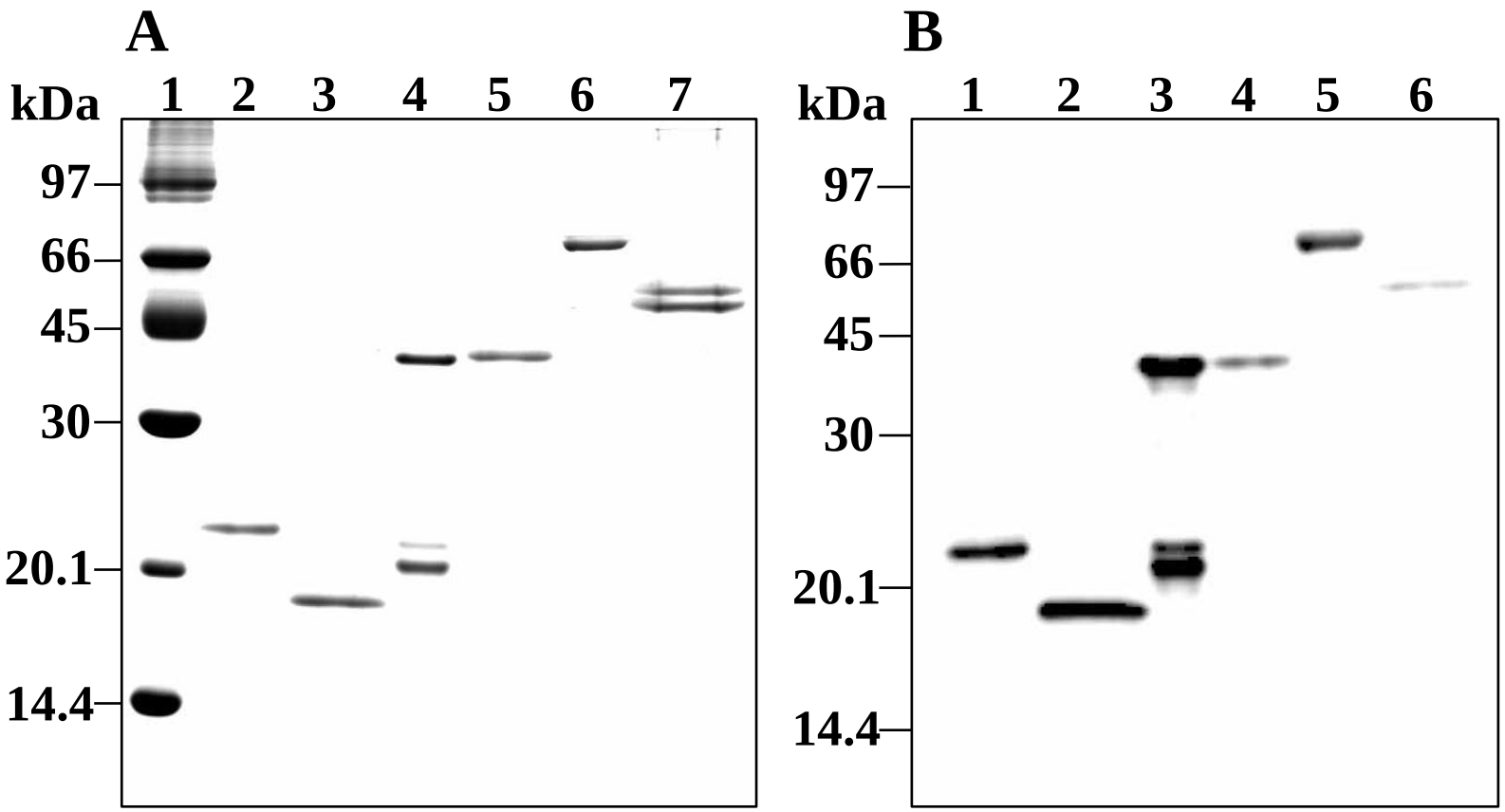

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Fig. 1, Ramadhani

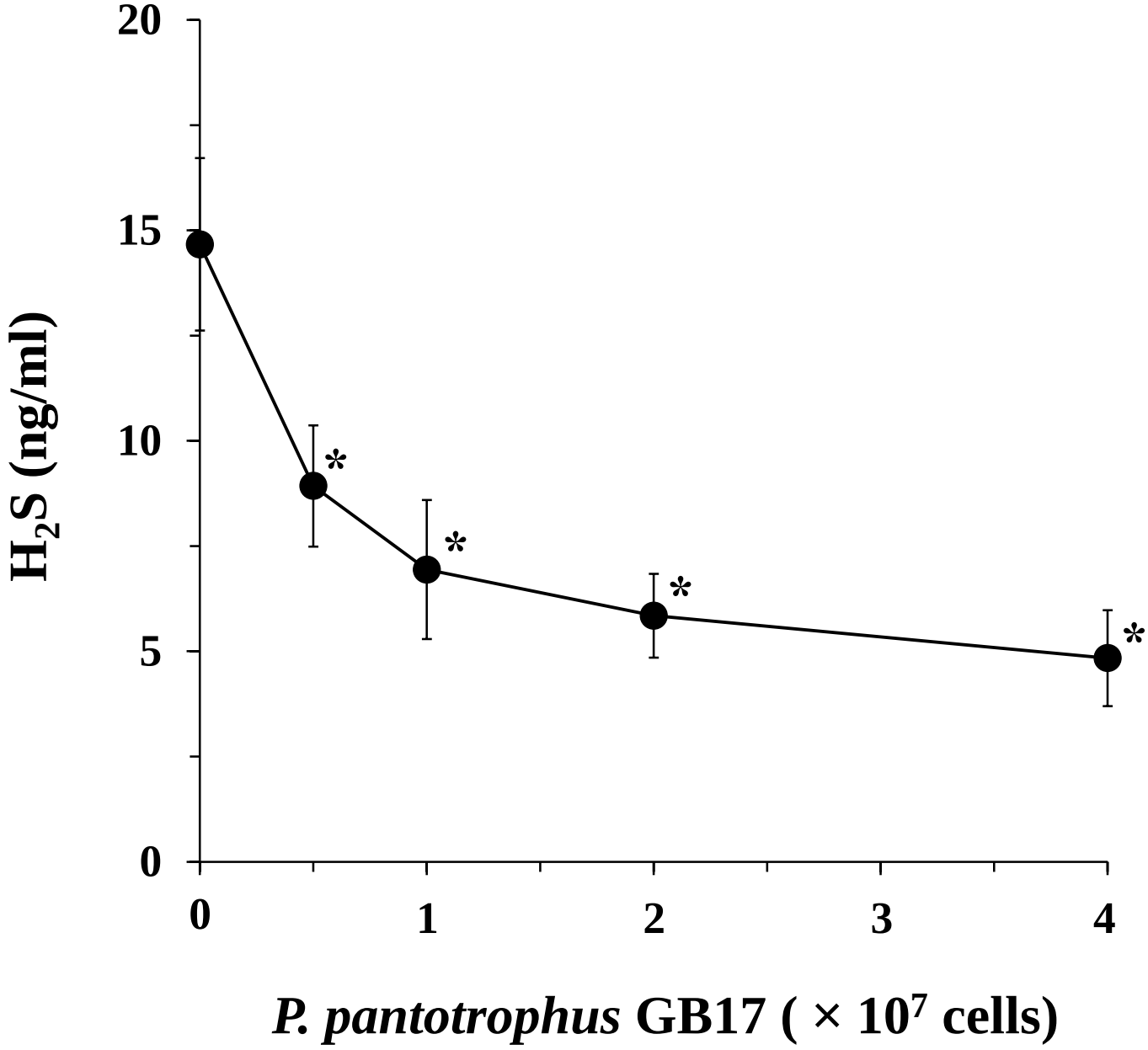

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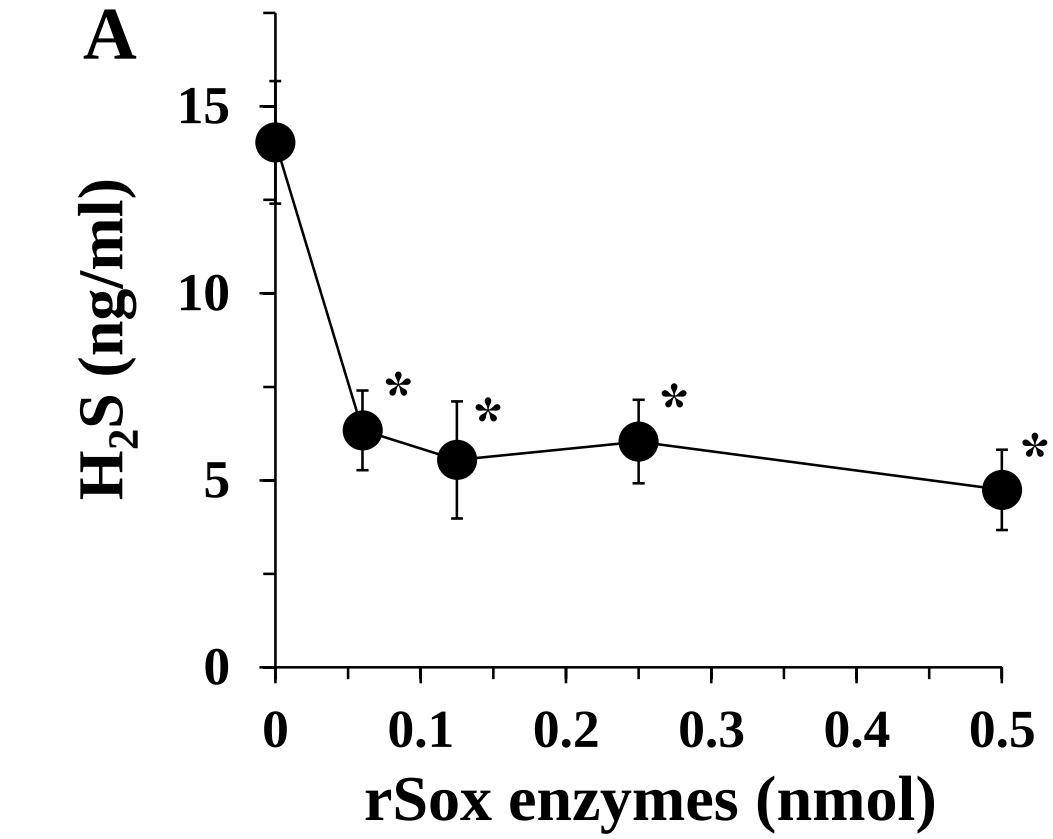
Fig. 2, Ramadhani



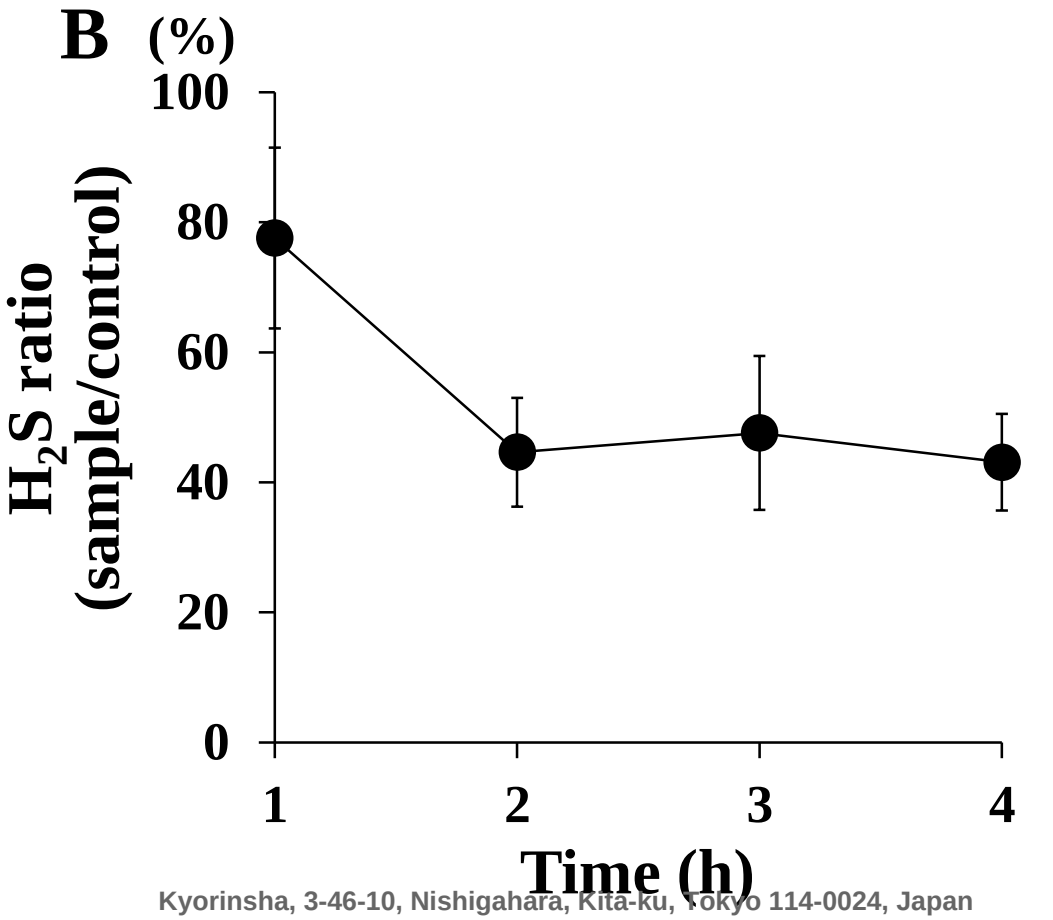
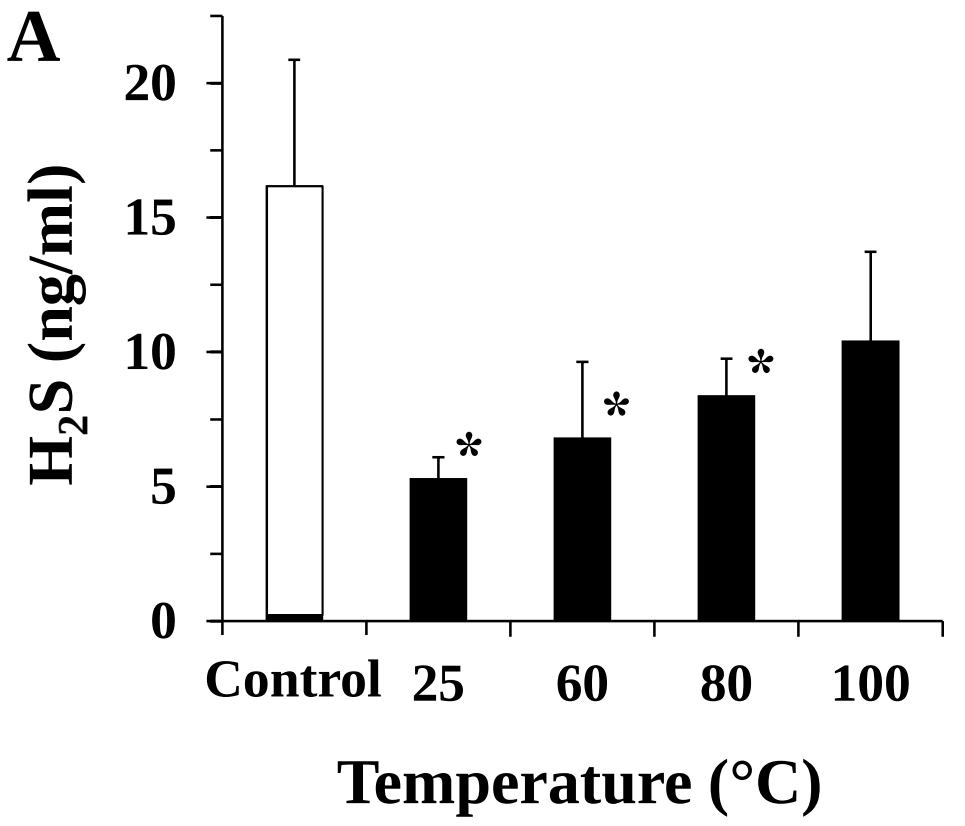


Fig. 3, Ramadhani



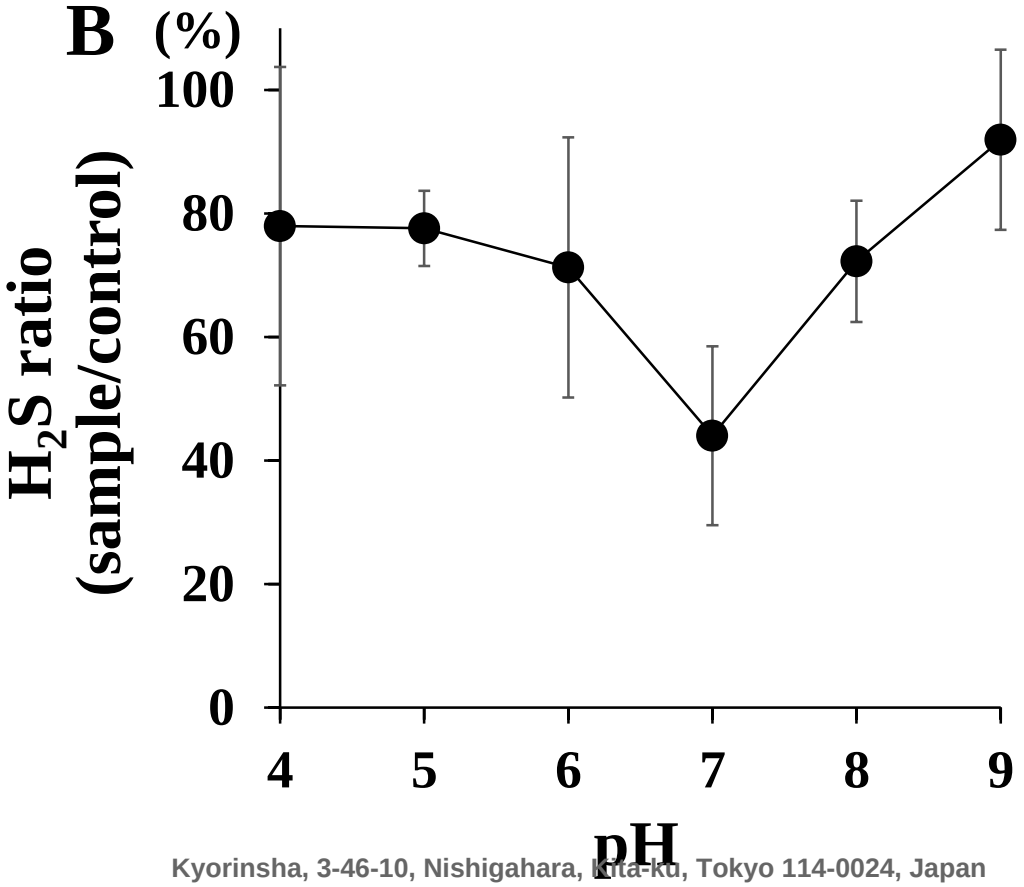


Fig. 4, Ramadhani