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A novel sulfate-reducing bacteria detection method based on inhibition of cysteine protease activity

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ABSTRACT

Sulfate-reducing bacteria (SRB) have been extensively studied in corrosion and environmental science. However, fast enumeration of SRB population is still a difficult task. This work presents a novel specific SRB detection method based on inhibition of cysteine protease activity. The hydrolytic activity of cysteine protease was inhibited by taking advantage of sulfide, the characteristic metabolic product of SRB, to attack active cysteine thiol group in cysteine protease catalytic sites. The active thiol *S*-sulfhydration process could be used for SRB detection, since the amount of sulfide accumulated in culture medium was highly related with initial bacterial concentration. The working conditions of cysteine protease have been optimized to obtain better detection capability, and the SRB detection performances have been evaluated in this work. The proposed SRB detection method based on inhibition of cysteine protease activity avoided the use of biological recognition elements. In addition, compared with the widely used most probable number (MPN) method which would take up to at least 15 days to accomplish whole detection process, the method based on inhibition of papain activity could detect SRB in 2 days, with a detection limit of 5.21×10^2 cfu mL^{-1}. The detection time for SRB population quantitative analysis was greatly shortened.

1. Introduction

Sulfate-reducing bacteria (SRB) are a large group of anaerobic microorganisms that use sulfate as a terminal electron acceptor for growth. They are widely distributed in anoxic habitats, such as oilfield waters and offshore sediments, where they have an important role in both the sulfur and carbon cycles [1,2]. SRB have been known as one of the key microorganisms in microbiological induced corrosion (MIC) [3,4], and the presence of SRB could cause severe environmental and industrial problems. On the other hand, this kind of pathogen has been found very useful in waste water treatment, and numerous reports have reported the possibility of taking advantage of SRB for sulfate and heavy metals removal [5–7]. Thus, establishment of selective and sensitive SRB detection method is crucial for corrosion analysis and environmental monitoring.

SRB population quantitative analysis is still relied mainly on conventional the most probable number (MPN) method at present. It involves a pre-enrichment step in SRB selective culture media followed by a biochemical test, and its detection principle is based on formation of black FeS precipitates by the reaction between ferrous ion and accumulated sulfide [8,9]. The distinct advantage of MPN method is low detection limit. Nevertheless, the sophisticated series of procedures would take up to at least 15 days to accomplish. A variety of other protocols have been developed for SRB detection, including biochemical test [10], enzyme-linked immunosorbent assays [11], molecular biotechniques [12], and biosensors [13–17]. These methods exhibited high detection selectivity by utilizing biomaterials, such as antibody, lectin, antibiotic and aptamer, as biorecognition elements, and bacterial detection time could be greatly shortened. However, these biorecognition elements are prone to lose their activity if not stored or used properly, and non-specific binding events of these biomaterials are inevitable in a detection process, which would affect the accuracy and reproducibility of bacterial detection. In addition, to date, more than 30 genera of SRB have been reported, and it is impossible to apply one kind of antibody, lectin or aptamer in recognition of SRB from different genera, different species or different strains of the same species.

During SRB growth, sulfide is the characteristic metabolic product metabolized by reducing sulfate in degradation of organic compounds. Much attention has been paid on sulfide for its toxicity and potential risks for human and environmental health.

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Exposure to concentrations as low as 10 ppm would lead to physical stress and at concentrations higher than 250 ppm it may cause death [18]. However, hydrogen sulfide has been recently recognized as the third gaseous messenger molecular (the other two are nitric oxide and carbon monoxide) that displays many physiological and pathological activities [19–23]. For example, hydrogen sulfide could moderate synaptic activity by improving the activity of *N*-methyl-D-aspartate receptors in neurons and activating astrocytes surround the synapse [24,25]. Vascular smooth muscle could be relaxed by hydrogen sulfide by activating K^+ channels [26]. In addition, hydrogen sulfide could also regulate insulin release, endoplasmic reticulum stress, and inflammation process [27–30]. The main hydrogen sulfide signaling model was through *S*-sulfhydrating the reactive cysteine residues of target proteins. During the *S*-sulfhydration process, the sulfhydryl group of cysteine would be modified to an –SSH group [22], and the protein activity would be affected as a result. Since sulfide is the characteristic bacterial metabolite of SRB, this has inspired us if we could establish a novel SRB detection method based on regulation of protein activity.

Cysteine proteases are proteolytic enzymes that involve an active cysteine in the catalytic sites. This kind of protease could cleave protein via nucleophilic attack on the carbonyl group of a peptide bond. Cysteine proteases are widely present in living organisms, and they are well-known in plant growth and development [31]. Recently, other applications have been explored in inhibition of bacterial attachment and growth [32,33], drug delivery [34], biosensors [35], etc. Since cysteine residue is an important component of the catalytic sites in cysteine protease, the protease activity would be greatly influenced if its sulfhydryl group be *S*-sulfhydrated to an –SSH group. Hence, in our research, cysteine protease was chosen to be *S*-sulfhydrated by SRB metabolic sulfide, and we proposed to explore a novel SRB detection method based on regulation of cysteine protease activity.

However, from a chemical viewpoint, directly conduct the cysteine protease *S*-sulfhydration process by sulfide is unfavorable, since both sulfide and the thiol group of reactive cysteine residue are particularly nucleophilic. It has been reported that the sulfane sulfur of persulfide is a reactive electrophile, and it is capable of transferring its sulfane sulfur to the protease active thiol [36]. Therefore, to conduct the cysteine protease *S*-sulfhydration process more efficiently, SRB metabolic sulfide was activated by oxidized glutathione (GSSG) before *S*-sulfhydration reaction, the resulting glutathione persulfide (GSSH) could easily attack the protein thiol to generate protein persulfide [37]. The persulfide modification of active thiol residue would inhibit protease activity largely.

In this research, we developed a novel and universal SRB detection method based on inhibition of cysteine protease activity. SRB were incubated in modified Postgate's culture at 30 °C to accumulate sulfide, the characteristic metabolite of SRB. GSSG was then introduced to active the accumulated sulfide, and the resulting GSSH could effectively attack the active thiol group of cysteine protease and thus inhibited the catalytic activity of protease. Since the amount of sulfide accumulated in culture media was highly related with the initial bacterial concentration of SRB, the cysteine protease *S*-sulfhydration process could be used for SRB detection. The procedures of SRB detection method based on inhibition of cysteine protease were schematized in Scheme 1.

2. Experimental section

2.1. Chemicals

Papain, a cysteine protease of the peptidase C1 family, was purchased from Solarbio Science and Technology Co., Ltd., Beijing, China. The catalytic triad of cysteine protease papain consists of cysteine-25, histidine-159, and asparagine-175. Papain was activated by incubated with 5 mM cysteine and 2 mM disodium EDTA before use. All other chemicals were obtained from Sinopharm Chemical Reagent Co., Ltd., Shanghai, China, and used without further purification. Magnesium sulfate, ammonium chloride, sodium sulfate, dipotassium hydrogen phosphate, calcium chloride, sodium hydroxide, sodium lactate and yeast exact were used to prepare the modified Postgate's culture medium. Milli-Q water (Millipore Co., Billerica, MA, USA) was used throughout.

2.2. Bacterial cultivation

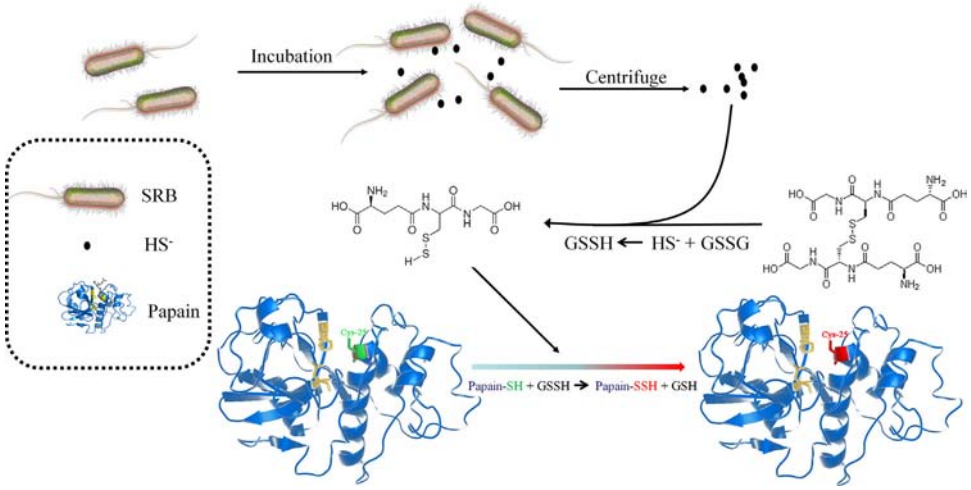
Seed SRB bacteria affiliated to *Desulforibrio caledoiensis* genus were isolated from marine bud of Bohai Sea, China. The bacterial cultivation and enumeration methods have been reported in our previous work [38,39]. Bacteria were cultivated in the modified Postgate's medium at 30 °C and visible bacterial number was determined according to the American Society of Testing Materials Standard D4412-84. A series of SRB decimal dilutions was cultivated in selective culture mediums at 20 °C for 21 days, and presence of SRB was indicated by producing FeS black precipitate. The initial bacterial population could be concluded by combining the degree of SRB sample dilution and number of positive results. Bacterial cells were harvested through centrifugation at 6000 rpm (relative centrifugal force (RCF)=2503*g*) for 15 min, and rinsed twice with 0.2 M phosphate buffer saline (PBS, pH 7.4). Bacterial solutions of various concentrations were obtained by serially dilution with PBS. *Escherichia coli*, *Staphylococcus aureus* and *Vibrio alginolyticus* were used as control microorganisms to investigate the detection specificity of the proposed method.

2.3. The sensing procedures for SRB detection

As shown in Scheme 1, the whole procedures of the bacterial detection method based on inhibition of protease activity consisted of three steps. Firstly, a series of SRB suspensions with concentrations from 1 to 10^8 cfu mL^{-1} were prepared by serial dilution and cultivated at 30 °C to accumulate the characteristic metabolite, sulfide. Then, after cultivation for a period of time, bacterial cells and nutrition substances were removed by centrifuging the SRB culture solution (2 mL) at 10,000 rpm (RCF=6953*g*) for 20 min, and 900 μL of upper aqueous solution was transferred into a centrifuge tube containing 100 μL of 20 mM GSSG solution (prepared in 50 mM PBS, pH 7.4). The mixture was incubated at 37 °C for 15 min to synthesize electrophilic inhibitor, GSSH. Finally, after reaction for 15 min, 100 μL of the resulting GSSH containing solution was immediately added to 100 μL of 2 mg mL^{-1} papain solution to evaluate its influence on papain catalytic activity. The activity of papain was highly related with initial concentration of SRB, because the bacterial concentration could decide the amount of GSSH inhibitor by control of sulfide concentration accumulated in culture medium. Thus, the catalytic activity inhibition process of cysteine protease papain could be used for SRB detection.

2.4. Determination of papain activity

The catalytic activity of papain was measured by using the hydrolytic reaction of casein substrate [40,41]. 100 μL of GSSH inhibitor containing solution was added to 100 μL of 2 mg mL^{-1} papain solution, and the mixture solution was incubated at 37 °C for 30 min to *S*-sulfhydrate the sulfhydryl groups of cysteine-25 residue to –SSH groups. After that, 200 μL of 1% aqueous casein solution (freshly prepared in 50 mM PBS) was added. Hydrolysis of casein was conducted for 10 min at 37 °C to release tyrosine



Scheme 1. Schematic diagram of procedures of the SRB detection method based on the inhibition of cysteine protease activity.

product, and then the reaction was terminated by adding 600 μL of 10% trichloroacetic acid solution. Finally, the suspension was centrifuged at room temperature and the absorbance of the supernatant was measured at 275 nm with a Hitachi U-2900 spectrophotometer (Hitachi Co., Ltd., Tokyo, Japan).

The inhibition ratio (%) was used to evaluate the inhibition ability of inhibitors, including GSSH, GSSG and SRB metabolic sulfide, against the activity of papain, and it was calculated as

$$\text{Inhibition ratio } (\%) = (A_0 - A)/A_0 \times 100$$

where A and A_0 are the absorbance at 275 nm of hydrolysis products with and without the addition of inhibitor, respectively.

3. Results and discussion

3.1. Optimization of working conditions for enzyme reaction

In order to obtain the best analytical performance for SRB detection, the optimized working conditions of cysteine protease papain have been evaluated.

Effect of pH on the catalytic ability of papain was shown in Fig. 1A. Papain solution was prepared in PBS with pH varied from 5.4 to 10.4, and then used for hydrolysis of casein into tyrosine. The catalytic activity of papain was evaluated by measuring the absorbance of the supernatant at 275 nm, and the relative activity was calculated by assuming that the maximum absorbance of the measurements as 100%. Since papain is a kind of neutral protease, the best catalytic performance was appeared at pH 7.4 in the experimental pH range. In fact, as seen from Fig. 1A, the relative activity of papain maintained at high levels over the testing pH range, indicating papain has high chemical stability and broad application pH range.

Another important factor on papain activity is buffer concentration, which could decide the buffering capacity and ionic strength of the system. The influence of buffer concentration on the hydrolytic activity of papain was evaluated by adjusting the PBS buffer from 0.01 M to 0.2 M (the pH was kept constant at 7.4). As can be seen in Fig. 1B, with the increase of buffer concentration, the relative activity of papain decreased as a result. Considering both buffering capacity and papain activity, 50 mM PBS was chosen in our subsequent experiments.

The concentration of papain was also optimized to achieve a high catalytic activity, and the result was presented in Fig. 1C. In the concentration range of 10 μg mL^{-1} to 2 mg mL^{-1}, a rapid increase of enzyme activity was observed with the increase of papain concentration. This is because more papain molecules were able to strike with casein substrate and thus more tyrosine product was formed in a given time. Nevertheless, when papain concentration was higher than 2 mg mL^{-1}, the increasing of protease amount would not increase the catalytic activity any more, and the relative activity reached a stable platform. The reason is that all casein substrate molecules have been combined with papain, and the catalytic activity of papain was constrained by the casein substrate concentration. Therefore, the optimized papain concentration of 2 mg mL^{-1} was used for our research.

3.2. Characterization of GSSH

Previous studies have reported that persulfide could be generated by reaction between sulfide and disulfide bonds as follows:

$$R\text{–}S\text{–}S\text{–}R + H_2S \rightarrow RSSH + RSH \quad (1)$$

By taking advantage of this reaction, cysteine persulfide and GSSH have been synthesized from cystine (Cys–S–S–Cys) and GSSG, respectively [37,42,43]. According to these literatures, UV–vis spectroscopy is a facile and direct approach to characterize the presence of persulfide functional group (Fig. 2). In this work, GSSH was synthesized by reaction between SRB metabolic sulfide and GSSG at 37 °C for 15 min as described in Section 2.3, and UV–vis absorption of GSSH was recorded immediately after the 15 min reaction time (curve a). Then the pH of the reaction solution was adjusted to pH 9 with 200 mM NaOH solution, and a new absorbance at 350 nm was detected (curve b). This absorbance cannot be due to disulfide, or to cysteine ion [44], and it can be only attributed to the deprotonation of GSSH to GSS^- under basic conditions. This result was consistent with previous reports [37,42,43], and could be considered as an obvious and direct evidence of the formation of GSSH.

3.3. Inhibition of papain activity by glutathione persulfide (GSSH), oxidized glutathione (GSSG), and SRB metabolic sulfide (HS^-)

Papain was first incubated with GSSG, GSSH and SRB metabolized sulfide for 30 min at 37 °C (as described in Section 2.3), followed by papain activity measurements. Thanks to the active cysteine-25 *S*-sulfhydration process, the catalytic ability of papain was inhibited, and the inhibition ability was presented with inhibition ratio (calculated as described in Section 2.4). The comparison of the inhibition ability of GSSG, GSSH and SRB metabolized sulfide

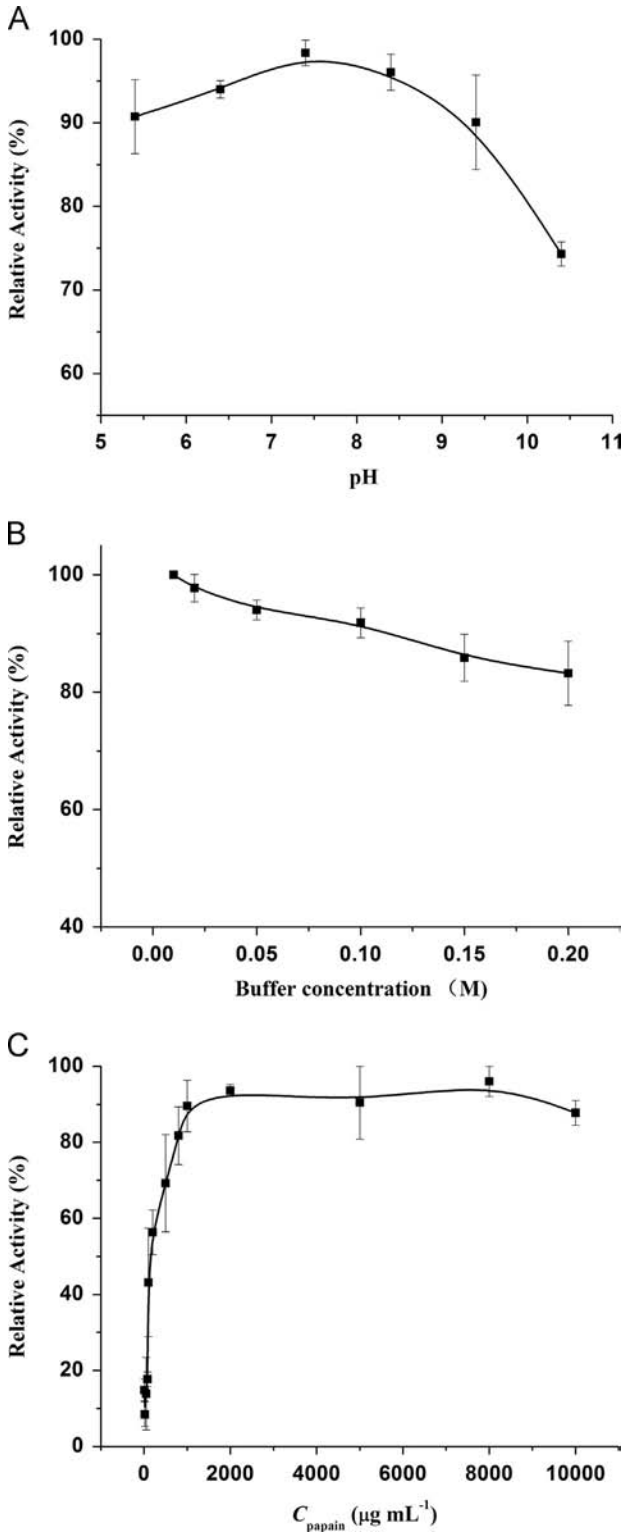


Fig. 1. Effect of pH (A), buffer concentration (B) and papain concentration (C) on the catalytic activity of papain. Mean value and standard deviation were calculated from triplicate experiments.

against the hydrolytic activity of papain was presented in Fig. 3. Under the experimental conditions, the inhibition ratio of GSSG against papain activity was only about 10%. SRB metabolized sulfide could attack the protease thiol groups to generate papain persulfides and thus result in a decrease of catalytic activity. However, as mentioned above, both sulfide and the thiol group of reactive cysteine residue are particularly nucleophilic, so the reaction

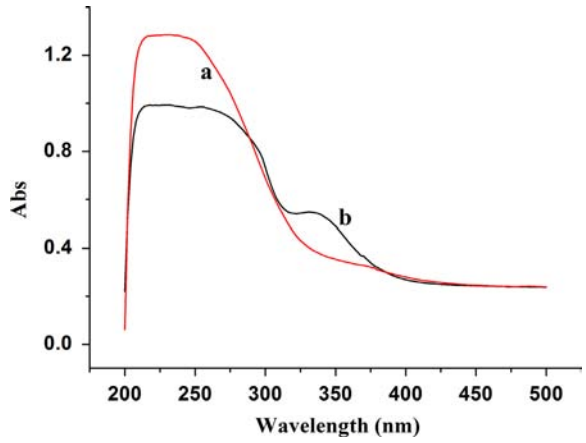


Fig. 2. UV–vis absorption of GSSH before (a) and after (b) addition of sodium hydroxide. Experimental conditions; GSSG concentration: 2 mM, SRB concentration: 1.0×10^7 cfu mL^{-1} and cultivation time: 96 h.

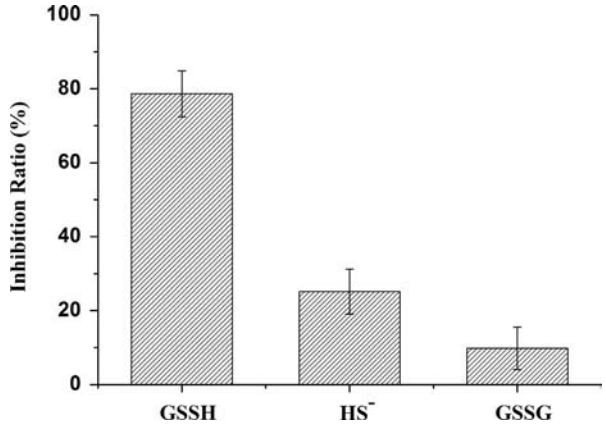


Fig. 3. Comparison of the inhibition ability of GSSH, SRB metabolized sulfide and GSSG against papain. Experimental conditions; papain concentration: 2 mg mL^{-1}, PBS concentration: 50 mM, pH: 7.4, temperature: 37 °C, catalytic reaction time: 10 min, SRB concentration: 1.0×10^7 cfu mL^{-1} and cultivation time: 96 h. Mean value and standard deviation were calculated from triplicate experiments.

efficiency of *S*-sulfhydration process was low and the inhibition ratio of SRB metabolized sulfide was not high. On the other hand, owing to the formation of electrophilic persulfide sulfane sulfur, GSSH could efficiently transfer its sulfane sulfurs to the active thiol groups of papain (Reaction (2)).

$$\mathrm{GSSH + Papain\text{–}SH \rightarrow Papain\text{–}SSH + GSH} \quad (2)$$

Thus, the catalytic activity of papain would be largely inhibited, and the inhibition ratio of GSSH could reach up to 80%. Moreover, we expected this inhibition effect on papain activity could be applied for marine SRB detection.

3.4. SRB detection performances

SRB solutions of various concentrations were cultivated in modified Postgate's culture, and then metabolized sulfide was isolated and pipetted to an aqueous GSSG solution. The mixture was incubated for 15 min at 37 °C, and the resulting GSSH could efficiently attack the active thiol of cysteine-25 and thus inhibit the hydrolytic ability of papain. Since SRB cultivation time could directly influence the amount of sulfide accumulated in SRB medium and therefore affect the formation of GSSH inhibitor, so the catalytic activity of papain was highly related with bacterial cultivation time. Effect of cultivation time on papain activity inhibition ratio was investigated, and the result was shown in

Fig. 4. At the initial stage of cultivation, the bacteria had to adapt to new culture environment, so the amount of sulfide produced by SRB of different concentrations were almost the same, resulting in the slight differences in the inhibition ratio. When the initial lag phase was over, SRB began their exponential growth, and higher bacterial concentrations resulted in faster sulfide metabolic rate. Thus, the amounts of sulfide accumulated by SRB of varied concentrations were different, which caused the different inhibition ratio against papain activity. After a period of cultivation, the SRB populations would reach saturation, and the sulfide metabolic rate slowed down as a result. Hence, similar inhibition ratios could be observed when SRB was cultivated for 72 and 96 h. The linear relationships between the inhibition ratio against papain activity and the logarithm of bacterial concentration varied with the prolongation of the cultivation time. The effect of increase in cultivation time from 48 to 96 h on the main analytical parameters was summarized in Table 1. After cultivation for 48 h, the proposed method based on inhibition of papain activity could detect SRB in the concentration range from 10^4 to 10^8 cfu mL^{-1}. When the cultivation time was extended to 96 h, a linear bacterial detection range up to 10^2 cfu mL^{-1} was obtained.

In order to investigate the specificity of the SRB detection method based on inhibition of cysteine protease activity, *E. coli*, *S. aureus* and *V. alginolyticus* were used as control microorganisms. SRB, *E. coli*, *S. aureus* and *V. alginolyticus* of the same concentration were cultivated for 4 days in modified Postgate's medium, and then treated for papain activity inhibition as described in Section 2.3. Fig. 5 shows the specificity of this SRB detection method, background signal was corresponded to sample prepared from a sterile culture medium without bacterial cells. The inhibition ratio showed a distinct increase when SRB was analyzed, indicating this method was highly specific for detection of SRB.

The MPN method is still the most widely used approach for SRB detection [8,9]. The whole detection process involves a pre-enrichment step followed by the biochemical test. Growth of SRB was determined by the appearance of FeS black precipitate, and bacterial population could be concluded from combining the degree of sample dilution and number of positive results. The MPN method possesses an ultra-low detection limit, but it would take up to at least 15 days to accomplish the whole procedure. The long-time delay would affect the adjustments of engineering construction and sterilization operations. Many other methods have been developed for SRB detection, including enzyme-linked immunosorbent assays [11], biochemical test [10], molecular biotechniques [12], and biosensors [13–17]. The high specificity of these methods was realized by using biological recognition elements, such as lectin, antibody and aptamer. However, these biological materials were usually expensive and unstable, and the accuracy of detection would be affected by inevitable non-specific absorption. Besides, the characteristic of high specificity of these methods would conversely restrain their universal application, since the diversity of SRB is enormous, and only a specific genus or species of SRB could be recognized by one kind of antibody or nucleotide sequence. The proposed method based on inhibition of cysteine protease activity avoided the use of biorecognition elements, and the detection selectivity was achieved by taking advantage of the characterized bacterial metabolic product, sulfide, which led to better universality of the method. In addition, compared with the most widely used MPN method, which take up to 15 days to accumulate SRB metabolized sulfide product, the detection time of this novel SRB approach was greatly shortened.

4. Conclusions

Inspired by a recent research hotspot in life science that sulfide has been considered as an important endogenously generated signaling molecular and it could modulate a range of physiological activities, we explored a novel SRB detection approach based on inhibition of cysteine protein activity. This SRB detection method

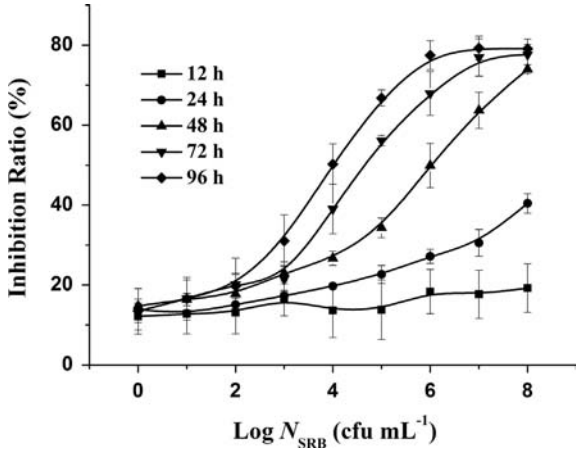


Fig. 4. Effect of bacterial cultivation time on the inhibition of papain activity. Catalytic reaction conditions; papain concentration: 2 mg mL^{-1}, PBS concentration: 50 mM, pH: 7.4, temperature: 37 °C and reaction time: 10 min. Mean value and standard deviation were calculated from triplicate experiments.

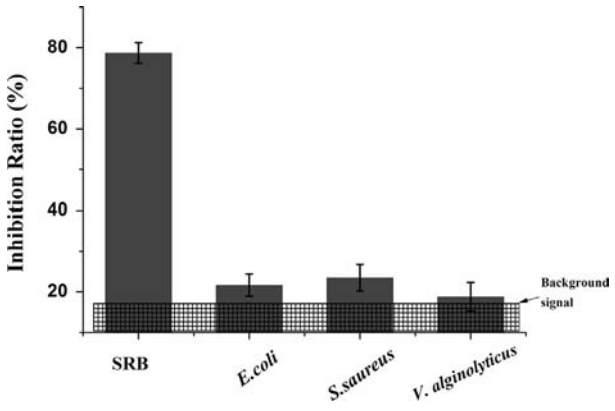


Fig. 5. Specificity of the SRB detection method based on the inhibition of papain activity. Experimental conditions; papain concentration: 2 mg mL^{-1}, PBS concentration: 50 mM, pH: 7.4, temperature: 37 °C, hydrolytic reaction time: 10 min, bacterial concentration of SRB, *E. coli*, *S. aureus* and *V. alginolyticus*: 1.0×10^7 cfu mL^{-1}, and cultivation time: 96 h. Mean value and standard deviation were calculated from triplicate experiments.

Table 1
The main analytical parameters for SRB detection after incubation for 48, 72 and 96 hours.

Cultivation time (h)	Bacterial concentration range (cfu mL^{-1})	Calibration equation	Limit of detection (cfu mL^{-1})[a]	R^2
48	10^4–10^8	Inhibition ratio = 12.40 log N_{SRB} − 24.67	5.21×10^2	0.9951
72	10^3–10^7	Inhibition ratio = 13.97 log N_{SRB} − 17.56	3.10×10^2	0.9907
96	10^2–10^6	Inhibition ratio = 15.12 log N_{SRB} − 11.42	1.59×10^1	0.9956

[a] Calculated by $3.3 \times SD/S$ (SD = the standard deviation of blank samples; S = the slope of the calibration curve).

avoided the use of biological recognition elements, and its detection principle was that sulfide, the characteristic metabolic product of SRB, was utilized to *S*-sulfhydrate the active cysteine thiol in protease catalytic center and thus inhibited the catalytic activity of cysteine protease. In order to conduct the active thiol group *S*-sulfhydration process efficiently, GSSG was introduced to synthesize electrophilic inhibitor GSSH. Since initial bacterial concentration could control the formation of GSSH inhibitor by accumulating the metabolic sulfide in culture medium. Thus, the activity of papain was highly related with initial concentration of SRB, and the cysteine protease activity inhibition process could be used for SRB detection. The detection performances of the SRB detection method varied with SRB cultivation time. SRB in the concentration range from 10^4 to 10^8 cfu mL^{-1} could be detected after SRB was cultivated for 2 days. When cultivation time was extended for 4 days, a linear bacterial detection range up to 10^2 cfu mL^{-1} was obtained with a detection limit of 1.59×10^1 cfu mL^{-1}. SRB detection time was greatly shortened compared with the widely used MPN method, which would take up to at least 15 days to accomplish whole detection process.

Acknowledgments

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