Use of sulfur-oxidizing bacteria as recognition elements in hydrogen sulfide biosensing system

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Abstract

Four sulfur-oxidizing bacteria (Thiobacillus thioparus, Acidithiobacillus thiooxidans PTCC1717, Acidithiobacillus ferrooxidans PTCC1646, and Acidithiobacillus ferrooxidans PTCC1647) were used as biorecognition elements in a hydrogen sulfide biosensing system. All the experiments were performed in 0.1 M phosphate buffer solution containing 1–20 ppm H\textsubscript{2}S with optimum pH and temperature for each species. Although H\textsubscript{2}S was applied to the biosensing system, the dissolved O\textsubscript{2} content decreased. Dissolved O\textsubscript{2} consumed by cells in both free and immobilized forms was measured using a dissolved oxygen sensor. Free bacterial cells exhibit fast response (<200 Sec). Immobilization of the cells on polyvinyl alcohol was optimized using an analytical software. Immobilized A. ferrooxidans and A. thiooxidans retained more than 50% of activity after 30 days of immobilization. According to the data, A. thiooxidans and A. ferrooxidans are appropriate species for hydrogen sulfide biosensor.

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Keywords: biosensing system, biosensors, dissolved oxygen, hydrogen sulfide, immobilization, microbial communication, polyvinyl alcohol, sulfur oxidizing bacteria

1. Introduction

Hydrogen sulfide is a colorless, flammable, and extremely toxic gas to living organisms [1]. It is one of the most important gases in the nature, which is produced by anaerobic sulfur-reducing bacteria or in the industrial processes as a by-product [2, 3]. H\textsubscript{2}S is harmful for human health at levels greater than 10 ppm and it can cause death at levels of more than 600 ppm [4, 5], so detection of H\textsubscript{2}S is absolutely necessary. Classical physical and chemical analysis techniques are usually limited because they are time consuming, expensive, and laboratory bound [6]. Sometimes these methods need sample pretreatment and they may be unable to detect trace amount of samples [7]. Biosensors have more advantages in comparison with physical or chemical analyses because of their sensitivity, specificity, portability, and economical aspect [8].

Biosensors are analytical devices, which consist of biological sensing elements and transducers to output signals to obtain information [9]. Various biological materials including DNA molecules, cofactors, antibodies, enzymes, microorganisms, tissues, and animal or plant cells have been used as recognition elements in biosensing systems [9]. Microbial species are widely used in the fabrication of biosensors for their special properties such as stability and resistance in wide range of pH and temperatures and easy production through cell culturing with low cost [10]. Whole-cell bacterial biosensors can respond to the various environmental changes and are especially suitable for the detection and monitoring of toxic components in soil and water [11].

Sulfur cycle bacteria especially sulfate-reducing and sulfide-oxidizing bacteria have a major role in the industrial
processes and environmental treatments [3]. Although sulfate-reducing bacteria cause some problems for the petroleum industry by the production of H₂S, they can be used in the treatment of acid mine drainage [12]. Sulfide-oxidizing bacteria carry out the biological oxidation of sulfur compounds and this feature is immensely important for the bioleaching of refractory minerals, biotreatment of laden sulfide water, and removal of H₂S from oil reservoirs [3, 13, 14]. The metabolic pathway of sulfide oxidation for aerobic sulfur chemolithotrophs has two steps. At first step, six electrons from sulfide transfer to the oxygen as the terminal electron acceptor through a cell electron transfer system. Oxidation of sulfite produced in previous step to sulfate occurs in two different pathways. In the most common pathway, ATP formation occurs as a result of electron transfer from sulfite to cytochromo c by the enzyme sulfite oxidase. In the other pathway, the reversal activity of the enzyme adenosine phosphosulfate reductase causes oxidation of sulfite. In this reaction, adenosine monophosphate is converted into adenosine diphosphate [3, 14, 15].

Although there are many examples of H₂S chemical sensors including optical sensors, semiconducting metal oxide sensors, electrochemical sensors, conducting polymer sensors, and piezoelectric sensors [16], there are very rare examples of biosensors for detecting H₂S and most of them are enzyme base biosensors [7, 17]. This research used sulfide-oxidizing bacteria as a biorecognition element in a H₂S detection system. The ability of O₂ consumption during sulfide oxidation described above is the main criteria for using these strains in the biosensing system. Performance of these bacteria was compared with each other as well. The objective of this novel study was to determine the most proper species for the H₂S biosensing system among the species that were studied.

2. Materials and Methods

2.1. Microorganisms

Bacteria used in this study included *Thiobacillus thioparus*, which was purchased from the University of Tehran Microorganisms Collection, and *Acidithiobacillus thiooxidans* PTCC1717, *A. ferrooxidans* PTCC1646, and *A. ferrooxidans* PTCC1647 (native strain), which were obtained from the Persian Type Culture Collection.

2.2. Culture medium

2.2.1. *Thiobacillus thioparus*

Cell culture was done in medium DSM 486 containing (in g/L): KH₂PO₄, 2.00; K₂HPO₄, 2.00; NH₄Cl, 0.4; Na₂CO₃, 0.4; MgCl₂·6H₂O, 0.2; Na₂S·O₃·5H₂O, 5; and vitamin and trace metals solutions. The process was carried out in shaking incubator at 30 °C, 180 rpm, and initial pH 7.

2.2.2. *Acidithiobacillus thiooxidans*

Microorganism was grown on medium DSM9463 containing (in g/L): (NH₄)₂SO₄, 2.00; MgSO₄·7H₂O, 0.25; K₂HPO₄, 0.1; KCl, 0.1; sulfur powder, 5.00; and initial pH 3.5. Incubation was done in shaking incubator at 28 °C and 180 rpm.

2.2.3. *A. ferrooxidans* PTCC1646 and *A. ferrooxidans* PTCC1647

Bacterial cells were grown on a slightly modified 9K medium with S as an energy source containing (in g/L): (NH₄)₂SO₄, 3.00; KCl, 0.10; K₂HPO₄, 0.50; MgSO₄·7H₂O, 0.50; Ca(NO₃)₂, 0.01; and sulfur, 10.00. The initial pH was adjusted to 2.5 using sulfuric acid, and cultures were incubated at 28 °C and 180 rpm. The amount of inoculums was 10% v/v for all species.

2.3. Microbial biosensing system for monitoring hydrogen sulfide

A microbial biosensing system similar to a biosensing system design by Wen and co-workers for monitoring methane [18] was utilized in this research. This biosensing system is illustrated in Fig. 1. It consists of a reaction chamber with volume of 500 mL containing 300 mL 0.1 M phosphate buffer with optimum pH for each bacterium, and a dissolved oxygen meter (model 8403; AZ Instruments, Heng Xin, Taiwan, People’s Republic of China) measured O₂ consumption by bacteria. An air pump injected air into phosphate buffer till saturation level was reached, then air flow was stopped, and H₂S (which was supplied by a gas container containing 1% H₂S and 99% N₂) was introduced into O₂-saturated phosphate buffer. H₂S gas flow rate was carefully adjusted with a mass flow controller. When specific concentration of H₂S injected into the reaction chamber, bacterial suspension or immobilized beads were added to the chamber as well. The buffer solution was agitated by a magnetic bar during the whole procedure. The decrease in dissolved oxygen concentration caused by bacterial cells during oxidation of H₂S was recorded by the O₂ sensor.

2.4. Bacterial growth and evaluation of the best time to harvest cells

Bacterial population was followed by optical density using a spectrophotometer (model Ce1010; Cecil Instruments, Cambridge UK). One hundred microliters of bacterial medium culture with 10% v/v inoculum was incubated in 250-mL flasks at an optimum temperature. Different phases of growth curve were determined for all four species. Ten microliters of bacterial cell suspensions belong to different times of log phase containing 2 × 10⁹ cell/mL were prepared by cell culture centrifugation at 7,700 g for 15 Min, and cell pellet was resuspended in 0.9% sodium chloride solution. The suspension for each species examined in the H₂S biosensing system at 10 ppm of H₂S and the culture with the most dissolved oxygen consumption selected as the best culture in the biosensing system for all species. For those media that contained sulfur powder, cultures were filtered through a filter paper to eliminate residual sulfur before optical density measurement or centrifugation.

2.5. Examination of cell suspension in different concentration of H₂S dissolved in phosphate buffer

Ten milliliters of cell suspension containing 2 × 10⁹ cell/mL was prepared by cell culture centrifugation at 7,700 g for 15 Min, and then precipitated cells were resuspended in 0.9%...
sodium chloride solution. Centrifugation was repeated two times. The \( \text{H}_2\text{S} \) biosensing system was prepared as described above. Various concentrations of \( \text{H}_2\text{S} \) (1, 3, 5, 10, 15, and 20 ppm [mg/L]) were injected into phosphate buffer solution, and then cell suspension was added to the solution. A decrease in dissolved oxygen was recorded during 900 Sec as well. These experiments were done for all species.

A negative control test was conducted for all species to ensure that bacterial species do not utilize dissolved oxygen in the absence of \( \text{H}_2\text{S} \) in the biosensing system. For this purpose, 10 mL cell suspension containing \( 2 \times 10^9 \) cell/mL was added into the reaction chamber containing 300 mL \( \text{H}_2\text{S} \) free phosphate buffer (with optimum pH and temperature for each species). Changes in dissolved oxygen concentration were monitored too.

2.6. Immobilization of sulfur oxidizing bacteria by the PVA–boric acid method

For developing stability and reuse possibility, bacterial cells were immobilized on a polymeric support, polyvinyl alcohol. A method described by Hashimoto and Furukawa [19] was used in this study. Eight percent, 10%, and 12% of PVA aqueous solution were prepared by adding 0.8, 1, or 1.2 g of PVA to 10.0 mL of distilled water, which was heated to the temperature of 80 °C. When a homogenized solution of PVA was readied and temperature decreased to around 60 °C, 1 g sodium alginate was mixed with the PVA solution. The PVA–alginate mixture was gently stirred for 45 Min, cooled to room temperature (20–25 °C), then followed by the addition of different amount of bacterial cells (2 \( \times 10^8 \), 4 \( \times 10^8 \), and 6 \( \times 10^8 \) cell/mL) harvested from specific time in log phase by centrifugation of cell culture, and mixed well. The mixture was dropped into a cool and gently stirred 4% boric acid and 2% \( \text{CaCl}_2 \) solution to form spherical beads. These beads were kept in boric acid solution for 24 H at 4 °C. The beads were then removed and washed with distilled water.

2.7. Optimization of immobilization of bacterial cells on PVA using statistical analysis

The response surface methodology was applied to understand the interaction of concentration of PVA aqueous solution and cell number and their effects on response. Also the optimum concentrations of PVA and cell number in immobilization procedure were found.

Central composite design (CCD) was carried out to optimize the concentration of PVA and cell number for the two factors on three levels. This design was augmented with five replications of the center points. An analytical software was used for the experimental design and data analysis. Table 1 shows factors and levels used in the experimental design. PVA beads could not be formed in concentrations of less than 8% and do not have spherical shape in concentrations of more than 12%. So, 8% and 12% were chosen as the minimum and maximum levels of PVA concentration as well. Table 2 shows the designed experiments totally.

### Table 1: Different levels of variables in experimental design

<table>
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<th>Level</th>
<th>Polyvinyl alcohol concentration (%, w/v)</th>
<th>Cell number (mL)</th>
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<td>8</td>
<td>( 2 \times 10^9 )</td>
</tr>
<tr>
<td>0</td>
<td>10</td>
<td>( 4 \times 10^9 )</td>
</tr>
<tr>
<td>1</td>
<td>12</td>
<td>( 6 \times 10^9 )</td>
</tr>
</tbody>
</table>
TABLE 2
Central composite design for optimization of two variables (each on three levels)

<table>
<thead>
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<th>Run</th>
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<th>Cell number (mL)</th>
</tr>
</thead>
<tbody>
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<td>0</td>
<td>1</td>
</tr>
<tr>
<td>2</td>
<td>1</td>
<td>−1</td>
</tr>
<tr>
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<td>1</td>
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<tr>
<td>6</td>
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<tr>
<td>13</td>
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</tbody>
</table>

2.8. Examination of immobilized beads stability and activity during 30 days
PVA beads with optimum concentration of PVA and cell number were examined in the biosensing system at 5, 10, 20, and 30 days, and a decrease in their O$_2$ consumption activity was measured. After each test, the beads were removed from phosphate buffer and washed with distilled water quickly, and then stored in 0.5 M phosphate buffer solution at 4°C.

3. Results
3.1. Growth curve and best time to harvest cells
To understand the time frame of log phase, optical density of inoculated flasks was measured at 600 nm and growth curves of the four sulfur-oxidizing bacteria were obtained. Culture condition and the measurement method are described in Sections 2.2 and 2.4. Lag, log, and stationary phases for each bacterium are shown in Fig. 2. Log phase is important in this study because during this phase, nutrients are metabolized at maximum speed by bacterial cells [20]. For characterizing the best culture to use in the biosensing system, log phase of growth curve for each strain divided into four or five equal time frames (depending on log phase length) and cells obtained from these points were examined in the H$_2$S biosensing system to find the most active cell culture. The cultures with the most amount of O$_2$ consumption were selected as the best cultures as well. T. thioparus that was harvested after 96 H and tested in the biosensing system consumed the most amount of O$_2$ in comparison with other T. thioparus cultures at different point of log phase, so 96 H has been chosen as the best time for cell harvesting. For A. thiooxidans, this time is 116 H and for A. ferroxidans 1646 it is 102 H. According to the results, A. ferroxidans 1647 did not consume H$_2$S in the biosensing system efficiently and subsequent experiments were not performed for this species. Data for O$_2$ consumption by four strains at different point of log phase are not shown.

3.2. Control test
The negative control test has shown that bacterial cells do not utilize dissolved oxygen when there is no H$_2$S in the biosensor chamber, because during the control test no decrease in O$_2$ concentration was recorded by O$_2$ sensor.

3.3. Performance of the bacterial cells in different concentrations of H$_2$S
O$_2$ consuming by bacterial cells was measured at six different concentrations of H$_2$S (1, 3, 5, 10, 15, and 20 ppm) for 900 Sec. Figures 3 and 4 show the results. H$_2$S solubility in aqueous NaCl solution at 30°C is about 3,000 ppm [21] so the solubility of H$_2$S was considered 100% in this work. A linear relationship between total decrease in dissolved O$_2$ and increase in H$_2$S concentration was considered in the range of 1–20 ppm H$_2$S for A. thiooxidans, 1–15 ppm H$_2$S for T. thioparus, and 1–10 ppm H$_2$S for A. ferroxidans. $R^2$ is about 0.95 for these three linear relationships. Although response time had been defined differently in other studies, in this article, response time has been defined as the time at which O$_2$ consumption for each hydrogen sulfide concentration can be distinguished separately. It seems that this definition is the best according to biosensor performance. According to the data shown in Fig. 4, response time for T. thioparus is 160 Sec, 200 Sec for A. ferroxidans 1646, and 190 Sec for A. thiooxidans. These response times could be improved by increasing the number of the cells in the reaction chamber.
FIG. 3  Decrease in dissolved oxygen concentration at different H₂S concentration:
(a) Acidithiobacillus ferrooxidans 1646, (b) Acidithiobacillus thiooxidans, and (c) Thiobacillus thioparus.

3.4. Optimization of immobilization of bacterial cells
When spherical PVA beads with different levels of cell number or PVA concentration were prepared, they were tested in H₂S biosensor separately and total O₂ consumption during 900 Sec for each type of beads recorded as well (Table 3). Scanning electron microscope images proved that entrapment of bacterial cells into PVA polymer was successful (Fig. 5). According to the results, T. thioparus was deactivated after immobilization and no O₂ consumption was detected for this bacterial strain. For other species (A. ferrooxidans 1646 and A. thiooxidans), the total decrease in dissolved oxygen concentration after 900 Sec was considered as the response of the experiments. The optimum concentration of PVA, the cell number for reaching the optimum response, and the equation for these parameters were calculated by Design Expert software. The quadratic polynomial model was established on the experimental results of CCD to identify the relationship between responses and variables. For A. thiooxidans, the equation was
\[ \text{response} = 0.7 - 0.23A + 0.3B - 0.25B^2 \] and for A. ferrooxidans the equation was \[ \text{response} = 0.83 - 0.21A + 0.19B. \] In these equations, A is PVA concentration and B is cell number per milliliter. \( R^2 \) for the first equation was 0.95 and for the second one was 0.8. The parameters obtained through the modeling indicate that the linear effects of A and B and squares effect of B also were found to be significant (\( P < 0.05 \)). A positive sign of the coefficient in the equation represents a synergistic effect, whereas a negative sign indicates an antagonistic effect. According to the software prediction for A. ferrooxidans, optimum response is 0.904 (ppm), optimum cell number per milliliter is \( 4.68 \times 10^9 \), and optimum PVA concentration is 10.74% (w/v). For A. thiooxidans, optimum response is 1.05 (ppm) and optimum cell number per milliliter is \( 5.2 \times 10^9 \) and optimum PVA concentration is 8% (w/v), respectively.

Figure 6 indicates actual versus predicted responses at different amount of PVA and cell number. To demonstrate discrepancy between predicted optimum response and actual response, PVA beads with optimum concentration and optimum cell number were prepared and examined in the biosensing system. This procedure was repeated three times and percentage error of model was calculated. Percentage error of optimum model for A. thiooxidans was 6.6% and for A. ferrooxidans it was 9.3%.
Moreover, Design Expert software indicates the effect of two variables (PVA concentration and cell number) on the response time of the biosensing system as well (which is not shown in this study).

3.5. Stability of immobilized beads during 30 days
The variation of \(O_2\) consumption was monitored in immobilized cells during 30 days after immobilization. The results revealed that \(A.\ thiooxidans\) retained 99% of its activity after 10 days of preparation and kept 70% of the activity after 30 days, and \(A.\ ferrooxidans\) retained 91% of the activity after 10 days and 54% of activity after 30 days (Fig. 7).

4. Discussion
In this study, we fabricated a preliminary form of \(H_2S\) biosensor, using sulfur-oxidizing bacteria. The basis of this system was measurement of the dissolved oxygen content in the buffer solution containing a particular concentration of \(H_2S\). Bacterial cells gain energy by turning \(H_2S\) into sulfuric acid using \(O_2\). This reaction leads to a decrease in dissolved \(O_2\) concentration in the biosensing system. The bacteria were applied in the system when they were in the specific moment in log phase and had the most sulfur utilization activity during their cell cycle.

Among the strains that were tested in biosensor apparatus at the different concentrations of \(H_2S\), the fastest response time belongs to \(T.\ thioparus\), and the widest linear range is related to \(A.\ thiooxidans\) but the difference between the amount of these two features for \(T.\ thioparus\) and \(A.\ thiooxidans\) is not too much; so, in this case, we did not consider any preference for these two strains. On the contrary, the quantity of \(O_2\) utilization by \(T.\ thioparus\) was less than \(A.\ ferrooxidans\) and \(A.\ thiooxidans\). For example, in the presence of 10 ppm \(H_2S\), \(T.\ thioparus\) consumed 0.4 ppm \(O_2\) but \(A.\ thiooxidans\) and \(A.\ ferrooxidans\) consumed 1.4 and 2.5 ppm \(O_2\), respectively. It seems that \(T.\ thioparus\) used in this study has a weak ability to utilize \(H_2S\). In a study on the subject of biofilters, Abdehagh and co-workers reported that although this bacterial strain showed a reasonable performance in the removal of \(H_2S\) from air stream containing different level of \(H_2S\), performance of other similar biofilter systems using different microbial cultures is higher [22]. Dissolved oxygen was fairly consumed by \(A.\ Thiooxidans\) and \(A.\ ferrooxidans\) in our biosensing system. According to other related studies, an acceptable performance for these two species was expected. \(A.\ thiooxidans\) and \(A.\ ferrooxidans\) indicated high efficiency (about 90%) in \(H_2S\) removal biosystems [1, 2]. In a similar study, the abilities of \(H_2S\) oxidation of these
TABLE 3
Decrease in dissolve oxygen concentration for immobilized A. thiooxidans and A. ferrooxidans beads in optimization experiments

<table>
<thead>
<tr>
<th>Experiment number</th>
<th>PVA concentration (%)</th>
<th>Cell number (per mL)</th>
<th>Response for A. thiooxidans (ppm)</th>
<th>Response for A. ferrooxidans (ppm)</th>
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<tbody>
<tr>
<td>1</td>
<td>12</td>
<td>$2 \times 10^9$</td>
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<td>nd</td>
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<tr>
<td>2</td>
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<td>0.48</td>
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<td>0.52</td>
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<td>0.49</td>
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<td>0.7</td>
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<td>0.08</td>
<td>0.2</td>
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<td>0.83</td>
</tr>
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<td>0.74</td>
<td>0.94</td>
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<td>0.78</td>
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<td>10</td>
<td>$4 \times 10^9$</td>
<td>0.75</td>
<td>0.93</td>
</tr>
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</table>

nd, not detectable.

two species were compared and no differences in oxidation efficiency between these two species were detected [23].

Because we could not find any example of H$_2$S microbial biosensor, it is not possible to compare the performance of our system with other similar microbial biosensors. However, the present microbial biosensing system was compared with some enzymatic biosensors. Savizi et al. [7] fabricated an amperometric sulfide detecting system using Coprinus cinereus peroxidase. In this system, the determination of sulfide can be achieved in a linear range of 1.09–16.3 µM and the response time is 43 Sec. It seems that this system is much more sensitive than our biosensing system.

Immobilization of bacterial cells makes the biosensor reusable. PVA is a water-soluble and nontoxic polymer, which has got stable texture after polymerization, so it was chosen as the immobilization support. T. thioparus becomes deactivated after immobilization. Deactivation may occur because of the remaining beads into acidic solution (pH 4) for 24 H, whereas optimum pH for T. thioparus is around 7. In this case, immobilization of T. thioparus on PVA was not successful. As we expected for the other strains, the amount of O$_2$ consumption decreased after immobilization because bioavailability of O$_2$ had been reduced.

During the optimization of immobilization, when PVA concentration was at highest level (12%) and cell number was at lowest level ($2 \times 10^9$ cell/mL), no response was recorded for A. ferrooxidans 1646 and A. thiooxidans. It seems that in this condition, PVA beads are too condensed, and mass transfer is slow. According to the $R^2$ and percentage error of the models, the optimum model for A. thiooxidans is more accurate. The diagram of discrepancy between predicted optimum response and actual response for A. thiooxidans is shown in Fig. 6.

In comparison with other microbial biosensing systems, the stability of O$_2$ consumption activity of this biosensing system after 30 days is in an acceptable level. For example, a whole-cell microbial biosensor designed by Wen and co-workers for detecting methane, lost 50% of its activity after 1 month [18]. The performance of another microbial methane biosensor, which was prepared by Okada and co-workers, was constant during 20 days [24]. A whole-cell algae biosensor designed to
determine vapors of solvents such as methanol kept at 50% of its activity after 30 days [25].

In conclusion, this paper demonstrated the feasibility of fabrication as a hydrogen sulfide biosensing system based on sulfur-oxidizing bacteria in collaboration with a dissolved oxygen sensor. This microbial biosensing system exhibits fast response (<200 Sec) and if bacterial cells be used in immobilized form, it will be reusable and stable for more than 1 month. Some of the most important features of this microbial biosensing system are simple sensing design, ease of operation, and the low cost of production. Among four bacterial species that were studied, *A. ferrooxidans* PTCC1647 did not utilize H$_2$S detectably and *T. thioparus* was deactivated after immobilization; however, *A. thiooxidans* and *A. ferrooxidans* PTCC1646 displayed a reasonable level of O$_2$ consumption related to H$_2$S concentration in both free and immobilized form. Therefore, these two species are proper bacterial species to be used as biorecognition elements in a H$_2$S biosensing system.

5. Acknowledgement

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6. References


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FIG. 6

Actual versus predicted responses at different levels of PVA and cell number: (a) *A. thiooxidans* and (b) *A. ferrooxidans*.

FIG. 7

The variation of O$_2$ consumption for immobilized cells.