Biosensors and Bioelectronics 70 (2015) 106–114

Contents lists available at ScienceDirect

Biosensors and Bioelectronics

journal homepage: www.elsevier.com/locate/bios

# Investigating the effect of design parameters on the response time of a highly sensitive microbial hydrogen sulfide biosensor based on oxygen consumption

Amin Vosoughi [a,b], Fatemeh Yazdian [b,c], Ghassem Amoabediny [a,b,*], Maziar Hakim [d]

[a] Department of Biochemical and Pharmaceutical Engineering, School of Chemical Engineering, College of Engineering, University of Tehran, P.O. Box 11365-4563, Tehran, Iran
[b] Research Center for New Technologies in Life Science Engineering, University of Tehran, P.O. Box 14395-1374, Tehran, Iran
[c] Department of Life Science Engineering, Faculty of New Sciences and Technologies, University of Tehran, P.O. Box 14395-1561, Tehran, Iran
[d] Department of Chemical Engineering, Sharif University of Technology, P.O. Box 11365-11155, Tehran, Iran

ARTICLE INFO

*Article history:*
Received 19 January 2015
Received in revised form
9 March 2015
Accepted 10 March 2015
Available online 11 March 2015

*Keywords:*
Hydrogen sulfide
*Thiobacillus thioparus*
Design parameters
Agarose
Sodium alginate
Response time

ABSTRACT

A novel hydrogen sulfide microbial biosensor was developed based on investigating the influence of four design parameters: cell concentration, immobilization bed type, hydrogen sulfide concentration, and geometrical shape of the biosensor. *Thiobacillus thioparus* was used as the recognition element and it was immobilized on sodium alginate as well as agarose bed. The results were optimized by the application of statistical optimization software based on response time of the system. Oxygen reduction was considered as the detection sign. Sodium alginate solution with a concentration of 2.3% (w/v) and optical density of 10 at 605 nm was found as the optimum conditions for immobilization with response time of 72 s. Optimum response time of immobilized *T. thioparus* on agarose was also found equal to 120 s at agarose concentration of 1.2% (w/v) and optical density of 10.83. Performance of the biosensor in different temperatures, pH and agitation speeds was also analyzed. The designed biosensor could detect concentrations of hydrogen sulfide as low as 0.5 ppm. *T. thioparus* could retain 99% of the original activity in both systems, after ten days passing the fabrication. A fractal analysis was also done theoretically to investigate the diffusion of oxygen in immobilized cells which showed a satisfactory value of oxygen take up by the immobilized cells.



## 1. Introduction

Since the last four decades, an immense research has been done in the field of detecting hydrogen sulfide. $H_2S$ is a venomous, flammable and colorless gas with a malodor of rotten egg. It is swiftly absorbed by the respiration system causing neurological sequelae which finally leads to death. It can also cause a malodor nuisance problem even at relatively low concentration of 2 ppm. At moderate concentrations of 50–150 ppm it causes headaches, eye irritation and dizziness. It is largely produced in many industries such as petroleum and gas refining (Pandey et al., 2012; Kim et al., 2008). Hence, a preliminary step toward handling this hazardous is to monitor and detect its existence in the environment. Therefore, novel and accurate techniques such as biosensors have been suggested for detection of low concentrations of $H_2S$. Proper selection of the biosensor receptor is of great importance. Both phototrophic and chemotrophic bacteria are good choices for receptor, since they use hydrogen sulfide as the energy source and through this process they consume oxygen (Oprime et al., 2001).

Bacteria such as *Thiobacillus denitrificans*, *Thiobacillus ferroxidans* and *Thiobacillus thioparus* can use $H_2S$ as energy source (Pancrazio et al., 1999). *T. thioparus* is chosen among them, due to its low nutrients requirements and high rate of $H_2S$ consumption. Moreover, *T. thioparus* has the ability to grow under rough environmental conditions such as oxygen deficiency and acidic solutions, which is a great privilege in fabrication of biosensors. Its optimum pH range is 6.6–7.2 and it grows well in 28 °C. The overall equation for oxidation of $H_2S$ is as following (Oyarzun et al. 2003; Caceres et al., 2010):

$$H_2S + 2O_2 \rightarrow SO_4^{2-} + 2H^+ \quad (1)$$

Along this reaction $H_2S$ acts as electron donor and *T. thioparus*

* Corresponding author at: Department of Biochemical and Pharmaceutical Engineering, School of Chemical Engineering, College of Engineering, University of Tehran, P.O. Box 11365-4563, Tehran, Iran.
*E-mail address:* amoabediny@ut.ac.ir (G. Amoabediny).

http://dx.doi.org/10.1016/j.bios.2015.03.025
0956-5663/

consumes oxygen. Hence, the oxygen concentration of the solution declines. The existence of $H_2S$ in the solution can be perceptible with the aim of this detection sign. A biosensor system based on this logic was designed and investigated (Kubo et al., 1995).

In the present study, the effect of four designing parameters, including cell, bed and $H_2S$ concentration, along with system scale, on the response time of the biosensor was studied. Response time is defined as the time when the concentration of dissolved oxygen in the system starts to decline until it approaches to a constant value. Enduring harsh conditions is of the main traits of a good biosensor. Thus, *T. thioparus* species were immobilized on sodium alginate and agarose. In addition to being an excellent matrix, agarose and sodium alginate have high porosity which results in high capacity for cell entrapment (Yujian et al., 2006; Parkash and Jaisawal., 2011). Moreover, biosensors should possess low detection limits; therefore, concentrations of 0.5–20 ppm of $H_2S$ were injected to the system. Eventually, the effect of bacteria's interference in the system was investigated through the volume. In this regard, two systems of 300 and 500 ml volume were used for the tests and mathematical explorations have been discussed in each system.

The main scope of the present study was to obtain an optimized biosensing system based on analyzing the effect of four different parameters affecting the response time of the system. In order to set up the system, a gas fed batch reactor was used, which is a stirred bilayer bioreactor. The bacteria was immobilized on two different surfaces of sodium alginate and agarose. The biosensor could effectively respond to low concentrations of hydrogen sulfide swiftly and with high specificity.

## 2. Experimental

### 2.1. Cell culture and media

The original strain of *T. thioparus* (PTCC 1668 taken from Ministry of science, Research and Technology of Iran) was used in all experiments. For long storage of the strain (up to 3 years), 850 μl of the media containing bacteria was put in 1.5 ml vials, and 150 μl Glycerol was added as the anti-frost solution. The solution was put in −86 °C ultra-low temperature freezer (Kaltis, Taiwan) for further use.

*T. thioparus* was grown in DSM 486 containing (in g/l): $KH_2PO_4$, 2.00; $K_2HPO_4$, 2.00; $NH_4Cl$, 0.4; $Na_2S_2O3 \cdot 5H_2O$, 5; $MgCl_2 \cdot 6H_2O$, 0.2; $Na_2CO_3$, 0.4; in addition to vitamin and trace metal solutions. The cultivation was done in 500 ml Erlenmeyer flasks for 1–3 days at 30 °C and 180 rpm in a shaking-incubator (Kuhner, Switzerland) and the pH was adjusted to 7. The cell growth curve of the bacteria was also plotted by the application of optical density method in 605 nm. Cell harvesting from the media was achieved by centrifugation in 4000*g* for 15 min at 4 °C. The resulting bacteria was washed 2–3 times by normal saline and was again centrifuged to remove the remaining media from the cell. The volume of the obtained cell suspension was about 50 ml $H_2S$.

### 2.2. Determination of the best time for cell harvesting

In order to find the best time for cell harvesting, bacteria was taken out from shaking-incubator after 56, 67, 96, 120 h. Cells were tested in the biosensor system, and the oxygen consumption rate curve was plotted for each sample. It was observed that cells which were harvested after 96 h, presented the best activity and also maximum rate of oxygen consumption in the system.

### 2.3. Design and operational parameters

The effect of four parameters, including cell concentration, immobilization bed, $H_2S$ concentration and geometrical shape, on the response time of the system was investigated. Additionally, the biological activity and the mechanical strength of the immobilized beads were also analyzed by the reference methods (Yujian et al., 2006; Myung et al., 2007). The whole achieved data were optimized by the Respond Surface Method.

#### 2.3.1. Cell concentration

The best response time for the biosensor mainly depends on the cell concentration owing to its high capability of oxygen consumption. Consequently, different concentrations of cell, designed by the Central composite design (CCD) method, were used to prepare the immobilized beads in the system. The concentration yielding the best result was acclaimed.

#### 2.3.2. Immobilization on sodium alginate

Different concentrations of sodium alginate beads in the range of 2–4% (w/v) were prepared by the following method. Sodium alginate and calcium chloride of analytical grade (Merck) were used. To prepare 3% (w/v) beads, 3 g of sodium alginate was added to 100 cc diluted water. The solution was heated for 30 min up to the time when homogeneity appeared. The 0.2% (w/v) solution of calcium chloride was also prepared by addition of 1.5 g calcium chloride to 100 cc diluted water. Hereafter, sodium alginate was cooled down to ambient temperature. 5 ml of Cell suspension with specific concentration was added to 100 cc sodium alginate solution. In order to obtain similar beads in terms of size, the yielded mixture was put in a syringe pump (JMS Syringe Pump, USA), and it was gradually dropped into the calcium chloride solution to form immobilized sodium alginate beads.

#### 2.3.3. Immobilization on agarose

The method of Parkash and Jaiswal was used for preparing agarose beads (Parkash and Jaisawal., 2011). 0.1 M Sodium acetate buffer with pH 5.5 was prepared by adding 28.82 ml of 1 M acetic acid (Merck) to 273.3 ml of sodium acetate and the volume was increased to 1 l. In order to form 2% (w/v) agarose beads, 0.2 g of agarose (Sigma-Aldrich) was added to 10 cc of sodium acetate buffer at 50 °C. Other concentrations between 1% and 3% (w/v) was also prepared by the same procedure. Afterward, 5 ml of cell suspension with a specific concentration was added to 100 cc (same as sodium alginate beads) of the agarose solution prepared in the previous step. The resulting solution was put into 100 cc syringe and injected into a plastic mesh with same sized cavities. The solution was then cooled down to ambient temperature for 3 h. Finally, the resulting beads were taken out of the mesh and preserved for 24 h at 4 °C in 25 mM sodium acetate solution before being used in the system.

#### 2.3.4. Measuring dissolved $H_2S$ concentration

Different concentrations of $H_2S$, ranging from 0.5 to 20 ppm, were injected to the system in order to acquire detection limit of biosensor. Theoretical and practical methods were both employed for measuring the concentration of dissolved $H_2S$. The variance between the results was less than 5%. The method of Duan et.al was used for theoretical approach as well as the titrimetric method which was used for the practical approach. In the theoretical approach the basis of calculation is on the equality of chemical potential between liquid and gas phase. The resulted equation can be seen below (Duan et al., 2007; Suleimenov, and Krupp 1994):

$$\exp\left(\frac{\mu^{l}_{H2S} - \mu^{v}_{H2S}}{RT}\right) = \frac{y_{H2S} p \varphi_{H2S}}{m_{H2S}\gamma_{H2S}} \quad (2)$$

In Eq. (2), $y_{H2S}$ is the molar concentration of $H_2S$ in gas phase and $m_{H2S}$ is the concentration of $H_2S$ in liquid phase in terms of mol/kg. In order to calculate $y_{H2S}$, the following equation can be used:

$$y_{H2S} = \left(P - P_{H2O}\right)/P \quad (3)$$

Application of Eqs. (2) and (3) leads to solubility diagrams for calculation of $H_2S$ concentration in normal saline solution in different temperatures and pressures (Suleimenov, and Krupp 1994).

In practical concept the titration method was applied (Suleimenov and Krupp, 1994). The required chemicals used in this method are as follows: 0.01 N standard sodium thiosulfate solution, 6 N HCL, 0.01 N iodine solution, and starch indicator. Compounds were added to the biosensor solution in accordance with the standard titration method. In this manner, the following equation was used to calculate the dissolved $H_2S$ concentration

$$H_2S = \frac{1000 \times 0.163(\text{ml blank titrate} - \text{ml sample titrate})}{\text{ml sample}} \quad (4)$$

In order to reach the desired concentration of $H_2S$ in the solution, the Guess-and-error method was used. In other words, $H_2S$ was injected to the system for 2 min in all experiments; however, the flow was varied between 2.3 and 9.1 (ml/min) by the mass flow controller, and the solution was titrated by the previous method.

#### 2.3.5. Geometrical shape and experimental set-up

The effect of shape was investigated through two different volumes of 300 and 500 ml. The experimental set-up is shown in Fig. 1. Air was supplied by a silent air compressor (Durr Technik, Model Sicolab, Germany) and was passed through a filter to remove the impurities. Air flow was measured by a rotameter (Omega, Model FL1500, England). The connections, tubing, and fittings were all made of Silicon. In all experiments normal saline was used as the solvent solution, where air and hydrogen sulfide were injected to and immobilized beads were immersed in it. Normal saline has a suitable osmotic pressure and a pH in the range of 7, which completely matches the value for *T. thioparus* (Ma et al., 2006; Ramirez et al., 2009). After that normal saline was completely saturated with oxygen, air flow was cut off and injection of hydrogen sulfide was initiated using a mass flow controller (Unit, Model 1661, USA). In all experiments an agitator with a constant speed was used and the dissolved oxygen was also measured by a portable dissolved oxygen meter (Mettler Toledo, SevenGo pro, Germany). The reaction chamber was a bilayer bioreactor in which the temperature was kept constant at 32 °C. By the time at which the desired $H_2S$ concentration was reached, flow of $H_2S$ was cut off and cells were pour into the solution. Eventually, the reduction of dissolved oxygen was recorder by the dissolved oxygen meter.

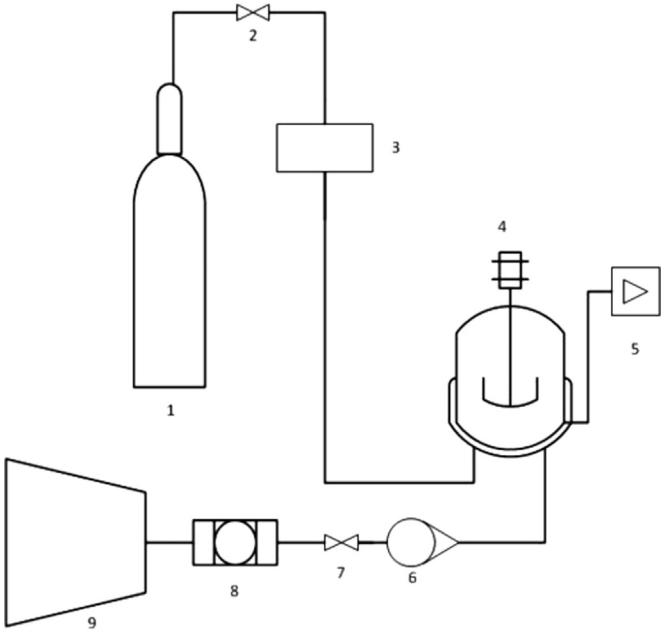


**Fig. 1.** Experimental set-up of the $H_2S$ biosensing system. (1) $H_2S$ reservoir; (2) needle valve; (3) mass flow controller; (4) Bi-layer bioreactor with Rushton turbine impeller; (5) Dissolved oxygen meter; (6) rotameter; (7) needle valve; (8) air filter and (9) air compressor.

### 2.4. Control tests

Two control tests were also carried out to investigate the respond of the system in absence of hydrogen sulfide and immobilized beads. First, 10 ppm of hydrogen sulfide was injected to the system in the absence of immobilized cell beads. Second, the cell beads were pour in the solution in absence of $H_2S$. It was observed that for both tests the reduction of dissolved oxygen was negligible in comparison with the actual tests.

### 2.5. Optimizing the design parameters

The optimization process for investigating the effect of four previously discussed parameters was carried out by the application of CCD method. The procedure for each immobilization bed consists of two steps: first, the simultaneous optimization of the bead and cell concentration; second, testing best results in different $H_2S$ concentrations and geometrical shapes. In the long run, to consummate our experiments thoroughly, the optimized results of the biosensor were tested in different temperatures, pH and agitation speeds; in order to analyze the effect of these parameters on the response time of the system, as they are some of the crucial factors influencing the efficiency of a biosensor.

### 2.6. Immobilized beads resistance

To investigate the resistance of the cells, an experiment under rough conditions using a 6-blade turbine at 50 °C and pH 4.2 was carried out on the immobilized beads. This procedure was done on both sodium alginate and agarose surfaces. The agitation speed was controlled using a tachometer (Davis instruments, Model DO-87700-01, USA) and was varied from 100 to 500 rpm. The beads were agitated in the bioreactor for 10 min. The cells were then used again in the biosensing system to determine the capability of the cells for oxygen consumption after this experiment.

### 2.7. Counting immobilized cells

In order to count the number of immobilized cells on each bed, cell counting was done before and after immobilization using a Neobar lamb under microscope (Olympus, Model CX21, Japan).

### 2.8. Beads preparation for scanning electron microscopy

Immobilized beads were stored in 1 M phosphate buffer solution for 12 h at 4 °C in order to be stabilized. They were washed with phosphate solution and dehydrated with different concentrations of ethanol (50%, 70%, 80%, 90% and 95%). Afterward,

they were washed again for 3 times with pure ethanol and incubated for 2 h at 30 °C to complete the dehydration process. Finally, they were cut into two pieces and were gold-coated and analyzed using SEM (Hitachi, Model S4160, Japan).

### 2.9. Fractal analysis for binding and dissociation rate of analyte to receptor

One of the pivotal factors that must be considered when designing biosensors, is the diffusional limitations and heterogeneity on the receptor surface. It must be noted that former to immobilization of receptors, the surface must be cleaned in order to remove any contaminants. By doing so, mass transport limitations will be eliminated or minimized if the system is either properly designed or properly operated (Halvin and Ben-Avraham, 2002; Giona, 1992). Nevertheless, there are still some restrictions for diffusion and heterogeneity considerations on the analyte surface. One approach for considering the effect of these two parameters is by using fractal analysis. In this manner, the diffusion of a particle within the media can be calculated by (Halvin and Ben-Avraham 2002)

$$r^2(t) = 2dD(t) \tag{5}$$

where $d$ is the dimensionality of space, $D$ is the diffusion coefficient, $r(t)$ is the distance which the particle passes through surface hole. Generally, there are two types of diffusion within porous structures: *anomalous diffusion* and *trapped diffusion* in which the particles are trapped in the surface holes in the latter one (Harder et al., 1987; Havlin, 1989). The diffusion coefficient is computed as follows:

$$D(t) = \frac{1}{4}t^{(2/Dw)} \tag{6}$$

For this purpose, fluorescence correlation spectroscopy (FCS) can be used to analyze the diffusion of the particles in solution. FCS is mainly employed in the field of optical microscopy. The technique analyzes the measured fluorescence intensity fluctuation by focus of light. By this method, both the concentration and size of the particles are determined. Many other approaches have been used for analyzing diffusion of particles in different media, such as agarose, however, most of them had some disadvantages (Hirota et al., 2000). FCS method has a great privilege of being used locally, meaning that not all the surface is needed to be analyzed (Winchil et al., 1982). FCS also has an advantage over other methods which is its complete selectivity to the fluorescently-labeled diffusing particles in the range of 1–150 nm (Berland et al., 1995; Schwille et al., 1999). By the assumption of *anomalous diffusion* being dominant in the solution, the FCS autocorrelation function can be described as (Aragon and Pecora, 1976)

$$G(\tau) = \frac{\left(1 - F_{tr} + F_{tr} \times e^{\left(\frac{-\tau}{\tau+T}\right)}\right)}{2\sqrt{2}N \times (1 - F_{tr})(1 + (\tau/\tau_c))\sqrt{\left(1 + \left(\tau/p^2\tau_c\right)\right)}} \tag{7}$$

where $F_{tr}$ is the triplet fraction, $\tau$ is the delay time, $\tau_c$ is the mean time that particle spends in the sample volume, $N$ is the mean number of particles in the sample, and $p$ is the ratio of longitudinal to transverse radius of the sample volume ($p = w_z/w_{xy}$). According to the above explained parameters, the present study can also be carried out as a new concept in devising new and optimized biosensor systems based on the diffusion rates and improvement of the sensitivity of the biosensor. By solving Eq. (7) based on the introduced parameters, it was found that by increasing the surface area of the beads, better diffusion of oxygen into the immobilized cells will happen, which consequently yields better response times for the biosensor. This was a big consideration through all experiments, meaning that the effort was mostly through expanding the fraction of surface to volume of immobilized beads. It was figured out that beads with larger surface areas presented a better performance in the system for consuming oxygen (Fatin Rouge et al., 2004).

## 3. Results and discussion

### 3.1. Investigating optimum time for cell harvesting

*T. thioparus* growth curve was plotted using optical density method in 605 nm. Also, in order to find out the best time of cell harvesting, different intervals of 56, 67, 96 and 120 h after incubation was chosen. It was observed that cells taken out of incubator after 96 h presented the foremost rate of oxygen consumption (Fig. 2).

### 3.2. Cell and BEd concentration optimization process

#### 3.2.1. Optimization of cell and BEd concentration on sodium alginate

In this stage two parameters including cell and bed concentration were optimized by the CCD method with a total number of 9 tests. The concentration of sodium alginate was 2–4% (w/v). It must be noted that concentrations above the mentioned levels, prevent oxygen to reach the cell surface (Yujian et al., 2006). Moreover, the $H_2S$ concentration was kept on constant value of 10 ppm through all experiments. The quadratic equation showing the calculated response time is given by Eq. (8). In this model, A and B stand for bed and cell concentration respectively, and P represents the importance of these factors

$$R_1 = 171 + 20.51A - 33.43B - 11.19A^2 - 22.44B^2 \tag{8}$$

Two other factors, namely $R$-Square and $P$-value were also incorporated. Values of $R$-Square near 1 showed a good compliance of theoretical and experimental results. Fig. 3a displays the effect of two parameters including bed and cell concentration, on the response time of the biosensor. Moreover, it displays a comparison between the actual and predicted response times of the biosensor (Fig. 3b). As it can be seen, the difference is less than 9% in all experiments. Optimized parameters are cited in Table 1. It was also observed that by increasing bed concentration up to a specific value (optical densities near 8), better response times were obtained. Nevertheless, increasing to more than this value resulted higher response times which were not preferable. The reason lying behind the increase of response time is that the bed acts as a barrier for oxygen to reach the cell surface, which is also cited in

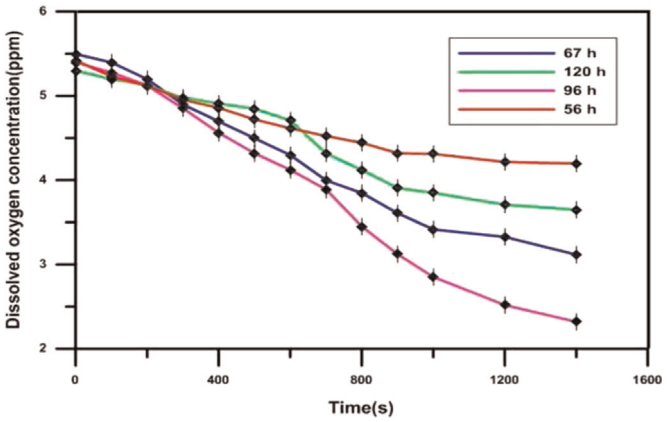


**Fig. 2.** Optimum time for cell harvesting.

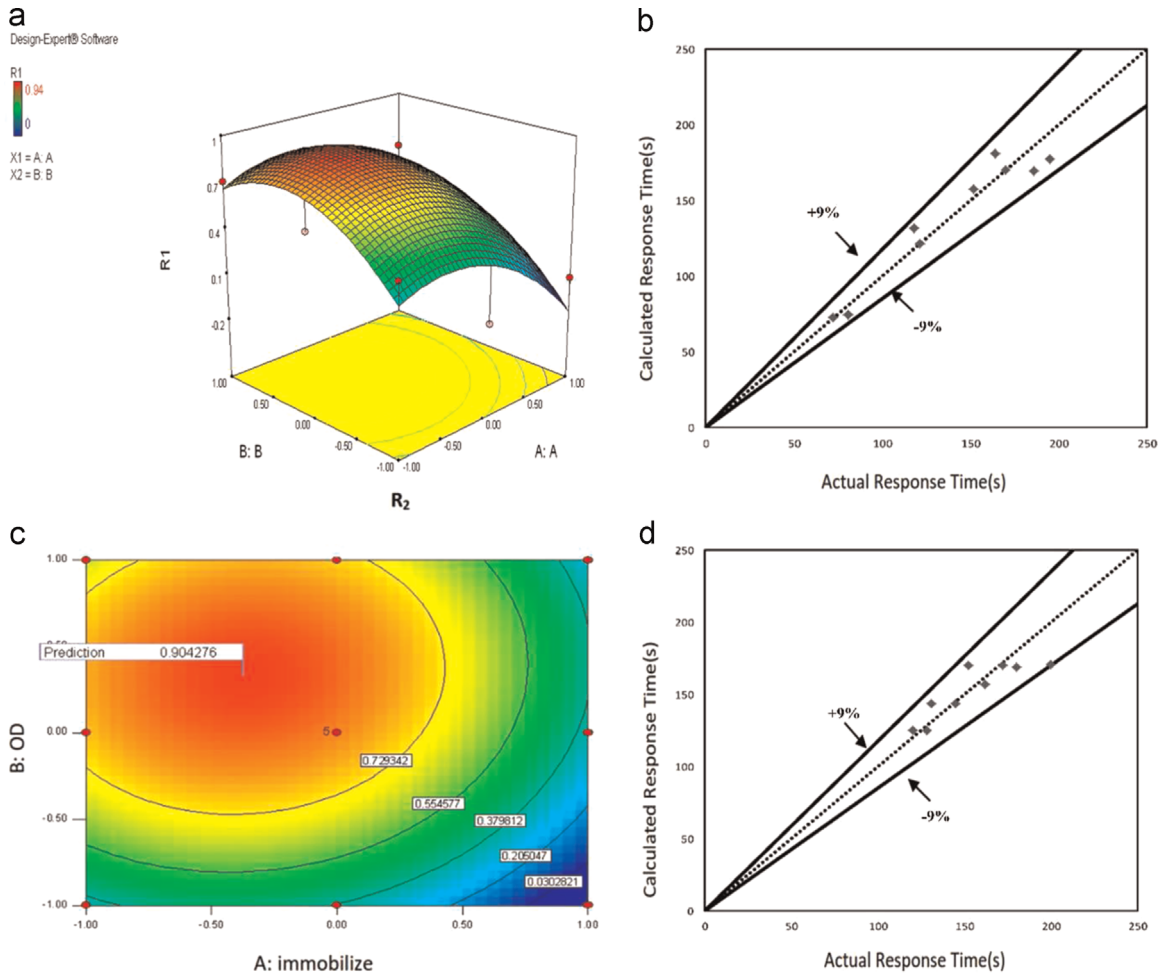


**Fig. 3.** (a) Effect of sodium alginate and cell concentration on response time. (b) Compliance of actual and calculated response times for sodium alginate. (c) Effect of agarose and cell concentration on response time. (d) Compliance of actual and calculated response times for agarose.

**Table 1**
Optimized parameters for sodium alginate and agarose bed.

| Surface | Model | Mean square | *F*-value | *P*-value |
|---|---|---|---|---|
| **Sodium alginate** | A | 3364.71 | 24.83 | 0.0016 |
| | B | 8939.27 | 65.96 | 0.0001 |
| | AB | 289 | 2.13 | 0.1876 |
| | $A^2$ | 870.68 | 6.42 | 0.039 |
| | $B^2$ | 4154.38 | 30.65 | 0.009 |
| **Agarose** | A | 687.9 | 8 | 0.0254 |
| | B | 4121.45 | 47.95 | 0.0002 |
| | AB | 100 | 1.16 | 0.3165 |
| | $A^2$ | 250.43 | 2.91 | 0.1316 |
| | $B^2$ | 43.48 | 0.51 | 0.4999 |

other similar experiments (Mirzaei et al., 2014; Ebrahimi et al., 2014). Meanwhile, increasing the cell concentration from 4 to 8 resulted to less and better response times.

### 3.2.2. Optimization of cell and bed concentration on agarose

Agarose has high porosity for cell immobilization (Parkash and Jaisawal, 2011). In the present section, the effect of bed and cell concentration were also investigated by the CCD method. All the parameters are same as the previous section. The effect of the two parameters on the response time as well as the comparison between experimental and predicted data are depicted in Fig. 3c and d, respectively. It has to be noted that the agarose concentration was 1–3% (w/v). Optimized parameters are also cited in Table 1. It was observed that, alike sodium alginate, by increase of cell and bed concentration up to a specific value, the response time decreases. The response time of the biosensor on agarose bed can be given with the following equation:

$$R_2 = 156.77 + 9.27A - 22.7B - 15.64A^2 - 44.56B^2 \quad (9)$$

### 3.3. Effect of $H_2S$ concentration on response time

16 experiments were designed for both systems of sodium alginate and agarose bed in order to achieve the best response time based on different $H_2S$ concentration. These experiments were again optimized by the CCD method and the resulted *R*-square value was near 1. The increase of the response time by augmenting the $H_2S$ concentration was observed. Accordingly, increment of $H_2S$ in the system which causes the waste and outgoing of the oxygen leads to lower amounts of oxygen to be present in the system as mentioned in similar works (Chung et al., 1996). Therefore, constant injection of oxygen has to be provided for concentrations of $H_2S$ higher than 10 ppm. Also, for values of $H_2S$ less than 1 ppm the response times were higher due to the lack of attainable nutrients for bacteria in the system.

### 3.4. Geometrical shape considerations

The effect of volume on the response time was investigated using two, bilayer bioreactors of 300 ml and 500 ml capacity. It was observed that the smaller bioreactor presented better and lower response times in that the oxygen and nutrients were more available for bacteria.

### 3.5. Immobilized beads resistance and viability

Agarose beads showed a better mechanical strength in comparison with sodium alginate beads. Agarose beads tolerated speeds up to 500 rpm; however sodium alginate beads were all broken down at speeds near 300 rpm due to the absence of methyl group in sodium alginate (Yujian et al., 2006; Ravichandra et al., 2009). In order to investigate the viability of cells after immobilization the colorization method with crystal violet was used. In this method the number of cells was counted before and after immobilization under an electronic microscope. It was observed that up to 90% of cells were immobilized on the surfaces.

### 3.6. Effect of temperature, pH and agitation speed on biosensor performance

#### 3.6.1. Effect of temperature and pH

One of the main criteria that must be considered in designing a biosensor is the "scale-up" ability of the system from Lab conditions to real-world conditions. Temperature and pH play a major role, as they affect many parameters, such as cell growth, hydrogen sulfide concentration, and oxygen concentration in the solution. Fig. 4a depicts the response of the biosensor to hydrogen sulfide for different temperatures between 15 °C and 50 °C. The best response time was achieved at 35 °C. The biosensor efficiency was diminished at higher and lower temperatures. The reason is principally laid under the inactivation of the bacteria at higher or lower temperature. Moreover, according to the dissolved oxygen concentration diagram, by increasing the temperature, the concentration will decrease drastically. The effect of pH was also investigated in the range of 3–10. The results are shown in Fig. 4b. The best response time was attained at the pH of 7. This is completely dialectic, since the optimum pH for the growth of *T. Thiobacillus* is 7. Additionally, the acidic solution tends to erode the immobilization surface which leads to dissemination of the bacteria in the solution; as a consequence, loss of cell in the system will take place.

#### 3.6.2. Impeller design and agitation speed optimization

Oxygen adequacy in the designed biosensor is of vital importance, since the detection signal is based on this factor. The oxygen transfer to the cells is a function of many variables in stirred tank bioreactors. Among all, the speed and type of impeller play the most significant roles. Fujasova, has studied the efficiency of seven types of impellers in 29 configurations (Fujasova et al., 2007). They have revealed that Rushton turbine with 6 vertical blades had the best performance in oxygen transfer rate. The studies were based on computing the value of $K_La$, which is the most reliable procedure in studying oxygen transfer. Hence, a 6 blade Rushton turbine impeller was used in the biosensing system. In order to peruse the effect of agitation speed, 10 different speeds between 100 and 900 rpm were tested, and the rate of oxygen consumption versus agitation speed was plotted. From the theoretical point of view, the higher the residence time and surface area of the bubbles mean a more efficient oxygen transfer in the bioreactor (Amaral et al., 2008). As it can be observed in Fig. 4c, the rate of oxygen consumption increases as the agitation speed is picked up; nevertheless, it suddenly waned after 600 rpm. It was seen that at speeds higher than 600 rpm, the bubbles were rapidly lost out of the system implying less residence time. Furthermore, the bubbles which were created at these speeds had a diminutive size, which also means less surface area. Therefore, our observations completely matches the theoretical part of the issue. The results are also in complete agreement with the same studies (Chen and Chen, 1999).

### 3.7. Biosensor stability

In order to determine the stability of the biosensor, bacterial beads fabricated at optimum conditions were washed with physiologic serum and stored at 4 °C. The sensitivity to $H_2S$ concentration and oxygen consumption of both types of beads were assessed 5, 10, 20, 30, 45, and 60 days after fabrication. Immobilized *T. thioparus* in sodium alginate and agarose retained 99%, 70%, and 37.39% of their activities after ten days, one month, and two months respectively. On the other hand, agarose showed a better stability as a result of its higher pore numbers. Moreover, better entrapment of bacteria was resulted compared to sodium

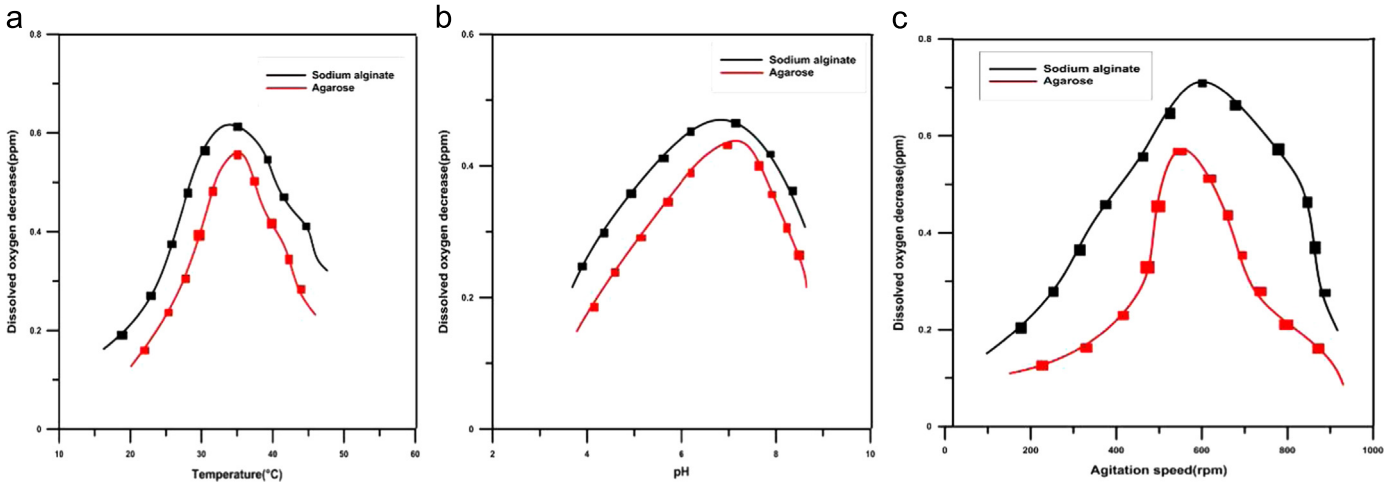


**Fig. 4.** (a) Effect of temperature on biosensor performance. (b) Effect of pH on biosensor performance. (c) Effect of agitation speed on biosensor performance.

alginate. This conclusion can be completely confirmed by considering this fact that sodium alginate provided a better response time owing to its less stiffness in its structure for bacteria entrapment and therefore more availability of oxygen to cell. On the other hand, agarose beads had better stability and also better resistance, mainly because of higher firmness for bacteria entrapment in its pores, but conversely, worse response times.

### 3.8. Determination of biosensor specificity through sulfate metabolites

Specificity can be best expressed as the Biosensor's "Discrimination threshold" (Esteban et al., 2013). Biosensors specificity chiefly depends upon the choice of biological receptor and transducer. Many enzymes are specific. Meanwhile, bacteria, yeast and tissue cultures are generally non-specific; however, most of the transducers, such as oxygen electrodes, pH electrodes and ISFETs show appropriate specificity. This enhanced specificity can be modified when these transducers are associated with receptors (Umezawa et al., 1995). Choosing a specific receptor for the biosensor is the key factor which can augment the specificity of it to a great extent. Hence, an approach has to be designed by deployment of both receptor and transducer in order to allege this issue.

The genus *T. thioparus* was first introduced by Beijernick in 1904 in order to characterize two species of chemolithiautotrophic sulfur oxidizing bacteria, *T. thioparus* and *T. denitrificans.* This strain of bacteria obtains its required energy from the oxidation of inorganic compunds, in the present case, sulfur compounds. The largest consumer of inorganic sulfur compounds is *T. thioparus* which oxidizes a wide variety of inorganic sulfur compounds, mainly thiosulfate and sulfate (Beijernick, 1904; Starkey, 1934; Fromageot, 1947). Hence, they are an appropriate choice for the study of inorganic sulfur metabolism. Based on the work done by vishniac, thiosulfate oxidation cycle with the aim of *T. thioparus* consists of the following reactions (Vishniac 1952):

$$S_2O_3^{2-} + \frac{1}{4}O_2 + \frac{1}{2}H_2O \rightarrow \frac{1}{2}S_4O_6^{-} + OH^{-} \quad (10)$$

$$3S_4O_6^{-} + \frac{5}{2}O_2 + H_2O \rightarrow 4S_3O_6^{-} + 2H^{+} \quad (11)$$

$$5S_2O_3^{-} + 4O_2 + H_2O \rightarrow 6SO_4^{-} + 4S + 2H^{+} \quad (12)$$

$$2S_4O_6^{-} \rightarrow S_5O_6^{-} + S_3O_6^{-} \quad (13)$$

$$S_5O_6^{-} \rightarrow S_4O_6^{-} + S \quad (14)$$

$$S_2O_3^{-} \rightarrow S_4O_6^{-} \begin{cases} \rightarrow S_3O_6^{-} \\ \rightarrow S_5O_6^{-} \rightarrow S \end{cases} \quad (15)$$

According to the above mentioned pathway, *T. thioparus* oxidizes thiosulfate with the intermediate formation of tetrathionate and thritionate; furthermore, by continuation of the reaction, precipitated elemental sulfur is produced in the *T. thioparus* culture.

Despite the fact that thiosulfate and sulfate are the main energy sources for *T. thioparus*, the strain can also make advantage of other sulfur compounds such as hydrogen sulfide (Kubo et al., 1995). As mentioned earlier, hydrogen sulfide has a high degree of solubility in normal saline. The reaction for the latter process is as follows:

$$H_2S_{(g)} \overset{K1}{\Leftrightarrow} H_2S_{(aq)} \quad (16)$$

$$H_2S_{(aq)} \overset{K2}{\Leftrightarrow} H^{+} + HS^{-} \quad (17)$$

$$HS^{-} \overset{K3}{\Leftrightarrow} H^{+} + S^{2-} \quad (18)$$

Based on the work done by (Suleimenov and Krupp, 1994), hydrogen sulfide has a high value of Henry constant $K_H$, up to 90 °C, which means it completely dissolves in normal saline. According to this the experiment, the value of $K_1$ in the above reaction, is about 5 times greater than $K_2$ and $K_3$; therefore, most of the hydrogen sulfide present in the system is in the form of aqueous hydrogen sulfide.

Bacteria that oxidizes sulfur-containing materials exist in both oxic and anoxic environments. The ones living in oxic conditions go through the above reactions. The bacteria will oxidize hydrogen sulfide until it runs out and then begin utilizing elemental sulfur. The logical explanation of this fact is that more energy can be acquired from oxidizing hydrogen sulfide in comparison with elemental sulfur (Ollock and Knox, 1943). *T. thioparus* can also obtain its required energy from sulfate oxidation; nevertheless there still exists an obstacle. Due to low reduction potential of Sulfate, its reduction generates low amount of energy. Hence, it is not practical to fix carbon using sulfate reduction as an energy source. Accordingly, in places where both $H_2S$ and $SO_4^{2-}$ are present simultaneously, *T. thioparus* prefers to use $H_2S$ rather than $SO_4^{2-}$. It also can be explained by comparison of Gibbs free energy law. The $|\Delta G|$ for oxidizing $H_2S$ is much larger than $|\Delta G|$ for oxidizing $SO_4^{2-}$(Oelkers and Helgeson, 1995), hence, the first reaction is much more desirable for the bacteria.

Apart from the theoretical approach, a practical approach was also designed to investigate the effect of interfering anions such as $SO_4^{2-}$, $Fe^{3+}$, $N_2$ and $CO_2$ gases, which are among the most common parasites and common gases present. It was observed that these anions and gases had less than 14% interference in the response times which completely avers the works done by same researchers (Motallebi et al., 2014; Kariminia et al., 2013).

Eventually, one can imagine that our biosensor is also sensitive to thiosulfate. This is to some extent true because as mentioned earlier, the best source of energy for *T. thioparus* is first of all thiosulfate. Apart from this fact, we must consider the place where our biosensor was designed to perform. The hydrogen sulfide biosensor was crafted to fulfill the necessities required in gas and petroleum industry. As the nature of such industries, hydrogen sulfide is the dominant sulfur compound present in these areas, compared to thiosulfate or others. Therefore, there would also be no conflict in this part too.

### 3.9. Comparison of the optimized biosensing systems with other biosensors and chemical sensors

Despite the fact that many researches have been done on biosensing systems, a small number of them are allocated to $H_2S$ detection in detail, as it was discussed here. Methane biosensing system, showed lower response times which was a result of using free cells; nevertheless, the use of free cells makes biosensors uneconomical (Damgaard et al., 2001; Karube et al., 1981). Polyvinyl alcohol was used as the immobilization bed which caused high initial lag time due to the high mass transfer resistance of PVA matrix (Mirzaei et al., 2014; Wen et al., 2008). Surface adsorption method was utilized for immobilization and a relatively low response time was achieved. However, microorganisms immobilized by surface adsorption for biosesning purposes possess lower stability than entrapped cells (Ebrahimi et al., 2014; Akyilmaz et al., 2006). A swift and sensitive inhibitor enzymatic based, hydrogen sulfide biosensor was also designed recently (Motallebi et al., 2014). In comparison with our biosensor, it has better

response time (55 s) and in some aspects better selectivity. Meanwhile, its disadvantages compared to a cell based biosensor is to great extent. First off, in spite of better selectivity and response time in enzymatic based biosensor, the cost and time consumption for manufacturing is much higher than a microbial one. Moreover, the reproducibility and life-time of a microbial biosensor is much better than an enzymatic one. The enzymatic biosensor retained only 73% of its original activity in comparison with the 99% of the microbial one. This characteristic makes the microbial biosensor more practical and useful in "situ-world" conditions, a parameter in which does make these devices to become more useful from the practical point of view. Fengjiao et al. have propounded a novel quartz crystal microbalance (QCM) biosensor for detection of hydrogen sulfide (Fengjiao et al., 2008). The biosensor was based on the piezo-immune method. The detection technique do have some analogy with the present study, that is, the response is introduced by the decline of frequency which is recorder by an oscillator.

Recently, a novel method has been propounded for hydrogen sulfide removal from a gas stream (Kim et al., 2008). They have designed a packed biofilter with sulfur oxidizing bacteria immobilized on sodium alginate and PVA. The immobilization method is same as ours, except mixing with the PVA. Moreover instead of using a mold tray for preparation of immobilized beads, we have employed a syringe pump in order to have more homogenous and regular beads. They have reported the efficiency removal of 45–100% for different loading rates of $H_2S$ between 0.1 and 13 g $H_2S\ m^{-3}\ h^{-1}$. They contend that due to immobilization, the biofilter was resistant to shock loads. Comparing to our procedure, it lacks rapid and fast response times to the existence of $H_2S$. Consequently, it needs a lot of preparation and set-up in order to run the system. Our biosensor mostly benefits from the smooth and handy set-up.

A more significant fact is the ability of the biosesnors to overcome the defects of the analogous chemical sensors. Hydrogen sulfide chemical techniques can be divided into three main categories: semiconducting metal oxide sensors, electrochemical sensors and optical sensors (Pandey et al., 2012). Semiconducting metal oxide sensors are very advantageous, due to their small size, simple construction, low weight, low power consumption, and low cost (Fine et al., 2010). Tanda et al. have constructed a portable sulfide monitor with a zinc-oxide (ZnO) semiconductor sensor for daily field studies. These authors were able to detect $H_2S$ samples within the range of 20 ppb along other reduced sulfur compounds (Tanda et al., 2007). Hence, the lack of accuracy for the specificity of some of the mentioned chemical sensors are felt. One of the other generally used chemical sensor for hydrogen sulfide is the $SnO_2$(or droped $SnO_2$) films, beside their advantages as small size, simple construction, low weight and low cost, they suffer mainly from limited specificity and selectivity against interfering gases such as $NO_2$(Vaishampayan et al., 2008). Therefore, most of the designed chemical sensors do act much better in their response time and detection range compared to biosensors, but they lack specificity as mentioned. Therefore, the future inquiries should be centered on optimizing the receptor, which is the bacteria in the present research. For instance, changing the media culture, ambient conditions, and immobilization bed. All these procedures where aimed to minimize the response time as we did. Nonetheless, still a long, but bright path is ahead for the future researchers.

### *3.10. Investigation of immobilized beads by Scanning electronic microscopy (SEM)*

SEM was utilized to investigate *T. thioparus* immobilization on both beds. As it can be observed in Fig. 5, polymeric beds possessed micro pores which provided proper place for microbial immobilization as well as substrate entrance. Rod-shaped *T. thioparus* can be clearly observed in Fig. 5a, at 5000× magnification. In both beds proper size of bacteria (1 μm length), entrapment, and dispersion was achieved. The photos clearly shows that sodium alginate has less pores for cell entrapment compared to agarose resulting less stability and better response times. On the contrary, agarose has more available pores for bacteria to be entrapped which leads to better stability and less availability of oxygen for cells and at last, worse response times compare to sodium alginate.

## 4. Conclusion

In this study, *T. thioparus* was immobilized on sodium alginate and agarose by entrapment method, and two proper $H_2S$ biosensing system were fabricated. The effect of four parameters including cell, immobilization bed, $H_2S$ concentration, and geometrical shape, on the response time of the system was investigated. The biosensor was optimized based on response time. Consequently, sodium alginate concentration of 3% (w/v) and optical density of 10 was determined as optimum condition for sodium alginate biosensor. Agarose concentration of 1.29% (w/v) and optical density of 10.83 was also found as optimum condition for agarose biosensing system. The response time of sodium alginate

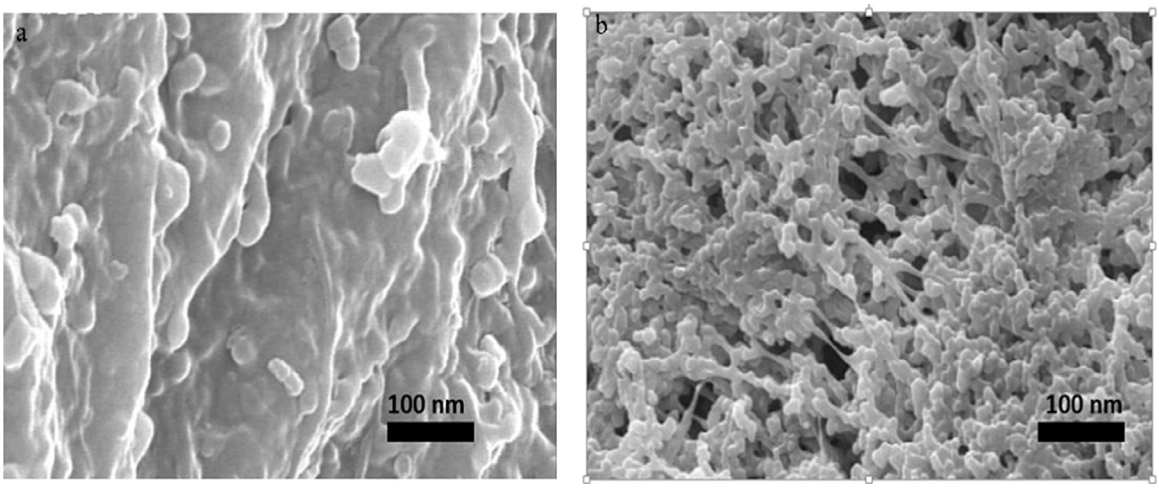


**Fig. 5.** SEM photography of immobilized *T. thioparus* on; (a) Sodium alginate bed. (b) Agarose bed.

biosensor and agarose biosensor was 72 s and 120 s, respectively, which shows a better response time for the sodium alginate bed. This was mainly achieved by more oxygen reaching the cell surface. It was observed that the biosensor could detect as low concentrations as 0.5 ppm. Also it was found that $H_2S$ has a good solubility in the normal saline and around 80% of the $H_2S$ injected to the system was dissolved in the solution. The immobilized beads of each system also presented a proper stability. The activity of the beads was reduced to 90% of the original activity, after two weeks of operation. Moreover, the biosensor showed a great specificity and selectivity in sensing the target. Besides, the response of the biosensor in different temperatures, pH and agitation speeds was also analyzed. The interfering anions and gases did not have a great impact on the response time of the biosensor. Comparison of technical data with previous studies also showed that both biosensors possess proper characteristics including, ease of operation, cost-effectiveness, short response time, and long stability.

## Acknowledgment

This project was supported financially by the Research Center for New Technologies in Life Science Engineering, University of Tehran.

## Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.bios.2015.03.025.

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