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Isolation of sulfur oxidizing bacteria from polluted water and screening for their efficiency of sulfide oxidase production

Yousef N.*, Mawad A., Aldaby E. and Hassanein M.
Department of Botany & Microbiology, Faculty of Science, Assiut University, 71516 Assiut, Egypt
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*to whom all correspondence should be addressed: e-mail: naeima@aun.edu.eg
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Abstract

Presence of sulfide in the environment represents huge concerns to biological life. The high costs and low safety of chemical and physical removal strategies lead to finding alternative strategies. Sulfur-oxidizing bacteria (SOB) are consider a promising alternative strategy, where they play a critical role in removal of such compounds from water and soil environments, as well as, they maintain the sulfur balance during natural sulfur cycle. The main objective of this study was to isolate sulfur oxidizing bacteria (SOB) from sulfide polluted water. In addition screening for sulfide oxidase producer bacteria was performed. Twenty isolates of sulfur oxidizing bacteria were isolated from eight different sulfide polluted water sources. During testing the isolates on thiosulfate broth medium, it was observed that, eleven isolates could efficiently reduce the pH of the media from 7.5 ± 0.2 to 5.0 ± 0.5 as a resulting of the oxidizing of sulfides to sulfate ion. The concentration of produced sulfate ion ranged from 76 to 155 mg/mL. The sulfur oxidase activity of the tested isolates ranged between 2.68 to 5.23 U/mL. The bacterial isolates were identified as *Bacillus* spp., *Pseudomonas* spp. and *Klebsiella* spp. based on their morphological and biochemical characterization.

Keywords: Sulfur-oxidizing bacteria, polluted water, biochemical characterization, sulfide oxidase, sulfate ion.

1. Introduction

Sulfide is generated as one of the waste chemicals in some industries, such as tanneries, rayon textile production, paper manufacturing, liquefied petroleum gas plants, petroleum and natural gas extractions and refining, agricultural and waste disposal produced inorganic sulfur compounds to the environment as by products (Park *et al.,* 2002). In nature, sulfur compounds are formed during various reactions, such as through volcanic eruptions, bacterial processes, evaporation of water or decay of dead organisms. Serious problems of sulfur are in reduced form as sulfides (Roth *et al.,* 1995). Hydrogen sulfide is widely known as the most undesirable component of biogas that causes not only serious sensory and toxic problems, but also corrosion of concrete and steel structures (Ruby *et al.,* 1981). Pulping site, petroleum refinery plant, sewage treatment facility and livestock rising farm emit very offensive odors and most of them contain sulfur compounds (Park *et al.,* 2002). Sulfides are detrimental agents in the environment because of their toxicity to human health and corrosive effect on concrete and metallic infrastructure, in addition to their strong unpleasant smell (Oprime *et al.,* 2001). Therefore, removal of sulfur compounds especially in the waste is necessary. Biological sulfide oxidation is currently the most widely used process for the treatment of sulfide wastewater (Liao *et al.,* 2008; Sorokin *et al.,* 2008). The oxidation reactions are performed mainly by sulfur oxidizing prokaryotes from the domains Archaea and Bacteria (Friedrich *et al.,* 2001). Recently, many new biotechnological approaches have been developed based on the biogeochemical cycle of sulfur in nature. Thus, sulfur-oxidizing bacteria play a critical role in these technologies (Pokorna and Zabranska, 2015).

Sulfur-oxidizing bacteria (SOB) play a vital role in bioremediation of sulfide-rich wastewater (Luo *et al.,* 2011). Distribution and abundance of SOB responsible for sulfide oxidation are of great importance for optimization of the treatment systems for sulfide biological degradation (Luo *et al.,* 2011). Different species of SOB apply several enzymes, pathways, and mechanisms of electron transport and energy conservation for oxidation of sulfide. Sulfur-oxidizing (Sox), dissimilatory sulfite reductase (Dsr), and sulfide quinone oxidoreductase (SQR) enzyme systems have been reported to associate with sulfide oxidation (Friedrich *et al.,* 2001; Sander *et al.,* 2006; Chan *et al.,* 2009).

Sulfide oxidase produced by sulfur-oxidizing bacteria (SOB) can be useful in deodorizing farm animal feces (Yun and Ohta, 1997), mitigation of sulfides from effluent streams (Visser *et al.,* 1997), landfills, wastewater facilities and also oil-field brine (Gevertz *et al.,* 2000). Graves *et al.* 2017 used natural consortium of SOB to convert reduced sulfur compounds to commercial valuable gypsum enriched product. Pokorna and Zabranska 2015 revealed that the sulfur oxidizing bacteria could remove the hazardous sulfides in water or gaseous phase.

Sulfur oxidizing bacteria (SOB) are capable of oxidizing sulfide to elemental sulfur (S^0) by partial oxidation, or sulfate (SO_4^{2-}). Also, hydrogen sulfide (H_2S), thiosulfate ($S_2O_3^{2-}$) and tetrathionate ($S_4O_6^{2-}$) can be converted to sulfate. Oxidation of sulfide and intermediate sulfur compounds by SOB are crucial for bioleaching and the removal of H_2S from polluted water. The majority of heterotrophic SOB bacteria belong to the genera *Pseudomonas, Xanthomonas, Halomonas, Escherichia coli* and *Klebsiella* (Gommers and Kuenen, 1988).

The main objective of this study is to isolation and identification of potential sulfur-oxidizing bacteria (SOB) from polluted water samples and screening of sulfide oxidase activity and sulfate ion produced by isolated strains. In addition to, the optimization of thiosulfate oxidation and sulfate ion production by SOB4 is a novel strategy to understand the microbial metabolism of sulfur in polluted water.

2. Materials and methods

2.1. Collection of samples

Eight water samples were collected from different polluted water sources (sewage and industrial wastes) in Assiut Governorate, Egypt. The samples were collected in clean and sterilized bottles and stored at 4 ± 0.1°C in a refrigerator for bacterial studies.

2.2. Isolation of sulfur-oxidizing bacteria

Sulfur-oxidizing bacteria were isolated from sulfide polluted water samples by using direct plating method (Visser *et al.,* 1997). The water sample (0.5 mL) was poured onto thiosulfate agar and broth media contained 5.0 g $Na_2S_2O_3$, 0.1 g K_2HPO_4, 0.2 g $NaHCO_3$, 0.1 g NH_4Cl and 1000 ml distilled water, pH 8.0. 0.0025 g of Bromo phenol blue (BPB) was added as an indicator (Vidyalakshmi *et al.,* 2007). Then incubated at 30°C up to 72 h, the pH of the broth inoculated medium were measured at 24 hrs intervals. The bacterial isolate which were found to able to reduce maximum pH and color of the broth medium were further selected for their sulfate ion determination ability test.

2.3. Screening of sulfur-oxidizing bacteria

It was performed by inoculating bacterial inoculums into sulfur-oxidizer broth medium (Asku *et al.,*1991). The cultures were then incubated with agitation at 200 rpm at 30˚C until the optical density (OD_{600}) of the cultures reached 0.6 – 0.8. The cultures were centrifuged at 4000 rpm, 4˚C for 30 minutes. Sulfide oxidase activity and protein concentration were assessed in the supernatant. The highest sulfide oxidase producers bacterial isolates were selected for further studies.

2.4. Sulfate ion production

The amount of sulfate ion ($SO4^{2-}$) produced during growth of sulfur-oxidizing bacteria on thiosulfate broth medium was measured spectrophotometrically. Barium chloride solution (10% w/v) was added (1:1) to bacterial culture supernatant; the mixture was mixed vigorously (Cha *et al.,* 1999). The positive results were indicated by white turbidity due to barium sulfate formation which measured with spectrophotometer at 450 nm. The obtained data was compared with the calibration curve using potassium sulfate (K_2SO_4) as standard (Kolmert *et al.,* 2000).

2.5. Sulfide oxidase assay

The enzyme activity was determined quantitively by measuring the product of enzymatic reaction in the form of sulfate ($SO4^{2-}$) (Hirano *et al*. 1996) method. The reaction mixture contain 4.5 mL of 0.1 M sodium acetate buffer (pH 5.6) and 1 mL supernatant, then 0.5 mL freshly prepared sodium sulfide (Na_2S) solution (0.06 g Na_2S, 0.16 g NaOH, 0.02 g EDTA Na_2. $2H_2O$, 2 mL glycerol and 40 mL distilled water). The mixture was incubated at 30˚C for 30 minutes and the reaction was subsequently stopped by the addition of 1.5 mL NaOH (1.0 M) with mixing. Concentration of sulfate ion formed during sulfide oxidase assay was assessed through white turbidity by measuring absorbance at 450 nm using spectrophotometer. The amount of turbidity formed is proportional to the sulfate concentration in the sample. One unit of sulfide oxidase activity was defined as an amount of the enzyme required to produce 1 µmol sulfate/hour/mL (U/mL).

2.6. Identification of the bacterial isolates

The bacterial isolates were presumptively identified based on morphological examination (colony characteristics, shape, spore, motility, Gram's reaction) and some biochemical characterization which described by MacFaddin (1980) (catalase production, lactose fermentation, Indole production, starch hydrolysis, gelatin hydrolysis, Growth at different pH and temperature), and growth on differential medium (MacConkey agar, Endo agar medium). The bacterial identification was carried out following the standard methods described in Bergey's Manual of Determinative Bacteriology (Holt *et al.,* 1994).

2.7. Optimization culture condition of bacterial isolate SOB4

2.7.1. Effect of initial pH values on sulfide oxidase activity and sulfate ion production by bacterial isolate SOB4

The bacterial isolate was grown on thiosulfate broth medium at different initial pH varied between 5 and 9. The initial pH was adjusted by 0.1 HCl or 0.1 NaOH.

2.7.2. Effect of different concentrations of thiosulfate on sulfide oxidase activity and sulfate ion production by bacterial isolate SOB4

Different concentrations of thiosulfate 5, 10, 15, 20, *25* and 30 mg per liter were supplemented into broth medium in 100 ml Flasks. Media were inoculated by bacterial isolate under investigation. The flasks were then incubated at 30°C for 48 h.

2.8. Statistical analysis

Statistical analysis was performed by SPSS, version 10 for windows (SPSS Inc; Chicago, IL, USA). Basic statistical parameters (mean and standard deviation) were estimated.

3. Results and discussion

3.1. Sulfur-oxidizing bacteria isolated from polluted water

Isolation of potential sulfur-oxidizing bacteria was initiated from sewage and also industrial wastes (Sawada, 2006). The bacterial isolation was initiated by direct plating of water sample onto solid thiosulfate medium using the spread plate technique. A total of 20 colonies of sustainable sulfur-oxidizing bacteria were isolated from 8 polluted water samples. Morphologically differentiated bacterial isolates forming different colony appearance on agar medium were isolated and inoculated on thiosulfate broth medium containing bromophenol blue (BPB) as an indicator (Figures 1). The bacterial isolates were picked and streaked onto thiosulfate agar medium to obtain a pure culture (Madigan *et al.,* 2000).

Figure 1. Growth of sulfur-oxidizing bacteria on thiosulfate broth supplied with bromophenol blue as an indicator

It was found that, bacterial isolates were able to change the color of the BPB in thiosulfate broth medium by reducing the pH of the medium from initial pH 8.0. From these only 2 isolates (SOB5 and SOB9) highly decreased the pH of the medium. The SOB isolates obtained from the polluted water samples could reduce the pH from 8.0 up to 5 in thiosulfate broth within 11 days of incubation. The ability of sulfur oxidizing bacteria to reduce medium pH was also reported by Donati *et al.* (1996). The pH reduction of the growth medium by sulfur oxidizing bacteria as a result of the production of sulfuric acid and oxidation of sulfide to sulfate ion (Behera *et al.,* 2014).

In total twenty isolates were obtained from different samples of the sulfur oxidizing medium plate. From their isolates 11 isolates were selected based on their pH reduction ability on bromophenol blue containing thiosulfate broth and agar media, turning the color of media from purple to colorless (Figure 1). These bacterial isolates were considered as efficient sulfur oxidizing bacteria (SOB 1-11).

3.2. Sulfate ion production

The ability of bacterial isolates to produce sulfate ion was investigated. It could be clearly seen from Figure (2) that, among the 11 isolates tested, isolate SOB-9 showed maximum sulfate ion concentration (155.12 mg/ml) followed by the isolate SOB-4 (125.73 mg/ml), while minimum sulfate ion concentration was produced by the isolate SOB-6 (79.65 mg/ml). In this respect, Ravichandra *et al*. (2007) reported the maximum sulfate ion production 14-150 mg/ml by a *Thiobacillus* spp., these finding were relatively lower than that obtained in the present study. Similar results were obtained by Babana *et al.* (2011) who reported the highest sulfuric acid concentration (243 mg/l) by a bacterial strain ATTC55128 followed by AHB436 (230 mg/l). The current results revealed that all the SOB produced high amount sulfate ion from $Na_2S_2O_3$ supplied in the medium.

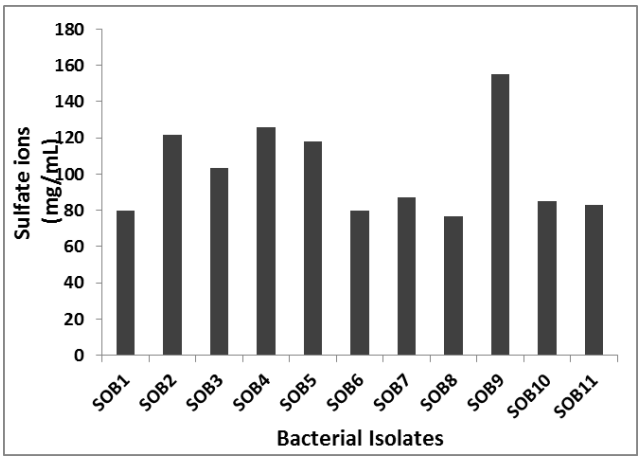

Figure 2. Sulfate ion production by eleven sulfur oxidizing bacterial isolates

3.3. Sulfide oxidase activity

Bacterial isolates were inoculated in the thiosulfate medium to detect their sulfur oxidase production (Figure 3). Generally, the enzyme activities of 11 isolates tested ranged from2.68 to 5.23 U/ml/min. From which isolates SOB9, SOB5 and SOB3 were the highest producers (5.23, 4.17 and 4.07 respectively). Rohwerdert and Sand (2003) reported that, sulfur dioxygenase activity produced by *Acidothiobacillus* and *Acidiphilium spp* of 5.0 ± 1.7 and 373 ± 90 nmol/min/mg respectively. Similarly Nakada and Ohta (2000) reported the crude sulfur oxidase extract activity of 11.7 units by *Bacillus spp.* Crude extract of thiosulphate oxidase from *Pseudomonas aeruginosa* showed maximum activity of 130 U/ml, as reported previously by Schook and Berk (1979). It is worthy to mention that, highly sulfide oxidase producers bacterial isolates (3 isolates) shown also maximum sulfate ion productions, resulting in reduction of media pH and this was clearly observed in SOB9, SOB5 and SOB4.

3.4. Identification of the bacterial strains

The eleven selected bacterial isolates were characterized by various morphological and biochemical features shown in Table (1). The Microscopic observation of the isolates revealed that most of them are rod shaped, motile and variable towards Gram's stain. Biochemical characteristics of microorganism were studied to identify the genus and species of unknown bacteria. Microorganisms are extremely versatile and their range of metabolic capabilities is very large (Norrell and Messley, 2003).

These characteristics such as, gram stain, spore formation, motility, catalase, indole, hydrolysis of gelatin and starch, can be used to demonstrate the exceptional metabolic diversity of prokaryotic organisms and aid in differentiation between closely related genera or species (MacFaddin, 1980). The bacterial isolates SOB1, SOB4, SOB7 and SOB11 were found to be *Bacillus* spp., SOB5 and SOB9 were tentatively assigned to the *Pseudomonas* spp. and SOB10 as *Micrococcus* sp., other three bacterial isolates SOB 2, SOB3, SOB8 belong to *Klebsiella* spp. Most of the heterotrophic bacteria, involve in sulfur oxidation belong to the genera *Pseudomonas* (Sorokin *et al.,* 1999), *Xanthobacter* (Cho *et al.,* 1992), *Escherichia coli* (Starkey *et al.,* 1935) were also reported earlier. Behera *et al.* (2014) suggested that the sulfur oxidizing bacteria isolated from Mangrove soil related to *Bacillus*, *Pseudomonas* and *Micrococcus* spp. Most of the heterotrophic SOB bacteria reported previously were found to belong to the genera *Pseudomonas, Paracoccus*, *Bacillus, Xanthomonas, Halomonas, Escherichia coli* and *Klebsiella* (Gommers and Kuenen, 1988; Mustafa *et al.,* 2015).

Table 1. Biochemical characteristics of Sulfur oxidizing bacterial isolates. SOB is Sulfur oxidizing bacteria,(-) is Not Detected

Characters	SOB1	SOB2	SOB3	SOB4	SOB5	SOB6	SOB7	SOB8	SOB9	SOB10	SOB11
Shape	Rods	Short rods	Short rods	rods	Short rods	rods	rods	Short rods	Short rods	cocci	Rods
Motility	-	+	+	-	+	-	+	+	+	-	-
Aerobic growth	+	+	+	+	+	+	+	+	+	+	+
Catalase	+	+	+	+	+	+	-	-	+	-	+
Gram stain	G-ve	G-ve	G-ve	G+ve	G-ve	G-ve	G+ve	G-ve	G-ve	G-ve	G-ve
Lactose fermentor	-	+	+	+	+	-	+	-	+	-	+
Indole production	-	-	-	-	+	-	-	-	+	-	-
Gelatin hydrolysis	+	-	+	-	++	-	+	-	++	-	-
Starch hydrolysis	++	-	+	+	-	+	++	++	-	-	-
Cellulase	-	-	-	+	-	-	-	-	-	-	+
Growth at pH 5-	+	+	+	+	+	+	+	+	+	+	+
Growth at 40°C	+	+	+	+	+	+	+	+	+	+	+

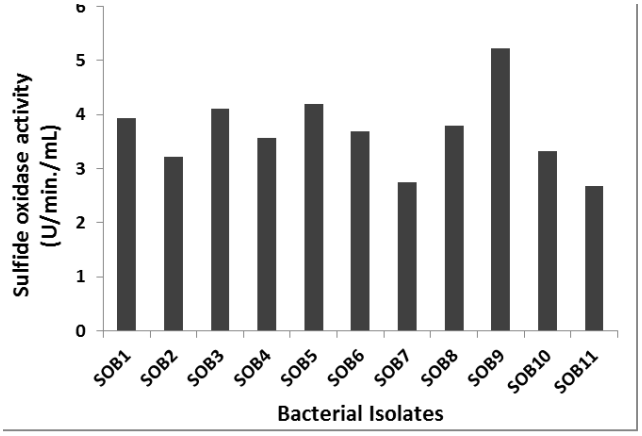

Figure 3. Sulfide oxidase activity of eleven sulfur oxidizing bacteria

3.5. Optimization culture condition of bacterial isolate SOB4

The growth rate of bacteria and their metabolism depend on the composition of the medium and the prevalent environmental conditions. Various bacterial strains differ in their nutritional requirements and cultural conditions for optimal growth and enzyme production. In the present study, an optimization for the production of sulfide oxidase enzyme by SOB4 was carried out. The study includes the effect of different pH values and different thiosulfate concentrations on sulfide oxidase activity and sulfate ion production by selected bacterial strain SOB4. SOB4 bacterial isolate was identified according to its morphological and biochemical characteristics as *Bacillus* sp.

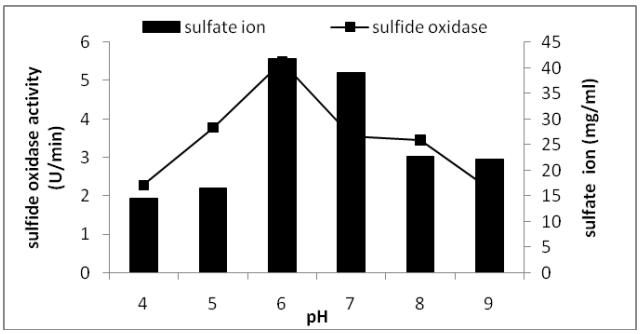

Figure 4. Effect of pH values on **s**ulfide oxidase activity and sulfate ion production of SOB4

3.6. Effect of different pH values on sulfide oxidase activity and sulfate ion production by Bacillus sp.

The concentration of hydrogen ion is the most important factor affecting the activity of sulfide oxidase enzyme as

well as the production of sulfate ion in the bacterial medium. The results in figure (4) showed that, the highest sulfide oxidase activity (5.5 U/min) was detected at pH 6.0. There was no significant difference between the values of sulfate ion concentration at pH 6.0 and 7.0 while its concentration at the first was slightly higher than the last (41.7 mg/ml). On the other hand, at high acidic and alkaline pH values, the induction of enzyme and production of sulfate ion were significantly low. Behera *et al.* (2016) mentioned that, the maximum production of sulfide oxidase by *Klebsiella* sp. was observed at pH 7.0.

From the previous results, it could be concluded that, the optimum pH value that suitable for induction of sulfide oxidase as well as production of sulfate ion was pH 6.0.

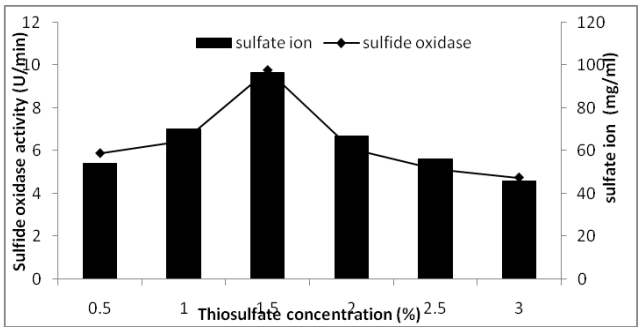

Figure 5. Effect of thiosulfate concentrations on **s**ulfide oxidase activity and sulfate ion production of SOB4

3.7. Effect of thiosulfate concentraction on sulfide oxidase activity and sulfate ion production by Bacillus sp.

Thiofulfate concentration is playing a critical role in the activity of sulfure oxidizing bacteria. This may be due to, thiosulfate is the stimulator of sulfide oxidase enzyme. The results in figure (5) showed that, the sulfide oxidase enzymes results was going parallel with the results of the produced sulfate ion. Meaning that, any increase in the enzyme activity was followed by increase sulfate ion. The optimum concentration of thiosulfate concentration was 1.5 %. At this concentration, the activity of sulfide oxidase was 9.78 U/min while the sulfate ion was 96.6 mg/ml. At 3% of thiosulfate, the lowest sulfate ion concentration was detected 45.9 mg/m. this may be due to the lowest induction of enzyme at this concentration. From the results, it could be observed that, there was a close relationship between the enzyme activity and sulfate ion production. Additionally, the thiosulfate concentration (substrate) is a critical factor that affecting the enzyme activity that subsequently affecting on sulfate ion production. Behera *et al.* (2016) observed that, The induction of sulfate ion and sulfide oxidase enzyme by *Klebsiella* sp. decrease by increasing the thiosulfate above 10 mg/ml. Many literatures mentioned that, the high concentration of thiosulfate decrease the bacterial capacity to convert it to sulfate ion because it may cause toxic affect on the bacterial growth (Skirnisdottir *et al.,* 2011).

4. Conclusion

From the previous results, it could be concluded that all tested bacterial isolates exhibited decrease in the pH value and capability to produce sulfate ion in the culture medium. The sulfur oxidizing bacteria isolated in this study by as well as the production of acid may encourage the utilization of these bacteria in reclamation of alkali soils. Using of these SOB as bio-inoculants can be incorporated to enhance sulfur oxidation in soil and to increase availability of sulfate to minimize S-fertilizers application and reduce environmental pollution and promotes sustainable agriculture.

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