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Isolation of *Ochrobactrum* sp.QZ2 from sulfide and nitrite treatment system

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ABSTRACT

A bacterial strain QZ2 was isolated from sludge of anoxic sulfide-oxidizing (ASO) reactor. Based on 16S rDNA sequence analysis and morphology, the isolate was identified as *Ochrobactrum* sp. QZ2. The strain was facultative chemolithotroph, able of using sulfide to reduce nitrite anaerobically. It produced either elemental sulfur or sulfate as the product of sulfide oxidation, depending on the initial sulfide and nitrite concentrations. The optimum growth pH and temperature for *Ochrobactrum* sp. QZ2 were found as 6.5–7.0 and 30 °C, respectively. The specific growth rate (μ) was found as 0.06 h^{-1} with a doubling time of 19.75 h; the growth seemed more sensitive to highly alkaline pH. *Ochrobactrum* sp. QZ2 catalyzed sulfide oxidation to sulfate was more sensitive to sulfide compared with nitrite as indicated by IC_{50} values for sulfide and nitrite utilization implying that isolate was relatively more tolerant to nitrite. The comparison of physiology of *Ochrobactrum* sp. QZ2 with those of other known sulfide-oxidizing bacteria suggested that the present isolate resembled to *Ochrobactrum anthropi* in its denitrification ability.

1. Introduction

Anoxic sulfide oxidation using nitrite as electron acceptor has been demonstrated in our laboratory to be a very successful biotechnological process capable of bioremediation of nitrite- and sulfide-rich wastewaters simultaneously employing mixed culture [1]. It was desirable to identify and characterize the bacterial species involved in the sulfide biooxidation found in mixed culture of anoxic sulfide-oxidizing (ASO) reactor.

Many microorganisms can use H_2S oxidation to gain energy [2]. Various groups of organisms can oxidize reduced sulfur compounds under aerobic or anaerobic conditions, including:

- Green sulfur bacteria (anaerobic, phototrophic, i.e., *Chlorobium*, etc.)
- Purple sulfur bacteria (anaerobic, phototrophic, i.e., *Chromatium*, *Thiocapsa*, etc.)
- Colorless sulfur bacteria (aerobic, i.e., *Thiobacillus*, *Beggiatoa*, *Thiothrix*, etc.)

Colorless sulfur-oxidizing bacteria are most widely used to oxidize H_2S using oxygen as an electron acceptor. This process is preferred because growth rates are significantly higher and there are no light intensity requirements. *Thiobacillus* species are thought to account for a majority of sulfide oxidation, via the sulfite-oxidase pathway [3].

Numerous other bacterial types are involved in the cycling of sulfur. For example, sulfur compounds of intermediate oxidation state (elemental sulfur, thiosulfate, and sulfite) can be actively disproportionated [4–6], and some organisms use disproportionation reactions as their sole means of energy conservation [7]. At present, all of the bacteria known to disproportionate sulfur compounds of intermediate oxidation state are found within the δ-subdivision of the purple bacteria.

The ability of organisms to oxidize sulfur compounds chemolithoautotrophically is widespread between the two prokaryotic domains (Bacteria and Archaea). Among the hyperthermophiles, deep-branching members of the Aquifex/Hydrogenobacter group in the Bacterial Domain can oxidize hydrogen sulfide using O_2 [8], as can do several known members of the Archaeal Domain [8]. Aquifex/Hydrogenobacter is a rather strange group of organisms, as its microaerophilic habitat is rather inconsistent with its deep-branching placement. In general, deeper-branching lineages house anaerobic organisms while aerobic organisms tend to occupy

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higher positions on the tree [9], reinforcing well-accepted notions [10] that life originated in an anaerobic environment. It has been suggested that the aerobic lifestyle of Aquifex/Hydrogenobacter developed over the course of evolution [11] and does not represent the ancestral metabolism of this group.

Of considerable significance for the process of sulfide oxidation is the large radiation of sulfide-oxidizing bacteria emerging from the base of the α- and β-subdivisions of the purple bacteria [12]. Most known non-photosynthetic, sulfide-oxidizing bacteria are found within these two subdivisions, and this radiation is, therefore, of some evolutionary significance. It has been proposed that evolutionary radiations of organisms are promoted by profound environmental changes [9].

1.1. Objectives of the study

Some bacterial strains belonging to *Ochrobactrum* sp. have been isolated previously capable of nitrite reduction in aerobic or anaerobic conditions [13,14]. In this paper, we report the isolation, identification and characterization of a chemolithoautotrophic sulfide-oxidizing, nitrite-reducing bacterial strain present in ASO reactor.

2. Materials and methods

2.1. Media and culture conditions

The minimal medium used for isolation contained (: $NaHCO_3$ ($1\,g\,L^{-1}$), trace element solution (1 mL), $NaHCO_3$ ($1\,g\,L^{-1}$), $MgCl_2$ ($1\,g\,L^{-1}$), KH_2PO_4 ($1\,g\,L^{-1}$), $(NH_4)_2SO_4$ ($0.24\,g\,L^{-1}$), agar (2%), $Na_2S.9H_2O$ ($512\,mg\,L^{-1}$), $NaNO_2$ ($528.75\,mg\,L^{-1}$), $(NH_4)_2SO_4$ ($0.5\,g\,L^{-1}$), KH_2PO_4 ($1\,g\,L^{-1}$), $MgCl_2$ ($1\,g\,L^{-1}$), and $NaHCO_3$ ($1\,g\,L^{-1}$). The trace element solution contained EDTA ($50\,g\,L^{-1}$, NaOH ($11\,g\,L^{-1}$), $CaCl_2{\cdot}2H_2O$ ($7.34\,g\,L^{-1}$), $FeCl_2{\cdot}4H_2O$ ($3.58\,g\,L^{-1}$), $MnCl_2{\cdot}2H_2O$ ($2.5\,g\,L^{-1}$), $ZnCl_2$ ($1.06\,g\,L^{-1}$), $CoCl_2{\cdot}6H_2O$ ($0.5\,g\,L^{-1}$), $(NH_4)_6Mo_7O_{24}{\cdot}4H_2O$ ($0.5\,g\,L^{-1}$), $CuCl_2{\cdot}2H_2O$ ($0.14\,g\,L^{-1}$), in distilled water. All experimental cultures are incubated at the fixed temperature designated by every test. The agar (2%) was added as solidifying agent.

The pH of the medium was adjusted between 7.0 and 7.5 following autoclaving and cooling. Then sulfide (1.0 mM) was added aseptically from a sterile stock solution of 1.0 M $Na_2S{\cdot}9H_2O$. The culture medium was poured in Petri plates in the presence of sterilized air and was allowed to cool down to 45 °C. The culture medium was flushed with oxygen free argon gas for 5 min to create anaerobic conditions. Then, 0.2–0.3 mL of sludge dilutions (10^{-2}, 10^{-3}, and 10^{-4}) were sprinkled over the cooled medium and incubated at 30 °C in anaerobic growth chamber.

2.2. Isolation of sulfide oxidizing–nitrite reducing bacteria

The sludge sample used to isolate the sulfide oxidizing–nitrite reducing bacterium was taken from the anoxic sulfide-oxidizing (ASO) bioreactor operated for more than 2 years in the department of Environmental Engineering, Zhejiang University Hangzhou, China. The sludge sample (5 mL) was diluted to 100, 1000 and 10,000 times. After dilution, the liquid culture was spread onto the solidified agar plates containing sulfide and nitrite using the dilution plate method, and was incubated at 30 °C for 48–72 h. Several rounds of endpoint dilution were used to further purify strains until pure isolates were obtained. The purified isolates, with higher capacity for sulfide oxidation and nitrite reduction were named as strain QZ2. Purified isolate obtained was transferred to liquid culture medium for enrichment and performing the *sulfide oxidation–nitrite reduction* ability test of the isolate. The composition of the minimal medium was same as described above.

2.3. Growth and maintenance

Routine growth and maintenance of both isolates were in mineral medium (composition described above). Stock culture of QZ2 was stored as lyophiles at −20 °C. Working cultures from the lyophiles were grown in mineral medium containing agar (2% agar). From the agar plates, the isolates were cultured into serum bottles containing mineral medium. Growth was detected from an increase in optical density (at 600 nm). In addition, growth on sulfide ($Na_2S{\cdot}9H_2O$) and nitrite ($NaNO_2$), as an electron donor and acceptor was detected as an increase in sulfate, sulfide and nitrite concentrations, respectively, relative to uninoculated controls.

2.4. Identification and characterization of bacterial isolates

2.4.1. Microscopic characterization

Colony structure and cell morphology of strain QZ2 was observed by a light microscope (Olympus BH-2, Japan) and transmission electron microscope (TEM) (JEM-1200EX, Japan). All cells used in microscopic characterization were grown in mineral medium containing sulfide and nitrite. The morphology and dimensions of isolate were determined from photomicrographs using scanning electron microscopy (SEM), and transmission electron microscopy (TEM). The widths and lengths given represent the averages of measurements of several cells. Motility and the presence of spores were determined using phase microscopy of wet mounts.

For SEM and TEM, 1 mL of QZ2 cells grown in mineral medium (containing sulfide and nitrite) for about 24 h was centrifuged at $10{,}000 \times g$ for 15 min. The pellet was resuspended in 1 mL of 0.1 M sodium phosphate (pH 7.0) containing 2.5% glutaraldehyde. After 30 min, the cells were washed three times with 0.1 M phosphate buffer and then fixed in 0.1% (w/v) osmium tetroxide. Following three more washes in 0.1 M phosphate buffer, a small portion of the cell suspension was removed and washed thrice with distilled water. This suspension was pipetted onto a glass slide. Twenty minutes later, the slide was immersed in a liquid nitrogen-cooled isopentane bath. The frozen slides were dried overnight in a lyophilizer, sputter coated with Au–Pd, and observed with a scanning and transmission electron microscope.

2.4.2. 16S rDNA sequence determination and phylogenetic analysis

Chromosomal DNA of the isolate was extracted by a slightly modified procedure of Wilson [15]. The 16S rDNA was amplified using PCR [16] with *Taq* polymerase (Boya) and the universal primer pair of 27f (5-AGAGTTTGATCCTGGCTCAG-3) and 1523R (5-GGTTACCTTGTTACGACTT-3) described by Weisburg et al. [17], and determined by the Shanghai Boya Biological Technique Company.

PCR reactions were performed in 100 μL reaction volumes containing 1 μL of Taq DNA polymerase (2.5 U/μL) (Sangon), 10 μL of 10 × PCR reaction buffer (Sangon), 2 μL of each of the primers (10 μM), 2 μL dNTPs (10 mM) (Sangon), 5 μL of the extracted DNA as the templates and 78 μL of sterile distilled water. The PCR amplification protocol was as follows: denaturation at 94 °C for 1 min, annealing at 52 °C for 1 min, and extension at 72 °C for 3 min, and all the three steps were repeated for 30 cycles.

Related sequences were obtained from the GenBank database (National Center for Biotechnology Information, NCBI) using the BLASTN search program. The 16S rDNA sequences determined and reference sequences obtained from GenBank databases were aligned using multiple sequence alignment software CLUSTAL W

ver. 1.81. Phylogenetic trees were constructed with MegAlign software of DNASTAR based on the 16S rDNA sequences of 10 strains closer to strain QZ2.

2.5. Growth kinetics

Growth tests were carried out in 65 mL serum bottles sealed with butyl rubber stoppers. Cell suspension (5%) of QZ2 strains (OD equal to 0.31) were inoculated and incubated at 30 °C in 45 mL of denitrifying medium with initial pH 7.5 containing sulfide and nitrite. The initial pH in theses vials was adjusted to 7.5 by adding Tris–HCl solutions. The bottles were flushed with oxygen-free argon to create anoxic conditions. Control experiments were carried out with cell-free medium. Three parallels were conducted for each experiment test.

2.5.1. Aerobic substrate tests

Aerobic growth in mineral medium minus acetate and nitrate was tested for the sulfide in 250-mL flasks containing 50 mL of medium with shaking on an incubator–shaker at 250 rpm.

2.5.2. Microaerophilic growth

O_2 (1%) growth with sulfide (1.0 mM) as an electron donor was tested in mineral medium without nitrite. The oxygen was added as 0.5 mL of sterile air (21% O_2) to the headspace of each bottle containing 10 mL of N_2–CO_2 (90:10, %). A control for chemical oxidation of sulfide was run in sterile mineral medium without nitrite containing 1% oxygen in the headspace.

2.6. Physiological characteristics

The isolate was tested for its ability to grow on various carbon sources and other substrates using Biolog and Vitek systems. The Vitek system (GNI+) (bioMérieux Vitek, USA) and the Biolog microstation (GN) (Biolog Hayward, CA, USA) were used for carbon source utilization and to identify physiological characteristics of the isolate.

2.7. Optimum growth pH

A 5 mL cell suspension of bacterial strain (OD = 0.3) was inoculated and incubated at 30 °C in 45 mL of sulfide–nitrite inorganic salt medium at various initial pHs of 5–11 containing sulfide–nitrite (3:8 molar ratio), respectively. The buffers (0.1 mM) used in pH experiments were citrate–phosphate (pHs 5.5 and 6.0), phosphate (pH 7.0), and Tris–hydrochloride (pH 8.5).

2.8. Optimum growth temperature

A 5 mL cell suspension of bacterial strain (OD = 0.3) was inoculated and incubated pH 7.5 in 45 mL of sulfide–nitrite inorganic salt medium at various temperatures of 20–45 °C containing sulfide–nitrite (3:8 molar ratio), respectively. The uninoculated sterile medium was used as a blank. Each test was replicated in triplicates. The OD of growth was monitored continuously. The natural logarithm of these measurements was plotted against the incubation time, and the growth rate was calculated from slopes of the plots.

2.9. Transformations of nitrite–nitrogen and sulfide–sulfur

2.9.1. Effect of sulfide concentrations

Strain QZ2 was grown in mineral medium with 92, 184, 276, 368, 460 and 552 mg S/L sulfide containing fixed nitrite concentration of 113.25 mg N/L in each culture bottle. After 48 h of incubation, samples of culture media were analyzed for sulfide, sulfate, nitrate, ammonium, pH and nitrite.

2.9.2. Effect of nitrite concentrations

The strain QZ2 was grown in mineral medium containing 113.25, 226.5, 339.75, 453, 566.25 and 679.5 mg N/L nitrite with fixed sulfide concentration of 92 mg S/L in each culture bottle. After 48 h of incubation, samples of culture media were analyzed for sulfide, sulfate, nitrate, ammonium, pH and nitrite.

2.10. Analytical methods

Ammonium nitrogen (NH_4^+–N) was measured by phenate method [18], nitrite nitrogen (NO_2^-–N) was measured by colorimetric method [18] and nitrate nitrogen (NO_3^-–N) was measured by ultraviolet spectrophotometric screening method [18] on daily basis using spectrophotometer (Unico UV-2102 PC and 722S, China). The sulfide was determined by iodometric method [18] and sulfate was measured by turbimetric method [18]. The pH measurement was performed according to the Standard Method [18]. A three-point calibration of pH meter was carried out daily.

2.11. Statistical and graphical work

Using computer program Sigma Plot v.10 carried out regression analysis, curve fittings and graphical work.

2.12. Nucleotide sequence data

The sequence determined in the present study was deposited in GenBank under the accession number EF542805.

3. Results

3.1. Characterization of isolates

The cells of *Ochrobactrum* sp. QZ2 were rod shaped with parallel sides and rounded ends measuring 0.5–1 × 1.5–2.0 μm in size (Fig. 1). Young cells were motile having a single polar flagellum, however, older cells usually non-motile. Colonies on agar medium were non-pigmented, round and small in size. Cells were oxidase and catalase negative. Growth could occur both aerobically and anaerobically, utilizing sulfide (electron donor) and nitrite (electron acceptor). The anaerobic enrichment resulted in a stable binary culture consisting of large rods with intracellular sulfur globules and younger straight rods that were occasionally motile. No spores were observed in the cells.

3.2. 16S rDNA sequencing and phylogenetic analysis

The partial 16S rDNA sequences of strain QZ2 (comprising 1394 nucleotides) were determined, and a phylogenetic tree was constructed based on 16S rDNA sequence (Fig. 2). The sequence was submitted to the GenBank database where accession No. EF542805 was allotted for the said sequences. The QZ2 strain was phylogenetically closely related to genus *Ochrobactrum*, having sequence similarities of 99%.

Presently the acceptable positional standard is that if the similarity of strain under investigation and a reference strain sequences is higher than 97–98%, they are regarded as belonging to the same genus [19,20]. Therefore, the result of this phylogenetic analysis together with that of phenotypic tests, the Vitek system, and the Biolog GP, the bacterial isolate QZ2 was identified as *Ochrobactrum* sp. QZ2.

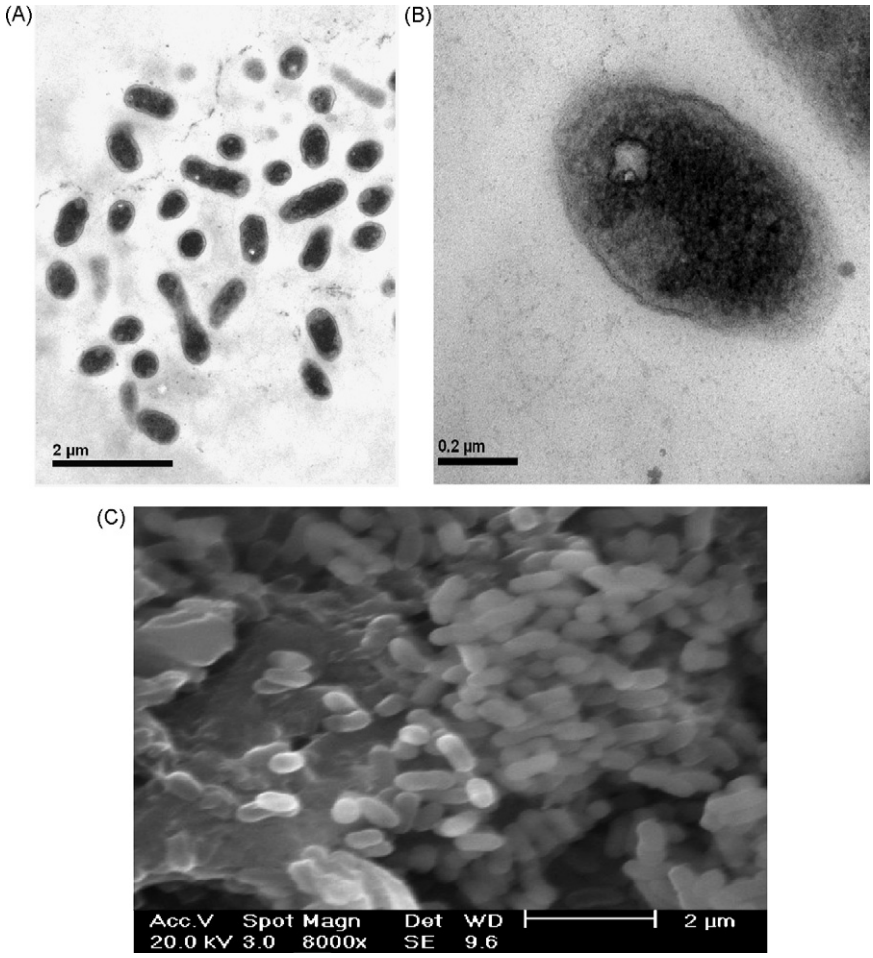

Fig. 1. Transmission electron micrograph of *Ochrobactrum* sp. QZ2 (A and B) scanning electron micrograph (C).

3.3. Physiological characteristics

3.3.1. Growth rates

As seen in Fig. 3, the OD_{600} value of the culture increased with the increase in sulfide and nitrite concentrations in medium from 0 to 0.6 for QZ2 after 72 h of incubation. The sulfide and nitrite removal was according to the reaction stoichiometry of Eq. (1), producing sulfate as the sulfide oxidation product (Fig. 4). However, complete oxidation of sulfide into sulfate was inhibited beyond certain substrate concentrations (Fig. 4). Beyond this substrate concentration, the sulfide might have been oxidized to elemental sulfur as the sulfide removal was not affected due to substrate inhibition. The correlation between sulfide and nitrite removal and bacterial growth indicated that these substrates served as source of energy for the isolates to carry out lithoautotrophic kind of denitrification:

$$3HS^- + 8NO_2{}^- + 5H^+ \rightarrow 3SO_4{}^- + 4N_2 + 4H_2O$$

$$\Delta G_m{}^\theta = -2944\,\text{kJ/mol} \quad (1)$$

Consumption of sulfide (200 mg S/L) and nitrite (250 mg N/L) in mineral medium was roughly proportional to growth for QZ2 (Fig. 3). Growth rate experiments were conducted at 30 °C and pH 7 at sulfide to nitrite molar ratio of 1.17, which was the proper proportion for simultaneous sulfide and nitrite removal using mixed

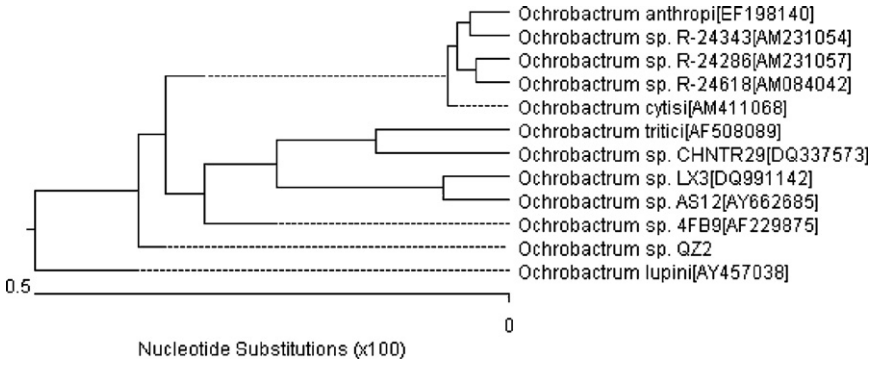

Fig. 2. Phylogenetic tree of *Ochrobactrum* sp. QZ2.

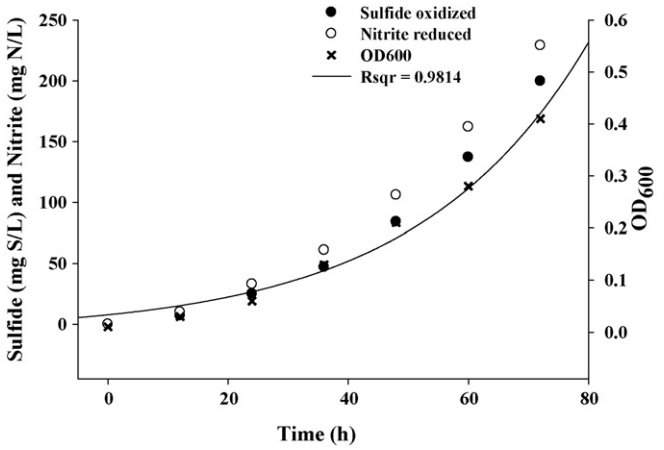

Fig. 3. The relation between growth (OD_{600}), nitrite and sulfide utilization with the passage of time growth for *Ochrobactrum* sp. QZ2.

cultures in anoxic sulfide-oxidizing reactor [1]. Fig. 3 illustrates that there was a significant ($p < 0.01$) monotonic increase in the bacterial growth rate (measured as optical density or OD_{600}) with the passage of time. Results showed that sulfide was almost completely removed by strain QZ2 within 72 h. The growth of isolate fitted well with the exponential growth rate equation as under:

$$y = ae^{bx} \quad (2)$$

where y = bacterial growth rate (OD_{600}), x = time factor, a and b = parameters of the model.

3.3.2. Effect of pH and temperature on substrate removal

The isolate was tested over a temperature range of 20 °C (i.e., the lowest tested) to 45 °C and a pH range from 6 to 11 (i.e., the highest tested). The growth and substrate removal of *Ochrobactrum* sp. QZ2 were optimal at an initial pH of 6.5–7.0 at 30 °C, but were slow at an initial pH of 8.0 or between pH 8.0 and 11.0 (Fig. 5). The strain QZ2 seemed more sensitive to highly alkaline pH.

The effect of temperature on the growth rate and substrate removal of strain QZ2 is shown in Figs. 6 and 7. *Ochrobactrum* sp. QZ2 grew well from 25 to 30 °C. Below and above this optimum temperature range growth and substrate utilization was affected badly.

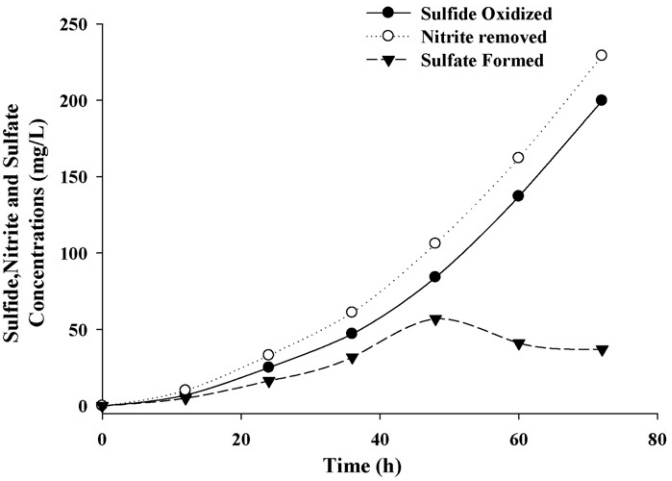

Fig. 4. Anoxic biooxidation of 200 mg sulfide/L by pure culture of *Ochrobactrum* sp.QZ2 with nitrite in batch cultures.

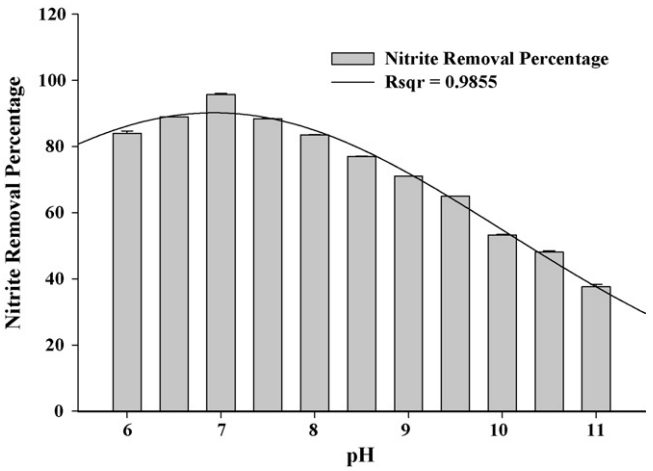

Fig. 5. The effect of different pHs on nitrite reduction (113.25 mg N/L) with sulfide (92 mg S/L) by pure cultures of *Ochrobactrum* sp.QZ2 in batch experiments.

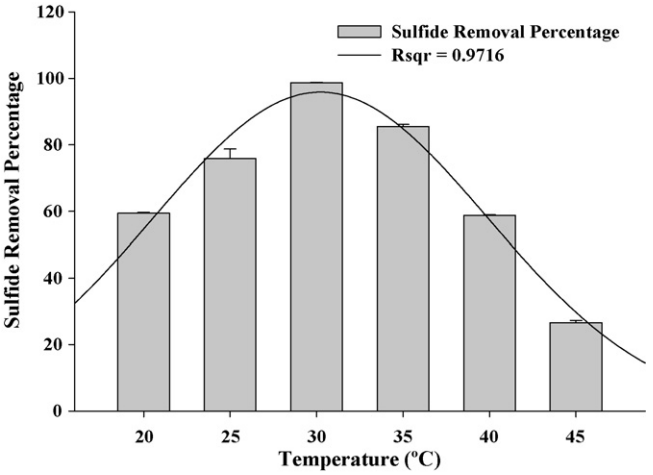

Fig. 6. The effect of temperature on anoxic sulfide (92 mg S/L) biooxidation with 113.25 mg nitrite/L by pure cultures of *Ochrobactrum* sp.QZ2 in batch experiments.

Quantitatively the relationship between the rate a reaction proceeds and its temperature is determined by the Arrhenius equation. At higher temperatures, the probability that two molecules will collide is higher. This higher collision rate results in a higher kinetic

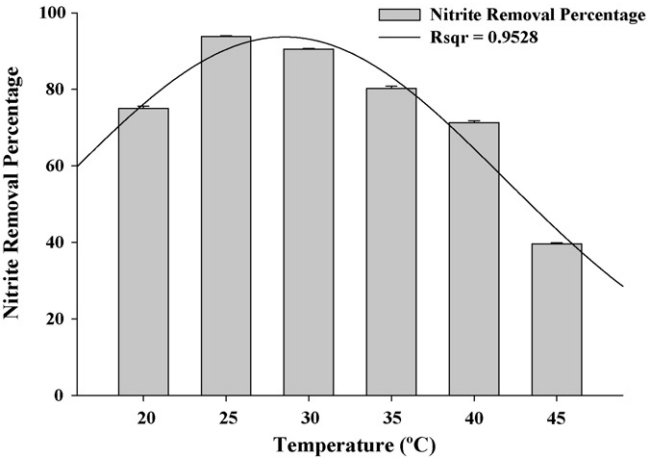

Fig. 7. The effect of temperature on 113.25 mg N/L nitrite reduction with sulfide (92 mg S/L) by pure cultures of *Ochrobactrum* sp.QZ2 in batch experiments.

energy, which has an effect on the activation energy of the reaction. The activation energy is the amount of energy required to ensure that a reaction happens. The activation energy can be calculated by using Arrhenius equation as under:

$$k = A\exp(-E_a/RT) \quad (3)$$

where k is the rate coefficient, A is a constant, E_a is the activation energy, R is the universal gas constant, and T is the temperature (in degrees Kelvin).

R has the value of 8.314×10^{-3} kJ mol^{-1} K^{-1}.

The activation energy for reaction shown in Eq. (1) was calculated at their optimum temperature for isolate QZ2. The results showed that isolate QZ2 had activation energy of 38.4 kJ/mol.

3.3.3. Biolog GN and vitek (GNI+) analysis

Appropriate Biolog GN Microplate was used to determine the relative capacity of substrate utilization of strain QZ2. The results illustrated that the isolate QZ2 could react strongly or even very strongly with 47 in nutrition pools of the Biolog GN system after 24 h of incubation. According to Biolog GN identification, the reaction profile was similar to that of *Ochrobactrum anthropi*, with 80% comparability and 0.629 of the similarity index.

The Vitek (GNI+) results demonstrated that the reaction profile of QZ2 gave positive results for growth on urea, hydrogen sulfide, growth control, glucose oxidative, and sucrose having 97% similarity with *Actinobacillus ureae*.

3.3.4. Aerobic, anaerobic and microaerophilic utilization of sulfide and nitrite

The strain QZ2 grew anaerobically using sulfide (3–6 mM) as an electron donor and nitrite as an electron acceptor. However, growth under purely anaerobic conditions was very slow. The isolate was capable of strictly autotrophic growth on sulfide and nitrite, accompanied by sulfate production and clumping of sulfur crystals as evident by electron microscopy. The strain was not capable of growth on sulfide and nitrite under fully aerobic (21% O_2) conditions. However, autotrophic growth was observed under microaerophilic conditions (1% O_2), using sulfide as an electron donor and nitrite as electron acceptor.

3.4. Metabolic kinetics

The results of nitrite (113.25 mg N/L) reduction with various sulfide concentrations (92–552 mg S/L) in the presence of different nitrite concentrations have been presented in Figs. 8–11. The results showed that the sulfide oxidation through denitrification continued until it was complete after 72 h. Stoichiometric analysis of sulfide oxidized and nitrite removal revealed that there might be some non-biological sulfide oxidation which could have arisen through chemical oxidation utilizing the residual O_2 present in the medium. The analysis showed that the rate of bacterial oxidation of sulfide followed a zero-order reaction.

For isolate QZ2, the sulfide oxidation was inhibited beyond 368 mg S/L (Fig. 8), while nitrite concentration of 340 mg N/L was effectively removed in 48 h using 92 mg S/L sulfide (Fig. 10). The initial sulfide concentration range of 92–368 mg S/L had no effect on the rate of sulfide oxidation with nitrite, though, at 400 mg/L and above, the rate declined.

IC_{50} value was calculated to observe the relative toxicity of sulfide and nitrite on the metabolic activities especially sulfide oxidation to sulfate (Figs. 9 and 11). IC_{50} value is the statistically derived estimate of a concentration of a substance resulting in 50% effect reduction within a specified time; decreasing IC_{50} values are indicating higher toxicity [21]. *Ochrobactrum* sp. QZ2 catalyzed sulfide oxidation to sulfate was more sensitive to sulfide compared with nitrite, as IC_{50} values of sulfide and nitrite were 331.55 mg S/L and 568.72 mg N/L. It implies that isolate QZ2 was relatively more tolerant to nitrite.

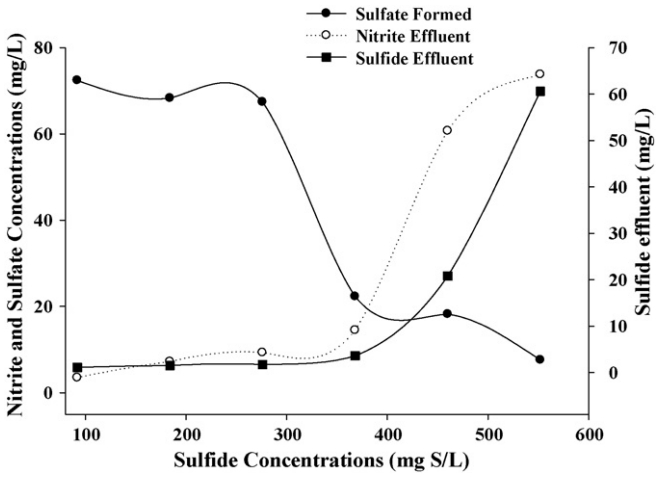

Fig. 8. The effect of various sulfide concentrations on nitrite (113.25 mg N/L) reduction by *Ochrobactrum* sp.QZ2 in batch cultures.

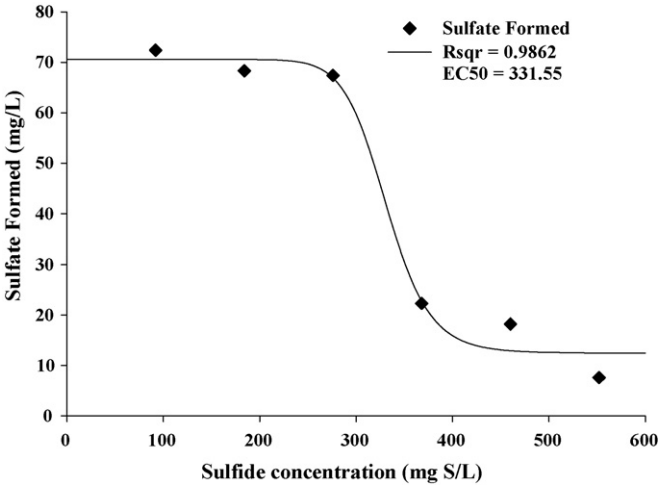

Fig. 9. The effect of sulfide concentrations on sulfate formation by pure cultures of *Ochrobactrum* sp. QZ2 in batch experiments.

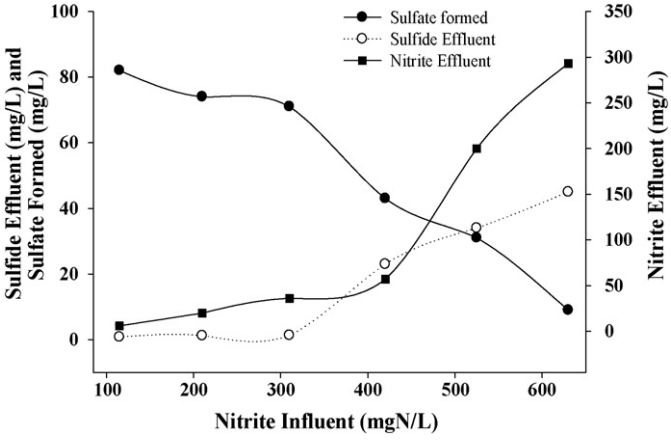

Fig. 10. Effect of various nitrite concentrations on anoxic sulfide (92 mg S/L) oxidation by *Ochrobactrum* sp. QZ2 in batch cultures.

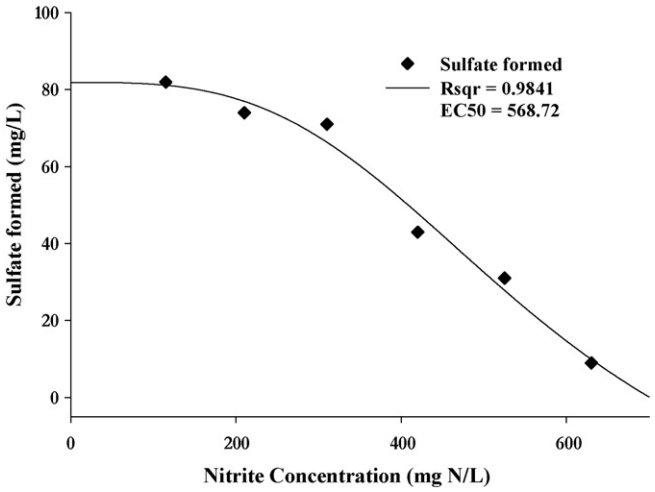

Fig. 11. Effect of nitrite concentrations on sulfate formation by pure cultures of *Ochrobactrum* sp. QZ2 in batch experiments.

4. Discussions

4.1. Isolation and growth

Present investigation dealt with the isolation, identification and partial characterization of some sulfide-oxidizing bacteria using nitrite as electron acceptor from ASO reactor. Strain QZ2 isolated from ASO reactor was able to grow in both liquid and solid plate minimal medium containing sulfide and nitrite. One of the difficulties in establishing the pure culture of QZ2 was that the isolates grew very slowly under purely anaerobic conditions, while the growth and appearance of colonies were relatively faster when grown under microaerobic conditions. Such better growth in microaerobic conditions can be explained on the basis of dual respiratory pathways. Such isolates might have ability to utilize limited amounts of oxygen as electron acceptor in the presence of sulfide (electron donor). Upon depletion of available oxygen in the culture medium, the metabolism might have shifted to utilize nitrite as electron acceptor instead of oxygen. This was strengthened by the fact that isolate was unable to grow and utilize sulfide under purely aerobic conditions implying that purely aerobic conditions might be toxic to these microaerobes.

4.2. Identification of isolates and comparison with other known sulfur-oxidizing bacteria

The phylogenetic tree developed from the 16S rDNA sequence analysis of the isolate QZ2 suggested its closely clustering with *Ochrobactrum lupine* and *Ochrobactrum* sp. According to Biolog GN identification, the reaction profile of QZ2 was similar to that of *Ochrobactrum anthropi*, with 80% comparability and 0.629 of the similarity index. The exploration for characteristics in Bergey's manual of determinative bacteriology revealed that most of the characteristics of the isolate contradicted those of *Ochrobactrum anthropi*. Thus, the identification of isolate QZ2 up to species level was relatively complicated; its identification up to genus level is retained here and will be used throughout this research.

The physiological characteristics of both isolates were compared with the characteristics of other sulfur-oxidizing bacteria belonging to the α-, β-, and γ-*Proteobacteria*: *Starkeya novella* [22] in the α-*Proteobacteria*; *Thiobacillus thioparus* [23] in the β-*Proteobacteria*; and *Halothiobacillus neapolitanus* [24], *Halothiobacillus hydrothermalis* [25], *Halothiobacillus halophilus* [12], and *Acidithiobacillus thiooxidans* [24] in the γ-*Proteobacteria*. It should be noted that all these bacteria had originally been classified as *Thiobacillus* but were recently reclassified by Kelly et al. [22] and Kelly and Wood [24]. The optimum pH for isolate QZ2 was in the range of 6.5–7.0. Though the 16S rRNA gene sequences of strain QZ2 and previously identified *Ochrobactrum* species were 99% similar at most, most of the physiological characteristics of the genus *Ochrobactrum* and isolate QZ2 are not analogous. However, the present isolate resembled to *Ochrobactrum anthropi* in its denitrification ability. Some bacterial strains belonging to *Ochrobactrum* sp. have been isolated previously capable of nitrite reduction in aerobic or anaerobic conditions [13,14].

4.3. Growth and anoxic sulfide oxidation of QZ2

Anoxic sulfide biooxidation by isolate followed a zero order. The growth was proportional to sulfide oxidation for QZ2, indicating that it was capable of electron transport from sulfide to nitrite (Figs. 3 and 4). Though the isolate oxidized sulfide to sulfate, the stoichiometric analysis of reaction (Eq. (1)) suggested that sulfide oxidation by isolate might be incomplete producing elemental sulfur as major sulfide oxidation product. Such results were obtained using mixed cultures in continuous mode ASO bioreactor [1]. A shift from sulfate to sulfur production was also observed for two aerobic sulfide-oxidizing *Thiobacillus* spp. at increasing sulfide concentrations [26–28]. Anoxic H_2S oxidation under denitrifying conditions produced sulfur and sulfate in almost equal proportions by an isolated *Thiobacillus denitrificans* [29]. *Thiomicrospira* sp. CVO was able to oxidize sulfide at concentrations as high as 19 mM [30]. Sulfide biooxidation occurred in two distinct phases, one resulting in the formation of sulfur and possibly other dissolved sulfur compounds rather than sulfate, followed by sulfate formation [30]. Sulfur production by aerobic, sulfide-oxidizing thiobacilli was determined to be maximal at an oxygen/sulfide uptake ratio near the expected theoretical value of 0.56 [28]. Sulfur production by QZ2 occurred at a nitrite/sulfide ratio of 1.17 (Eq. (1)). *S. deleyianum* oxidizes sulfide to elemental sulfur while reducing nitrate to ammonium [31]. Elemental sulfur was also the end product of fumarate-dependent sulfide oxidation by *Wolinella succinogenes* and *S. deleyianum* [32,33].

Nitrite and sulfite (intermediate during sulfide oxidation) toxicity mechanism was studied in yeast cells below pH 5.0 by Hinze and Holzer [34]. It was assumed that nitrite and sulfite penetrate the cell membrane in their undissociated forms as nitrous acid ($pK = 3.3$) or sulfurous acid ($pK = 1.8$), respectively. Due to the neutral intracellular pH they are trapped inside the cell in their anionic forms, which are impermeable to the cell membrane. The results showed that millimolar concentrations of nitrite decreased the ATP level to less than 10% of the initial value. Nitrite and sulfite in combination deplete the ATP content of yeast cells much stronger than expected for the sum of the separate effects of these compounds ("synergistic effect"). Although isolate QZ2 was relatively tolerant to sulfide and nitrite, these pollutants may have a similar toxic mechanism described for yeast cells, restricting their growth considerably.

5. Conclusions

A sulfide oxidizing, nitrite reducing facultative chemolithotrophic bacterial strain QZ2 was isolated from laboratory scale anoxic sulfide-oxidizing (ASO) bioreactor and was partially characterized. The 16SrDNA sequence analysis, the results of Biolog GN, Vitek GNI+ and morphological features of the isolate suggested the identification of isolate as *Ochrobactrum* sp. QZ2.

These isolate was able to grow under slightly acidic to neutral and neutral to slightly alkaline pH, mesophilic, and microaerophilic conditions. The growth and substrate removal of *Ochrobactrum* sp. QZ2 were optimal at an initial pH of 6.5–7.0 at 30 °C. The specific growth rate (μm) for isolate QZ2 was found as 0.06 h^{-1}.The doubling time for isolate was determined as 19.75 h.

The rates of sulfide and nitrite utilization for *Ochrobactrum* sp.QZ2 were 2.19 mg S/L h^{-1} and 2.43 mg N/L h^{-1}, respectively. *Ochrobactrum* sp. QZ2 catalyzed sulfide oxidation to sulfate was more sensitive to sulfide compared with nitrite, as IC_{50} values of sulfide and nitrite were 331.55 mg S/L and 568.72 mg N/L implying that isolate QZ2 was relatively more tolerant to nitrite.

The physiological characteristics of *Ochrobactrum* sp. QZ2 with the characteristics of other known Sulfide-Oxidizing bacteria were compared. The comparison suggested that the present isolate resembled to *Ochrobactrum anthropi* in its denitrification ability. The isolate QZ2 was among the significant populations involved in the internal sulfur cycle occurring in the wastewater and might be responsible for the oxidation of sulfide and/or elemental sulfur to sulfate under anoxic conditions.

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