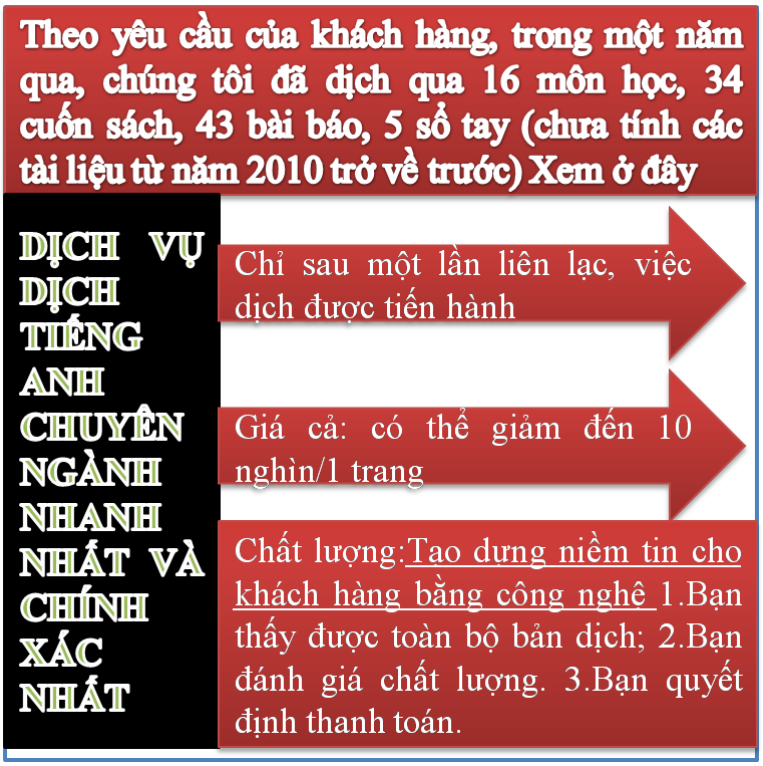

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Anaerobiosis: molecular biology, genetics and other aspects Isolation and characterization of Pseudomonas stutzeri QZ1 from an anoxic sulfide-oxidizing bioreactor Bacterial strain QZ1 was isolated from sludge of anoxic sulfide-oxidizing (ASO) reactor. Based on 16S rDNA sequence analysis and morphological characteristics, the isolate was identified as Pseudomonas stutzeri. The isolate was found to be a facultative chemolithotroph, using sulfide as electron donor and nitrite as electron acceptor. The strain QZ1 produced sulfate as the major product of sulfide oxidation, depending on the initial sulfide and nitrite concentrations. The isolate was capable of growth under strictly autotrophic conditions. The growth and substrate removal of Pseudomonas stutzeri QZ1 were optimal at an initial pH of 7.5-8.0 at 30 °C. The specific growth rate (m) was found as 0.035 h-1 with a doubling time of 21.5 h. For isolate QZ1, the EC50 values both for sulfide and nitrite were found to be 335.95 mg S L-1 and 512.38 mg N L-1, respectively, showing that the sulfide oxidation into sulfate by Pseudomonas stutzeri QZ1 was badly affected beyond these substrate concentrations.	Đời sống kỵ khí: sinh học phân tử, di truyền học và các khía cạnh khác Sự phân lập và xác định tính chất của Pseudomonas stutzeri QZ1 từ lò phản ứng sinh học oxy hóa sunfua thiếu oxy Chủng vi khuẩn QZ1 được phân lập từ bùn của lò phản ứng oxy hóa sunfua thiếu oxy (ASO). Dựa trên quá trình phân tích trình tự 16S rDNA và các đặc điểm hình thái học, chúng tôi đã xác định được dòng vi khuẩn phân lập đó chính là Pseudomonas stutzeri. Chúng tôi thấy dòng vi khuẩn phân lập này là một sinh vật hóa dưỡng vô cơ tùy ý, sử dụng sunfua như một chất cho điện tử và nitric như một chất nhận điện tử. Chủng QZ1 tạo ra sunfat như một sản phẩm chính của quá trình oxy hóa sunfua, phụ thuộc vào nồng độ sunfua và nitric ban đầu. Dòng vi khuẩn phân lập có khả năng phát triển trong điều kiện tự dưỡng nghiêm ngặt. Sự tăng trưởng và loại bỏ chất nền của Pseudomonas stutzeri QZ1 tối ưu ở PH ban đầu bằng 7.5-8.0 ở 30 °C. Tốc độ tăng trưởng đặc trưng (m) là 0.035 h-1 với thời gian tăng gấp đôi 21.5 h. Đối với dòng vi khuẩn phân lập QZ1, các giá trị EC50 đối với cả sunfua và nitric tương ứng là 335.95 mg S L-1 và 512.38 mg N L-1, chứng tỏ rằng sự oxy hóa sunfua thành sunfat bị ảnh hưởng tiêu cực khi vượt quá những nồng độ chất nền này.	

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 gratifying to identify and characterize the bacterial species involved in sulfide biooxidation in mixed culture of an anoxic sulfide-oxidizing (ASO) reactor.

Many microorganisms can exploit H2S oxidation to gain energy [2] . A variety of bacterial genera can oxidize reduced sulfur compounds under aerobic or anaerobic conditions, including:

- green sulfur bacteria (anaerobic, phototrophic, e.g. Chlorobium, etc.);
- purple sulfur bacteria (anaerobic, phototrophic, e.g. Chroma- tium, Thiocapsa, etc.);
- colorless sulfur bacteria (aerobic, e.g. Thiobacillus, Beggiatoa, Thiothrix, etc.).

Thiobacillus species are thought to account for the bulk of sulfide oxidation, via the sulfite-oxidase pathway [3].

1.1. Objectives of the study

In this paper, we report the isolation, identification and char-acterization of a chemolithoautotrophic sulfide-oxidizing, nitrite- reducing bacterial strain present in an ASO reactor.

2. Materials and methods

2.1. Media and culture conditions

The minimal medium used for isolation contained (grams per liter each): NaHCO3, 1; trace element solution, 1 mL; NaHCO3, 1; MgCl2, 1; KH2PO4, 1; (NH4)2SO4, 0.24; Na2S.9H2O, 512 (mg L-1); NaNO2, 528.75 (mg L-1); (NH4)2SO4, 0.5; KH2PO4,1; MgCl2,1; and NaHCO3,1. The trace element solution contained EDTA, 5; NaOH, 11; CaCl2.2H2O, 7.34; FeCl2.4H2O, 3.58; MnCl2.2H2O, 2.5; ZnCl2, 1.06; CoCl2.6H2O, 0.5; (NH4)6Mo7O24.4H2O, 0.5; CuCl2.2H2O, 0.14; in 1 L of distilled water. All experimental cultures were 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. The pH was adjusted using a PHS-9V pH meter (China). Then sulfide (1.0 mM) was added aseptically from a sterile stock solution of 1.0 M Na2S9H2O. The culture medium was poured into 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. Between 0.2 and 0.3 mL of sludge dilutions (10-2,10-3, and 10-4) were sprinkled over the sterilized 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 10-2,10-3 and 10-4 times. After dilution, the liquid culture was spread onto the solid-ified agar plates containing sulfide and nitrite using the dilution plate method, and these were incubated at 30 °C for 48-72 h. Endpoint dilution was carried out several times to further purify strains until pure isolates were obtained. The purified

isolate with a higher capacity for sulfide oxidation and nitrite reduction was named strain QZ1. The purified isolate obtained was transferred to liquid culture medium for enrichment and performing the sulfide oxidation-nitrite reduction ability test. The composition of the minimal medium was as described in Section 2. .

2.3. Growth and maintenance

Routine growth and maintenance of the isolate was carried out in mineral medium. Stock cultures of QZ1 were stored as lyophiles at -20 °C for 15 days. Working cultures from the lyophiles were grown in mineral medium containing agar (2% agar) for 3-5 days. From the agar plates, the isolate was 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, respectively, was detected as an increase in sulfate, sulfide and nitrite concentrations, respectively, relative to uninoculated controls.

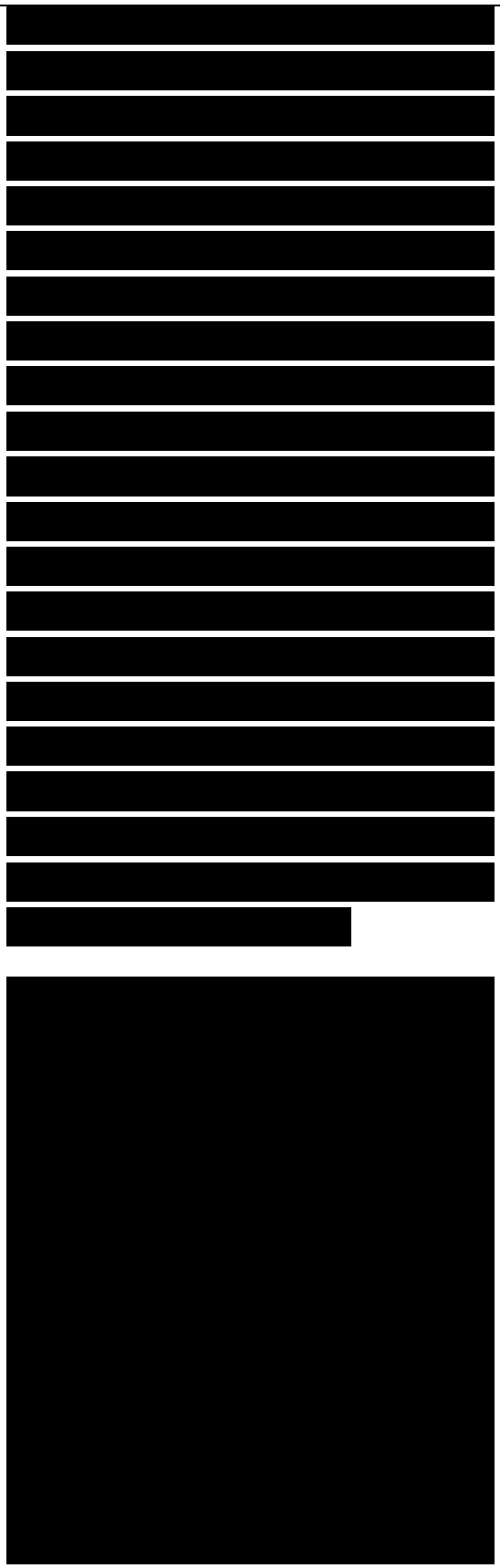
2.4. Identification and characterization of bacterial isolates

2.4.1. Microscopic characterization

Colony structure and cell

morphology of the strain QZ1 was observed under 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 the isolate were determined from photomicrographs using scanning electron microscopy (SEM), and transmission elec-tron microscopy (TEM). The width and length given represent the average measurements of several cells. Motility and the presence of spores were determined using phase microscopy of wet mounts. Gram staining was performed by the Hucker method [4].

For SEM and TEM, 1 mL of QZ1 cells grown in mineral medium (containing sulfide and nitrite) for about 24 h was centrifuged at10,0 x 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 thrice with 0.1 M phosphate buffer and then fixed in 0.1% (wt/vol) osmium tetroxide. Following three more washes in 0.1 M phosphate buffer, an aliquot of cell suspen-sion 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 [5]. The 16S rDNA was amplified using PCR [6] with Taq polymerase (Boya) and the universal primer pair of 27f (5-AGA GTT TGA TCC TGG CTC AG-3) and 1523R (5-GGT TAC CTT GTT ACG ACT T-3) described by Weisburg et al. [7], and determined by the Shanghai Boya Biological Technique Company.

PCR reactions were performed in 100 mL reaction volumes containing 1 mL of Taq DNA polymerase (2.5 U mL-1) (Sangon), 10 mL of 10x PCR reaction buffer (Sangon), 2 mL of each of the primers (10 mM), 2 mLdNTPs(10 mM), (Sangon), 5 mL of the extracted DNA as the templates and 78 mL 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 °Cfor3min,andall 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 Gene Bank databases were aligned using multiple sequence alignment software CLUSTAL W ver. 1.81. A phylogenetic tree was constructed with MegAlign soft-ware of DNASTAR based on the 16S rDNA sequences of 10 strains closer to QZ1.

2.5. Growth kinetics

Growth tests were carried out in 65-mL serum bottles sealed with butyl rubber stoppers. Cell suspension (5%) of QZ1 (OD = 0.31) was 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 these 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 replicates were conducted for each experiment.

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. Microaerophilic (1% O2) 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% O2) to the headspace of each bottle containing 10 mL of N2-CO2 (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 (GN1+) (bioMerieux Vitek, USA) and the Biolog microstation (GN) (Biolog Hayward, CA, USA) were used for carbon source utilization and to identify physiological	[redacted]	

characteristics of the isolate, respectively.

2.7. Optimum growth pH

A 5-mL cell suspension of bacterial strain (OD = 0.3) was inoc-ulated and incubated at 30 °C in 45 mL of sulfide-nitrite inorganic salt medium at various initial pHs between 5 and 11, containing sulfide-nitrite in a 3:8 molar ratio. The buffers (0.1 mM) used in pH experiments were citrate-phosphate (pH 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 inoc-ulated and incubated pH 7.5 in 45 mL of sulfide-nitrite inorganic salt medium at various temperatures between 20 and 45 °C con-taining sulfide-nitrite (3:8 molar ratio) and was cultivated for 23 days. The uninoculated sterile medium was used as a blank. Each test was replicated in triplicate. The OD of growth was monitored after every 12 h continuously. The natural logarithm of these measurements was plotted against the incubation time, and the growth rate was calculated from slopes of the plots.

[redacted]

2.9. Transformations of nitrite-nitrogen and sulfide-sulfur

[redacted]

2.9.1. Effect of sulfide concentrations

[redacted]

The strain QZ1 was grown in mineral medium with 92, 184, 276, 368, 460 and 552 mg S L-1 sulfide containing fixed nitrite concentrations of 113.25 mg N L-1 in each culture bottle. After 48 h of incubation, samples of culture media were analyzed for sulfide, sulfate, nitrate, ammonium, pH and nitrite.

[redacted]

2.9.2. Effect of nitrite concentrations

[redacted]

The strain QZ1 was grown in mineral medium containing 113.25, 226.5, 339.75, 453, 566.25 and 679.5 mg N L-1 nitrite with fixed sulfide concentrations of 92 mg S L-1 in each culture bottle. After 48 h of incubation, samples of culture media were analyzed for sulfide, sulfate, nitrate, ammonium, pH and nitrite.

[redacted]

2.10. Analytical methods

[redacted]

Ammonium nitrogen (NH^-N) was measured by the phenate method [8],

nitrite nitrogen (NO2--N) was measured by the colori-metric method [8] and nitrate nitrogen (NO—-N) was measured by the ultraviolet spectrophotometric screening method [8] on a daily basis using a spectrophotometer (Unico UV-2102 PC and 722S, China). The sulfide was determined by the iodometric method [8] and sulfate was measured by the turbimetric method [8]. The pH measurement was performed according to the standard method [8]. A three-point calibration of the pH meter was carried out daily.

2.11. Statistical and graphical work

Regression analysis, curve fittings and graphical work were carried out using the computer program Sigma Plot™ v.10.

3. Results

3.1. Characterization of isolates

The cells of Pseudomonas sp. QZ1 were slightly curved (almost straight) measuring 0.5-1 x 1.5-3 mm in size (Fig. 1 A,B,C). The individual cells were not surrounded by any sheath. Cells were Gram negative and motile having a single polar flagellum. Growth could occur both aerobically and anaerobically, utilizing sulfide (electron donor) and nitrite (electron acceptor). Cells were catalase and oxidase negative. No spores were

observed in the cells.

3.2. 16S rDNA sequencing and phylogenetic analysis

The partial 16S rDNA sequences of strain QZ1 (comprising 1454 bp) 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. EF542804 was allotted for the said sequence. The QZ1 strain is phylogenetically closely related to the genus Pseudomonas having sequence similarity of 99%.

Presently the acceptable positional standard is that if the simi-larity of the strain under investigation and a reference strain sequence is higher than 97-98%, they are regarded as belonging to the same genus [9,10]. Therefore, the result of this phylogenetic analysis together with that of phenotypic tests, the Vitek system, and the Biolog GP, the bacterial isolate QZ1 was identified as Pseudomonas sp. QZ1.

3.3. Physiological characteristics

3.3.1. Growth rates

As seen in Figs. 3 and 4, the OD600 value of the culture increased with

the increase in sulfide and nitrite concentrations in the medium from 0 to 0.5 for QZ1 after 72 h of incubation. The sulfide and nitrite removal was according to the reaction stoichiometry of Eq. (1), producing sulfate as the major sulfide oxidation product (Fig. 5). However, complete oxidation of sulfide into sulfate was inhibited beyond certain substrate concentrations (Fig. 5). Beyond this substrate concentration, the sulfide may 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 sources of energy for the isolate to carry out lithoauto- trophic denitrification.

3HS- + 8NO— + 5H+/3SO- + 4N2 + 4H2O
DGm = — 2944KJ/mol (1)

Consumption of sulfide (200 mg S L-1) and nitrite (250 mg N L-1) in mineral medium was roughly proportional to growth of QZ1 (Figs. 3 and 4). Growth rate experiments were conducted at 30 °C and pH 7 at a sulfide to nitrite molar ratio of

1.17, which was the proper proportion for simultaneous sulfide and nitrite removal using mixed cultures in an anoxic sulfide-oxidizing reactor [1]. Figs. 3 and 4 illustrate that there was a significant ($p < 0.01$) monotonic increase in the bacterial growth rate (measured as optical density or OD6oo) with the passage of time. Results showed that sulfide was almost completely removed by strain QZ1 within 72 h. The growth of the isolate fitted well with the exponential growth rate equation as under Eq. (2):

$y = aebx$ (2)

Where y is the bacterial growth rate (OD600); x is the time factor; a and b are the parameters of the model.

The specific growth rate (m) for isolate QZ1 was found to be 0.035 h—1. The doubling time was determined as 21.5 h. The R2 value (0.99) for QZ1 (Figs. 3 and 4), indicated that the growth rate was significantly ($p < 0.01$) dependent on substrate concentrations and time factor. The sulfide and nitrite utilization by QZ1 showed that the reaction was zero order, i.e. the sulfide and nitrite utiliza-tion had a

positive direct correlation with the biomass concentra-tion. Moreover, the sulfide and nitrite utilization was not influenced by the substrate concentrations. The rates of sulfide and nitrite utilization for Pseudomonas stutzeri QZ1 were 2.19 mg S L—1 h—1 and 2.43 mg N L 1 h 1, respectively. The rates of substrate utilization increased with the increase in biomass concentration.

3.3.2. Effect of pH and temperature on substrate removal

The isolate was tested over a temperature range of20-45 °Cand a pH range from 6 to 11. The growth and substrate removal of Pseudomonas stutzeri QZ1 were optimal at an initial pH of 7.5-8.0 at 30 °C, but were slow at an initial pH of 6.0 or between pH 8.0 and 11.0 (Figs. 6 and 7). The effects of temperature on the growth rate and substrate removal of strain QZ1 are shown in Figs. 8 and 9. Strain QZ1 grew well from 30 to 35 °C, but not at 45 °C, at pH 7.5.

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 mole-cules will collide is higher. This higher collision rate results in a higher kinetic 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 the Arrhenius equation as in Eq. (3):

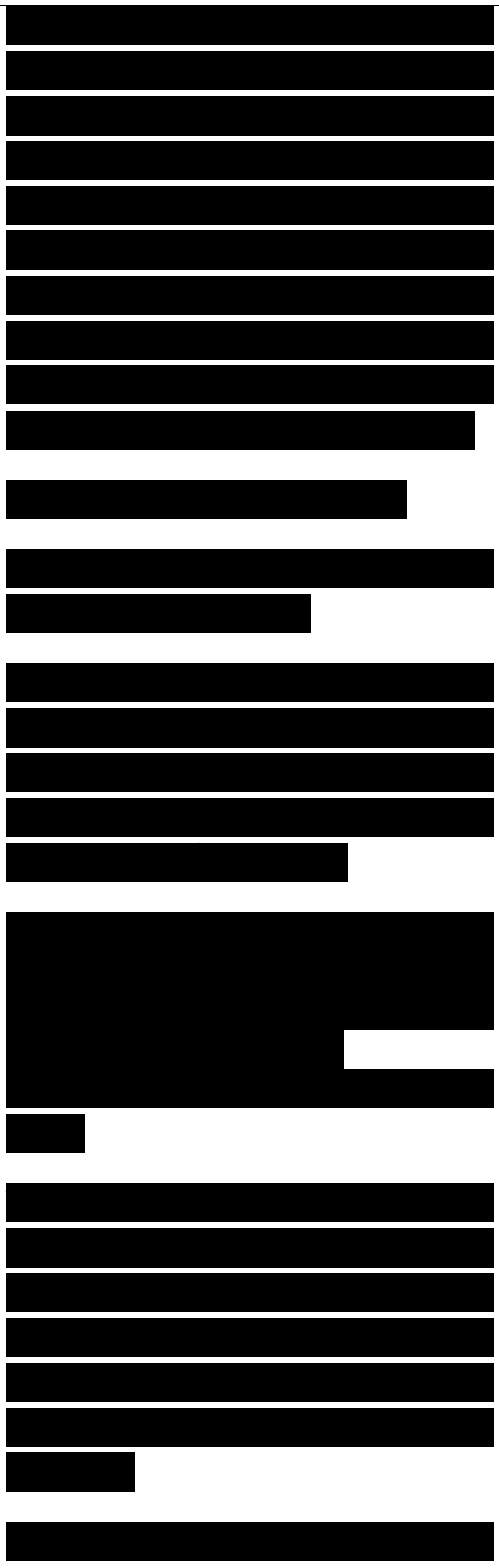
$$k = A*\exp(-Ea=R*T) \quad (3)$$

Fig. 2. Phylogenetic tree of Pseudomonas sp. QZ1.

Fig. 5. Anoxic biooxidation of 200 mg sulfide L—1 by pure culture of Pseudomonas stutzeri QZ1 with nitrite in batch cultures.

Where k is the rate coefficient, A is a constant, Ea 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 x 10—3 kJ mol—1K—1.

The activation energy for the reaction shown in Eq. (1) was calculated at the optimum temperatures for QZ1. The results showed that the isolate had an activation energy value of 39.5 KJ mol—1.



3.3.3. Biolog GN and Vitek (GNI+) analysis

The appropriate Biolog GN Microplate was used to determine the relative capacity for substrate utilization of the isolate. The result illustrated that the isolate QZ1 could react strongly or even very strongly with 50 in nutrition pools of the Biolog GN system after 24 h of incubation.

According to Biolog GN identification, the reaction profile of QZ1 did not match any of the bacterium. The Vitek (GN1+) results demonstrated that the isolate QZ1 gave positive results for growth with glucose oxidative, citrate, malonate, hydrogen sulfide, and mannitol. The results demonstrated that QZ1 had 87% similarity with Pseudomonas stutzeri, while it had 7% similarity with Bacillus sp.

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

The isolate QZ1 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 strain QZ1 was capable of strictly autotrophic growth on sulfide and nitrite,

accompanied by sulfate production and clumping of sulfur crystals as shown by electron microscopy. However, the isolate was not capable of growth on sulfide and nitrite under fully aerobic (21% O2) conditions. Nevertheless, autotrophic growth was observed under microaerophilic conditions (1% O2), using sulfide as an electron donor and nitrite as electron acceptor.

3.4. Metabolic kinetics

The results of nitrite (113.25 mg N L—1) reduction with various sulfide concentrations (92-552 mg S L—1) in the presence of different nitrite concentrations are presented in Figs. 10-13. The results show that sulfide oxidation through denitrification continued until it was complete after 72 h. Stoichiometric analysis of sulfide oxidized and nitrite removal revealed that there may be some non-biological sulfide oxidation which seemed to have arisen

through chemical oxidation utilizing the residual O2 present in the medium. The rate of bacterial oxidation of sulfide followed a zero

order reaction.

For QZ1, sulfide oxidation was inhibited beyond 400 mg S L-1 at fixed nitrite concentration (Fig. 10), while nitrite concentration of 350 mg N L-1 was effectively removed in 48 h using 92 mg S L-1 sulfide (Fig. 12). The initial sulfide concentration at 92-368 mg S L-1 had no effect on the rate of sulfide oxidation with nitrite, though, at 400 mg L-1 and above, the rate declined.

The IC50 value was calculated for both isolates to observe the relative toxicity of sulfide and nitrite on the metabolic activities, especially sulfide oxidation to sulfate (Figs. 11 and 13). The IC50 value is the statistically derived estimate of a concentration of a substance resulting in 50% effect reduction within a specified time; decreasing IC50 values indicate higher toxicity (http://pb. merck.de/servlet/PB/menu/1341610).

For isolate QZ1, the IC50 value for sulfide oxidation into sulfate was calculated both for sulfide and nitrite; it was found that the isolate was more sensitive to sulfide than to nitrite. The IC50 values of sulfide and nitrite were 335.95 mg S L and 512.38 mg N L-1, so the sulfide

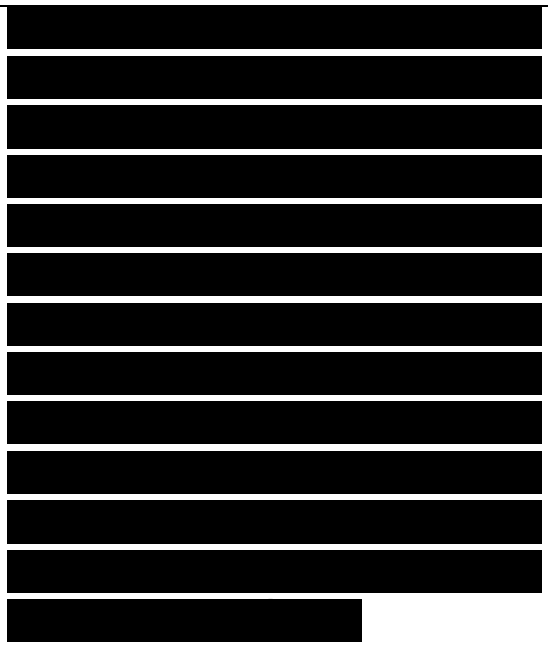
oxidation into sulfate by Pseudomonas stutzeri QZ1 was badly affected beyond these substrate concentrations.

4. Discussion

4.1. Isolation and growth of QZ1

The present investigation dealt with the isolation, identification and partial characterization of some sulfide-oxidizing bacteria using nitrite as electron acceptor from an ASO reactor. Strain QZ1 isolated from the 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 QZ1 was that the isolate grew very slowly under purely anaerobic conditions, while the growth and appearance of colonies were faster when grown under microaerobic conditions. Such better growth in microaerobic conditions can be explained on the basis of dual respiratory path-ways. Such isolates may have the ability to utilize limited amounts of oxygen as electron acceptors in the presence of sulfide (electron donor). We hypothesized that upon depletion of available oxygen in the culture medium, the metabolism might have shifted to utilize nitrite as the electron acceptor instead of oxygen.

This was strengthened by the fact that the isolate was unable to grow and utilize sulfide under purely aerobic conditions, implying that purely aerobic conditions may be toxic to microaerobes.



Sulfide concentration (mg S/L)

Fig. 11. Effect of sulfide concentrations on sulfate formation by pure cultures of Pseudomonas stutzeri QZ1 in batch experiments.

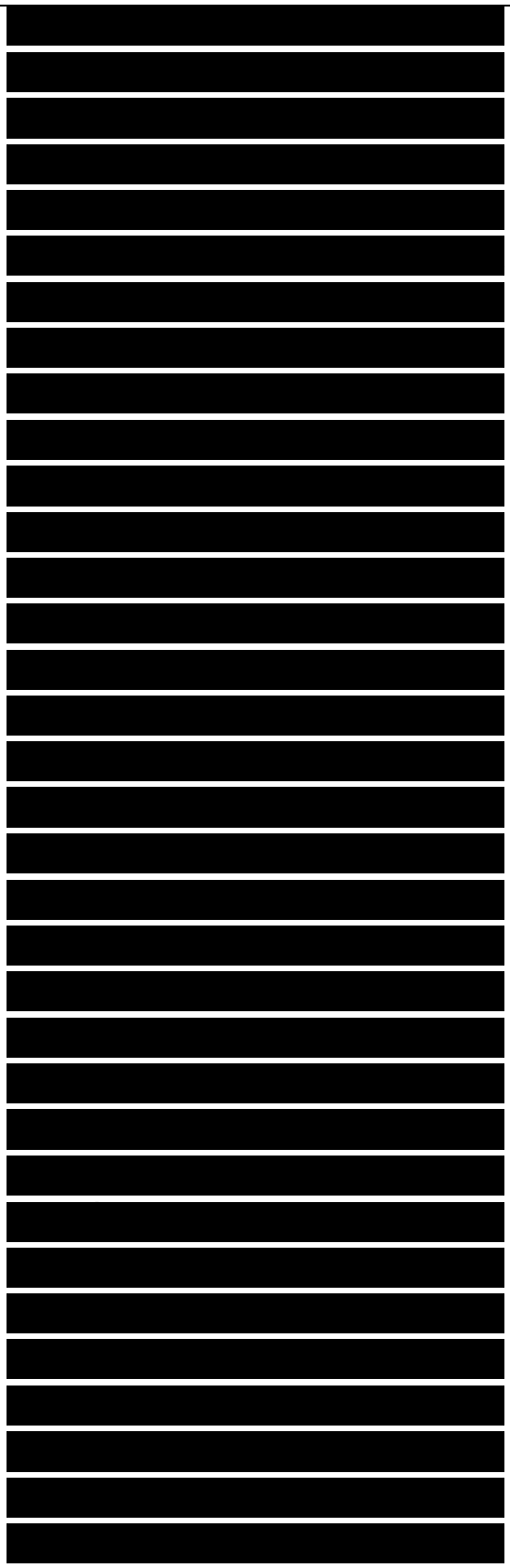
4.2. Identification of isolates and comparison with other known sulfur-oxidizing bacteria

The phylogenetic tree developed from the 16S rDNA sequence analysis of QZ1 suggested that the isolate clustered closely with Pseudomonas stutzeri and Pseudomonas fragi. However, Bergey's Manual of Determinative Bacteriology [11] suggested that the morphological and physiological features of QZ1 were closely related to Pseudomonas stutzeri. Thus,

isolate QZ1 was assigned the species Pseudomonas stutzeri. The Vitek GN1+ results also identified QZ1 as Pseudomonas stutzeri.

The physiological characteristics of the isolate were compared with the characteristics of other sulfur-oxidizing bacteria belonging to the a-, b-, and g-Proteobacteria: Starkeya novella [12] in the a-Proteobacteria; Thiobacillusthioparus [13] in the b-Proteobacteria; and Halothiobacillus neapolitanus [14], Halothiobacillus hydro- thermalis [16], Halothiobacillus halophilus [14], and Acidithiobacillus thiooxidans [15] in the g-Proteobacteria. It should be noted that all these bacteria had originally been classified as Thiobacillus but were recently reclassified by Kelly et al., [12] and Kelly and Wood [14]. Strain QZ1 differed from the other species in its inability to grow at low pH (no growth below pH 6.0).

4.3. Growth and anoxic sulfide oxidation by QZ1

Anoxic sulfide biooxidation by the isolate QZ1 followed a zero order. The growth was proportional to sulfide oxidation by QZ1, indicating that it was capable of electron transport from sulfide to nitrite. Though the isolate oxidized sulfide to sulfate, the stoichio-metric analysis of reaction Eq. (1) suggested that sulfide oxidation may be incomplete producing elemental sulfur as the major sulfide oxidation product. Such results were obtained using mixed cultures in a 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 [16-18]. Anoxic H2S oxidation under denitrifying conditions produced sulfur and sulfate in almost equal proportions by an isolated Thiobacillus denitrificans [19]. Thiomicrospira sp. CVO was able to oxidize sulfide at concentrations as high as 19 mM [20]. 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 [20].		

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 [18]. Sulforspirillum dele- yianum oxidizes sulfide to elemental sulfur while reducing nitrate to ammonium [21]. Elemental sulfur was also the end product of fumarate-dependent sulfide oxidation by Wolinella succinogenes and S. deleyianum [22,23].	[redacted]	
Nitrite and sulfite (intermediate during sulfide oxidation) toxicity mechanisms were studied in yeast cells below pH 5.0 by Hinze and Holzer [24]. 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 show 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 more strongly than expected from the sum of the separate effects of	[redacted]	

these compounds (“synergistic effect’’). Although isolate QZ1 was relatively tolerant to sulfide and nitrite, these pollutants may have similar toxic mechanisms to those described for yeast cells, restricting their growth considerably.

5. Conclusions

(1) A sulfide-oxidizing, nitrite-reducing facultative chemo-lithotrophic bacterial strain QZ1 was isolated from a laboratory scale anoxic sulfide-oxidizing (ASO) bioreactor and was partially characterized. The 16S rDNA sequence analysis, the results of Biolog GN, Vitek GNI+ and morphological features suggested the identification of the isolate as Pseudomonas stutzeri QZ1.

(2) The 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 Pseudomonas stutzeri QZ1 were optimal at an initial pH of 7.5-8.0 at 30 °C. The specific growth rate (m) of QZ1 and was found to be 0.035 h-1.

The doubling time for isolate QZ1 was determined as 21.5 h. The rates of sulfide and nitrite utilization for Pseudomonas stutzeri QZ1 were 2.19 mg S L-1 h-1 and 2.43 mg N L-1 h-1, respectively. The IC50 values of sulfide and nitrite were 335.95 mg S L-1 and 512.38 mg N L-1, so sulfide oxidation into sulfate by Pseudomonas stutzeri QZ1 was badly affected beyond these substrate concentrations. (3) The results indicate that isolate QZ1 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 oxic conditions.	[redacted] (3)Các kết quả cho thấy dòng vi khuẩn phân lập QZ1 là một trong những quần thể quan trọng tham gia vào chu trình lưu huỳnh nội bộ xảy ra trong nước thải và có thể giữ vai trò nhất định trong quá trình oxy hóa sunfua và và / hoặc lưu huỳnh thành sunfat trong điều kiện có oxy.	