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Isolation and characterization of sulphur oxidizing bacteria (*Halothiobacillus* sp.) from aquaculture farm soil

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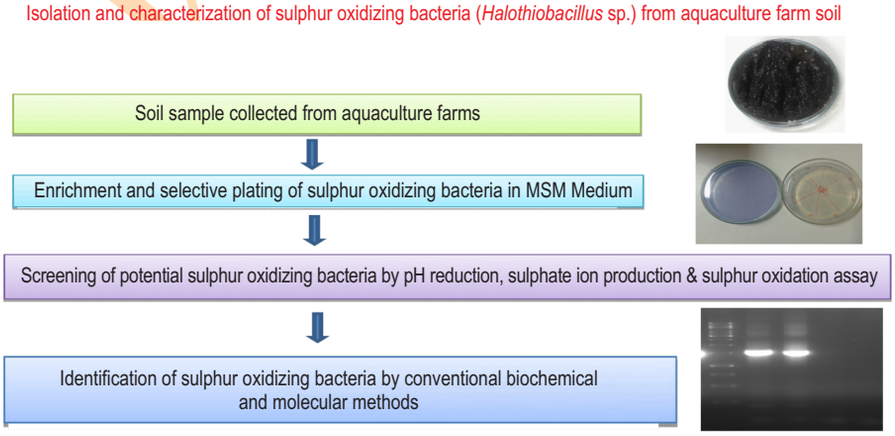
Abstract

Aim : Isolation and characterization of *Halothiobacillus* sp. from the shrimp aquaculture farm soil and their sulphur oxidation ability and utilization of H_2S in *in-vitro* model.

Methodology : Starkeys mineral salt medium was used to screen autotrophic sulphur oxidizing bacteria. For the qualitative screening, bacterial isolates were inoculated in mineral salt medium containing bromo phenol blue indicator to monitor change in pH. The isolates were studied further for their sulphate ion production, sulphur oxidase enzyme production and utilization of Na_2S. Identification was carried out by conventional biochemical and molecular methods.

Results : Fifty isolates showed distinct sulphur oxidizing ability on the mineral salt medium. The pH reduction test revealed that out of fifty isolates six could efficiently reduce the pH of the medium to 3.0 from an initial pH of 7 within 96 hr of incubation at 30°C. Maximum sulphate ion (12.65 mg ml^{-1}) and sulphur oxidase enzyme (16.64 mM sulphate hr^{-1} ml^{-1}) was produced by a bacterial isolate, *Halothiobacillus* sp. strain rk3. All the six isolates efficiently utilized Na_2S in *in-vitro* conditions. Conventional and molecular identification (16S rRNA sequence analysis) revealed that the sulphur oxidizing bacterial isolates belonged to *Halothiobacillus* spp. Furthermore, sequencing similarity calculation showed an average nucleotide identity (ANI) values higher than 99% which suggests that the isolates were not genetically different.

Isolation and characterization of sulphur oxidizing bacteria (*Halothiobacillus* sp.) from aquaculture farm soil



Interpretation : The present investigation revealed the presence of *Halothiobacillus* sp. as natural microflora of farm soils in shrimp aquaculture.

Key words: Aquaculture, *Halothiobacillus*, Sulphur oxidizing bacteria, Sulphur oxidase, Sulphate ion

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Introduction

Bacteria are the major participants in the biogeochemical cycles of carbon, sulphur, nitrogen and phosphorous (Rojas *et al.,* 2001) in natural environment. One such group of bacteria known as sulphate reducing bacteria uses sulphate ions as a terminal electron acceptor in their metabolism and produces sulphide which is toxic to the aquatic animals (Kawahara *et al.,* 2008). On the other hand, another group of bacteria known as sulphur oxidizing bacteria metabolizes sulphide to sulphate which is non-toxic (Friedrich *et al.,* 2001; Behra *et al.,* 2014). The autotrophic sulphur oxidizing bacteria, *viz.,* green sulphur bacteria and purple sulphur bacteria are facultative chemoautolithotrophs and are naturally found growing in environments with pH and temperature ranging between 1-9 and 4ºC-95ºC, respectively (Jorgensen and Revsbech 1983; Robertson *et al.,* 2006). Among the autotrophic sulphur oxidizing bacteria, the genus *Thiobacillus* comprise of rod shaped Gram negative, colorless bacteria that has the ability to use sulphur compounds as electron donor for their growth (Kelly and Harrison, 1989). These organisms play a crucial role in the natural sulphur-cycle in both freshwater and saline environments (Kuenen *et al.,* 1992).

In the present day, intensification of farming practices in shrimp aquaculture systems has led to the excessive use of highly proteinaceous feeds to increase production. This results in the deposition of unutilized feed in the pond bottom. The excess accumulation of unconsumed feed increases the biological oxygen demand in the aquatic system due to bacterial degradation that develops anaerobic condition. This favours the multiplication of sulphate reducing bacteria which utilizes sulphates for their metabolism and produce hydrogen sulphide (Boyd 1992; Abraham *et al.,* 2004). Hydrogen sulphide is highly toxic at very low levels and penetrates across the cell membranes of the fish and other aquatic invertebrates resulting in acute mortalities (Colby and Smith, 1967). This can be ameliorated by supplementing sulphate oxidizing bacteria as bio-inoculants in the aquaculture farms.

Earlier, several attempts have been made to isolate and characterize sulphur oxidizing bacteria from the rhizosphere soil of paddy and groundnut, soil and water from coal mine, sewage and biogas slurry (Rangaswamy *et al.,* 2005); cattle manure compost (Kumar *et al.,* 2014); marine water, sewage and rhizosphere soil (Veerender *et al.,* 2014) and different ecosystems (Sridar *et al.,* 2015). Though there are few studies where investigation have been carried out to enumerate these bacteria from various aquaculture systems (Rao and Karunasagar 2000; Devaraja *et al.,* 2002; Patil *et al.,* 2012, 2015), there is a dearth of information on the isolation and characterization of obligate autotrophic sulphur oxidizing bacteria in aquaculture, except for the study of Abraham *et al.* (2015) who reported effective conversion of H_2S to sulphur compounds due to increase of sulphur oxidizing bacteria counts in shrimp farms. Although, the presence of sulphur oxidizing bacteria in the aquaculture farms have been reported, however, no study has been conducted on the *in-vitro* utilization of hydrogen-sulphide by these bacteria. Therefore, the present study was conducted to isolate and identify sulphur oxidizing bacteria from aquaculture farm soil and characterize their sulphur oxidation potential in *in-vitro* model.

Materials and Methods

Collection of soil sample: Soil samples (top soil about 1 cm) were collected from mid-culture intensive shrimp farms located in Ernakulam District, Kerala, India. Soil samples collected in aseptic sample bags (Himedia, Mumbai, India) with proper labeling were brought to the laboratory in ice box and stored in a BOD incubator (Labline instruments, India) at 4 ± 0.1°C for further analysis.

Sulphur oxidizing bacteria media: Mineral salt medium was used for the isolation of sulphur oxidizing bacteria (Starkey 1935); For plating, the plates were prepared with the addition of agar (15 g l^{-1}) (BD Difco, India) and bromo phenol blue at 0.0025 g as an indicator for monitoring the pH changes of the medium.

Enrichment and isolation of pure culture: Ten gram of soil samples was added to the mineral salt medium (90 ml) and incubated at 30°C for 96 hr. The enriched broth (10 ml) was subsequently transferred to fresh broth for isolating pure culture and incubated under same conditions. Sulphur oxidizing bacteria were isolated by directly plating 0.1 ml of serially diluted enriched sample with 1X phosphate buffer saline and spread plated onto the sulphur-oxidizer agar plates and was incubated at 30°C for 96 hr. After incubation, well defined isolated colonies were randomly picked and streaked onto sulphur-oxidizer medium plate to check for purity. For qualitative screening, the isolates were grown in the broth for a period of 168 hr. Broth pH was estimated at 24 hr interval using pH meter (Eutech instruments, Mumbai, India). Bacterial isolates with ability to lower the pH of the broth were selected and preserved in glycerol at -80°C.

Sulphate ion production: The sulphate ion (SO_4^{2-}) produced in the medium was measured according to the method of Cha *et al.* (1999) with spectrophotometer (Cary 100, UV-VIS spectrophotometer, Thermofisher Scientific, USA) by reacting 1:1 of the bacterial culture supernatant (prepared by centrifuging the bacterial broth culture at 1000 rpm for 5 min) with barium chloride solution (10% w/v). The suspension was shaken vigorously and measured with spectrophotometer at 450 nm. The amount of sulphate formed was calculated from sulphate standard curve which was prepared by dissolving known concentrations (0 to 3 mM) of potassium sulphate in deionized water following the method of Kolmert *et al.* (2000).

Sulphur oxidation assay: The sulphur-oxidase activity was measured spectrophotometrically (Cary 100, UV-VIS spectrophotometer, Thermofisher Scientific, USA) (450 nm) in the reaction mixture according to Hirano *et al.* (1996). The entire reaction was performed in a sealed vial (10 ml) with the reaction

initiation by adding Na_2S (0.5 ml) to the mixture containing sodium citrate buffer (4.5 ml; pH 5.7 and 0.1 M) and 1.0 ml cell suspension and was incubated at 30°C. The reaction was terminated with addition of 1 M NaOH (1.5 ml). The cells were removed from the resultant solution by centrifuging at 10,000 rpm for 15 min. The concentration of sulphate ion was determined by adding equal volume of barium chloride solution (10% w/v). The reactant and sulphide-oxidase activity were measured spectrophotometrically at 450 nm. The enzyme oxidase activity (one unit) was defined as the amount required to produce 1µ mol sulphate hr^{-1} ml^{-1} (U ml^{-1}). For blank, sodium citrate buffer (pH 6.0) was used to carry the reaction replacing the cell suspension.

Utilization of Na_2S in *in-vitro* conditions: All the six bacterial isolates utilizing Na_2S in *in-vitro* conditions were studied with two-layer agar method (Nelson *et al.,* 1983). The chemolithoautotrophic growth was performed in fifty milliliter tubes with agar-based Starkeys mineral salt medium. The medium consisted of bottom agar (1.5% w/v) with Na_2S (sulphide source), the middle layer was filled with culture and the top layer with semisolid agar (0.7% w/v) which was free off sulphide. To monitor the pH change (color), 1 ml of phenol red indicator (0.1%) was added to the agar medium. Initially, the bottom layer was supplemented with 0.3 ml of sterile 1 M Na_2S (0.3 mM final concentration). The bottom agar was sterilized, cooled and added to Na_2S solution and allowed to solidify. Later, the grown sulphur oxidizing bacteria cultures were added (1 ml). Finally, the top sterilized semi solid agar was added and incubated at 30°C for 96 hr.

Identification of the bacterial isolates: Bacterial isolates in this study were identified as per standard methods (Brenner *et al.,* 2005). The bacterial isolates were presumptively identified by means of morphological examination (shape, spore and motility), staining reaction (Gram's reaction) and biochemical tests (catalase production, oxidase production, methyl red reaction, indole production, nitrate reduction, nitrite reduction, carbohydrate metabolism (acid-gas production), thiosulphate utilization. The growth was recorded at pH 5 and temperature 40°C.

16s rRNA sequencing analysis: The isolates with sulphur oxidizing capability were taken for 16S rRNA sequencing analysis. Bacterial cultures were pelleted and washed in 1X TE buffer (pH 8.0). Crude DNA was extracted by boiling lysis method. Amplification of 16S rRNA region was performed using primers 27F 5'-GAGTTTGCTCCTGGCTCAG-3' and 1492R 5'-GGTTACCTTGTTACGACTT-3'. The reaction was performed in a total of 25 µl reaction mixture consisted of 12.5 µl of 2X OneTaq Quick load PCR master mix (New England Biolabs, Germany), 0.5 µl (20 pmol) of each of the primers and 2 µl of DNA template and 9.5 µl distilled water. The PCR cycling conditions *i.e.,* 94°C (2 min) for initial denaturation were maintained followed by 35 cycles of denaturation, annealing and extension at 94°C (30 sec), 50°C (1 min) and 68°C (90 sec) with a final extension at 68°C (7 min). The amplicons were analyzed by agarose gel electrophoresis and visualized in gel documentation system (Biorad, Germany). GenElute Gel extraction kit (Sigma, USA) was used for purification of amplicons and outsourced for sequencing at Scigenome Pvt. Ltd. (Cochin, India). Chromatogram analysis was performed in Mega Version 4 and checked for sequencing similarity for calculation of average nucleotide identity (ANI) values by using EzBioCloud (http://www.ezbiocloud.net/). Phylogenetic analysis with related species and genus was performed in Clustal omega. Genetic relatedness among the sulphur oxidizing bacteria isolates were analysed using Clustal 2.1.

Statistical analysis: One-way ANOVA was used for data analysis SPSS V. 16 software (SPSS Inc., Chicago, Ilinois, USA). The means were compared using Duncan multiple range test to find the difference at 5% level of significance ($P< 0.05$).

Results and Discussion

Aquaculture farm soil samples were screened for sulphur oxidizing bacteria using enrichment culture technique in mineral salt medium. The enriched soil samples were able to produce colonies up to 10^{-5} ml dilutions on agar medium. A total of fifty distinct bacterial isolates were picked and checked for their purity by subsequent sub culturing and were maintained on slants. Out of fifty isolates, six isolates showed characteristic reduction of pH from 7 to 3 within 96 hr of incubation and were selected for further studies. Sulphur oxidizing bacteria can use sulphur as a fundamental substrate during oxidation process and oxidizes it to sulphates (Pokorna and Zabranska, 2015).

The genus *Thiobacillus* among sulphur oxidizing bacteria plays a very important role in the biological oxidation of sulphur in natural environment (Yang *et al.,* 2010). In previous study, sulphur oxidizing bacteria has been isolated from the freshwater fish farm soil (Kumar *et al.,* 2018). Sulphur oxidizing bacteria has also been isolated from pulses rhizosphere, biogas slurry, paddy rhizosphere, mine soil, sewage and tannery effluent (Vidyalakshmi and Sridar, 2006). Another study conducted by Behera *et al.* (2014) from mangrove soil of Mahanadi river have also reported screening of sulphur oxidizing bacteria. Thiobacilli generally enhances sulphur oxidation rate and reduces the level of toxic gases such as H_2S in aquaculture environment. Substantial quantum of phosphorous, nitrogen and organic matter are usually received and accumulated in aquaculture ponds during intensive farming (Vass, 2017).

This high load of organic matter contributes in the development of anaerobic conditions in soil water interface resulting in production of reduced toxic gases such as hydrogen sulphide, nitrite and ammonia (Muralidhar, 2017). These reduced compounds are toxic to aquatic animals and are majorly documented in intensively stocked and fed shrimp farms (Patil *et al.,* 2012, 2015; Abraham *et al.,* 2004, 2015). *Thiobacillus* spp. can oxidize sulphur at pH ranging from 1 to 9, but optimum growth is recorded only under acidic conditions of pH 1 to 5 (Robertson and Kuenen, 2006). Decrease in pH to 2.5-3.0 from neutral during growth of sulphur oxidizing bacteria varies within species. In

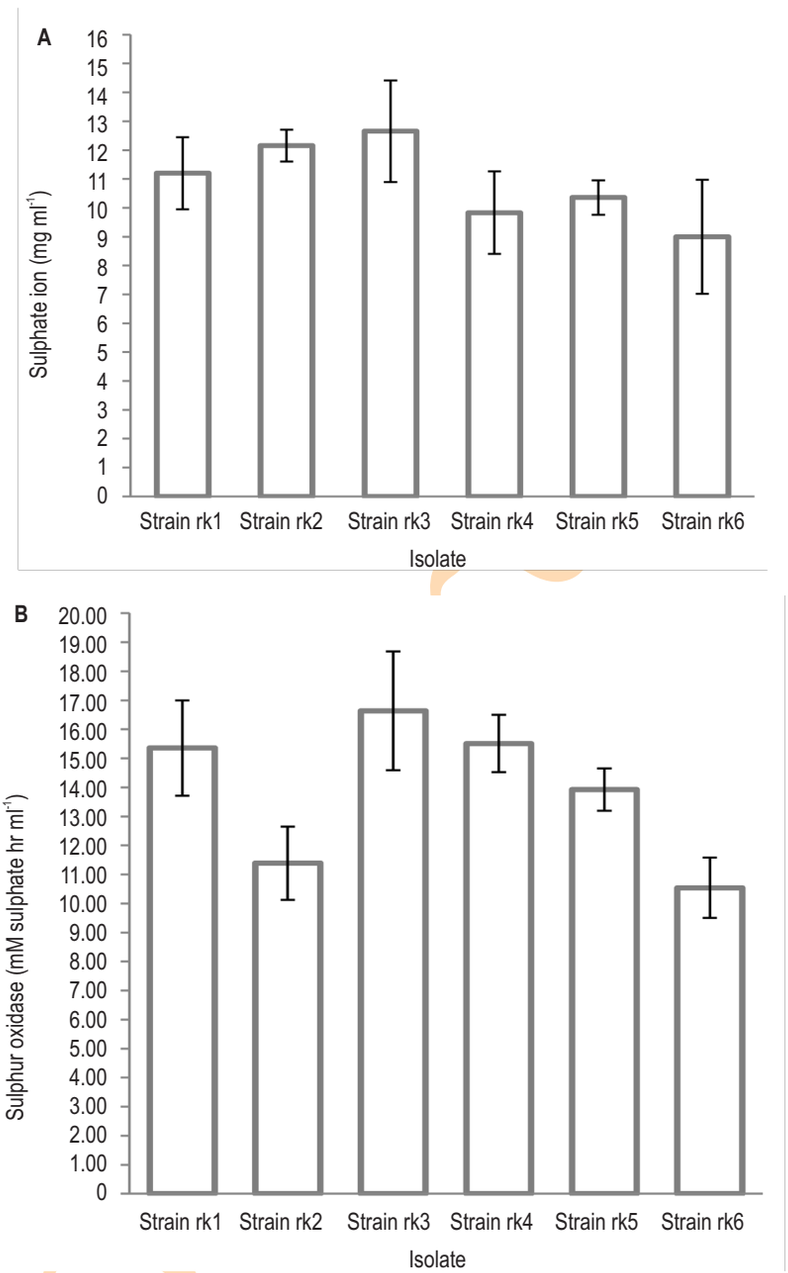

Fig. 1: Sulphate ion produced by the six sulphur oxidizing bacterial isolates in the medium. Maximum amount of sulphate ion (12.65 mg ml^{-1}) was produced by bacterial isolate *Halothiobacillus* sp. strain rk3. B) Sulphur oxidation activity of six sulphur oxidizing bacterial isolates. Maximum activity of sulphur oxidase enzyme (16.64 mM sulphate hr^{-1} ml^{-1}) was shown by bacterial isolate *Halothiobacillus* sp. strain rk3.

majority, optimum growth was noticed at pH 6.5 to 8.0 and temperature 30 to 42°C (Kelly and Wood, 2003). However, in our study all the six isolates were found to grow at 40°C. During growth of sulphur oxidizing bacteria, the amount of sulphate ion (SO_4^{2-}) produced is shown in Fig. 1A. Maximum sulphate ion (12.65 mg ml^{-1}) was produced by bacterial isolate *Halothiobacillus* sp. strain rk3. There was no significant difference (P>0.05) noticed for sulphate ion produced among the six isolates. Barium chloride test was used to measure the amount of sulphate ion produced during the oxidation of sulphur into sulphate by sulphur oxidising bacteria. In a recent study, sulphate production from thiosulphate by sulphur oxidizing bacteria (isolated from freshwater fish farm soil) was found to be 1.42 to1.62 mg ml^{-1} (Kumar *et al.,* 2018). Sulphate production by the isolates BGS2 and TRY2 was found to be 79.20 and 68.80 mg per 100 ml broth (Vidhyasri and Sridar, 2011). In another study, sulphate production

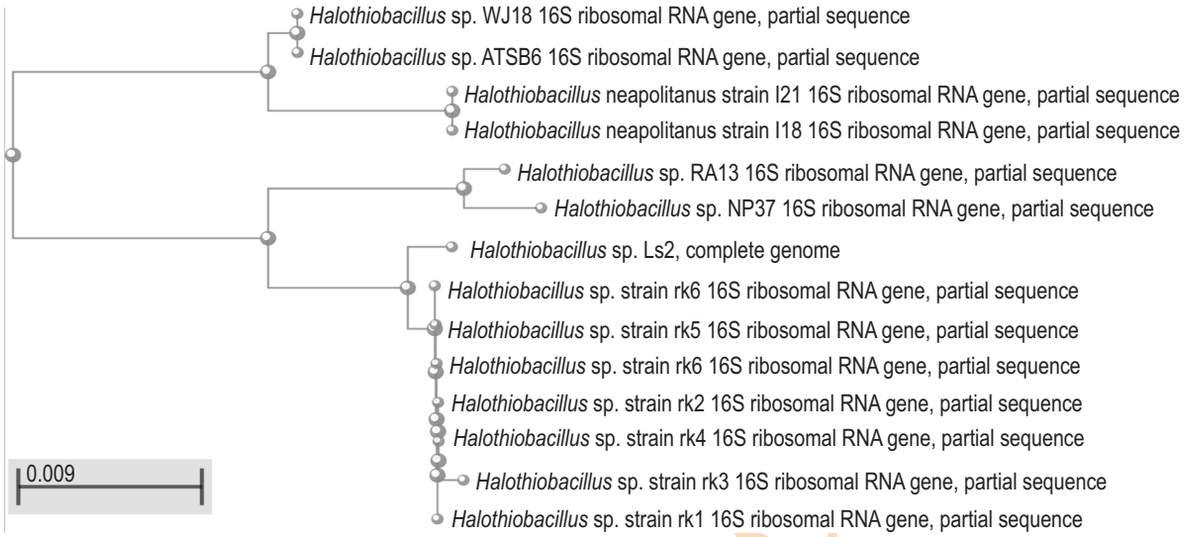

Fig. 2 : Phylogenetic tree of bacterial isolates constructed using 16S rRNA gene partial sequences by neighborhood joining method. The reference sequences of *Halothiobacillus* sp. and *Halothiobacillus neopalinatus* is selected from the public domain (NCBI) and compared with the six sulphur oxidizing bacterial isolates which forms a distinct clade. The bar represents a sequence divergence of 0.009%.

by ten bacterial strains isolated from industrial wastewater, sewerage water and sulphur mud varied from 13.4 to 571.6 mg l^{-1} after 8 days of incubation (Ullah *et al.,* 2014).

The sulphur-oxidase activity of six bacterial isolates is shown in Fig. 1B. The maximum activity of sulphur oxidase enzyme (16.64 mM sulphate hr^{-1} ml^{-1}) was shown by bacterial isolate *Halothiobacillus* sp. strain rk3. There was no significant difference (P>0.05) noticed for sulphide oxidation among the bacterial isolates. Sulphur oxidizing bacteria can oxidize sulphide and inorganic sulphur reduced compounds due to the production of sulphur oxidase enzyme (Ravichandra *et al.,* 2007), sulphate is formed from biological oxidation of the reduced inorganic sulphur compounds (Thatoi, *et al.,* 2012).

All the six sulphur oxidizing bacteria isolates were screened for the utilization of Na_2S in *in-vitro* conditions. There was change in color of the medium to yellow which indicates a decrease in pH. Oxidation of Na_2S was evidenced by the presence of white mat like structure between the two agar layers indicating that Na_2S is oxidized by sulphur oxidizing bacteria and forms precipitate which is deposited in the tube. Utilization of Na_2S has also been studied for MKH 41 isolate in *in-vitro* condition (Mustafa *et al.,* 2015). The change in pH observed in the present

Table 1: Morphological, physiological and biochemical characterization identification of sulphur oxidizing bacteria

Characters	Strain rk1	Strain rk2	Strain rk3	Strain rk4	Strain rk5	Strain rk6
Shape	Thin rod	Thin rod	Thin rod	Thin rod	Thin rod	Thin rod
Spore	-	-	-	-	-	-
Motile	+	+	+	+	+	+
Aerobic growth	+	+	+	+	+	+
Gram stain	-	-	-	-	-	-
Catalase test	-	-	-	-	-	-
Oxidase test	-	-	-	-	-	-
MR test	-	-	-	-	-	-
Indole production	+	+	+	+	+	+
Fermentation of glucose	+	+	+	+	+	+
Nitrate reduction	+	+	+	+	+	+
Nitrate reduction	-	-	-	-	-	-
Thiosulphate utilization	+	+	+	+	+	+
Growth at pH 5	+	+	+	+	+	+
Growth at 40°C	+	+	+	+	+	+

Table 2: Genetic relatedness among the sulphur oxidizing bacterial isolates

		Percent Identity						
		1	2	3	4	5	6	
Divergence	1		99.88	100.00	100.00	100.00	100.00	*Halothiobacillus* sp. strain rk1
	2	99.88		99.88	99.88	99.88	99.88	*Halothiobacillus* sp. strain rk2
	3	100.00	99.88		100.00	100.00	100.00	*Halothiobacillus* sp. strain rk3
	4	100.00	99.88	100.00		100.00	100.00	*Halothiobacillus* sp. strain rk4
	5	100.00	99.88	100.00	100.00		100.00	*Halothiobacillus* sp. strain rk5
	6	100.00	99.88	100.00	100.00	100.00		*Halothiobacillus* sp. strain rk6

study might be due to the utilization of Na_2S and formation of sulphuric acid (Friedrich *et al.,* 2001). Identification of these isolates was performed as per standard procedures using morphological, physiological and biochemical characters (Table 1). On mineral salt agar plates isolates produced small colonies (1±3 mm diameter) with white to yellowish centre. The isolates were Gram-negative, non-spore producing thin rod shaped single occurring (occasionally in pair) motile bacteria. All six isolates were obligate aerobes and showed chemolithoautotrophic growth with thiosulphate. Study on physiological and biochemical characteristics of these six isolates revealed that they were negative for catalase, oxidase, methyl red, produced indole/acid and reduced nitrate, but not nitrite.

The bacteria also showed heterotrophic growth at pH 5 and temperature 40°C. For molecular characterization, amplicons of 1500 bp size were purified and sent for sequencing. 16S rRNA sequencing analysis revealed that the sulphur oxidizing bacteria isolated in the present study belonged to the genus *Halothiobacillus* of *c-Proteobacteria* which are frequently isolated from the soil, freshwater, and marine environments (Kelly and Wood, 2003). Further, clustering and dendrogram revealed that the isolates were closely related. Phylogenetic tree of 16S rRNA gene was constructed with six isolates obtained in this study in comparison to the reference *Halothiobacillus* sp. and *Halothiobacillus neopalinatus* available in the public domain NCBI (Fig. 2). The study revealed that the reference strains (LS2) formed a monophyletic cluster with 99% identity with these bacterial isolates. The genetic relatedness among the sulphur oxidizing bacteria is depicted in Table 2. The percent identity observed among the isolates was nearly 100%, suggesting that there was not much variation in the 16S rRNA sequences.

The nucleotide sequence data are available in public domain under the Genbank accession numbers [MG014236.1: *Halothiobacillus* sp. strain rk1 (ANI-99.42%); MG014237.1: *Halothiobacillus* sp. strain rk2 (ANI-99.65%); MG014238.1: *Halothiobacillus* sp. strain rk3 (ANI-99.51%); MG014239.1: *Halothiobacillus* sp. strain rk4 (ANI-99.39%); MG014240.1: *Halothiobacillus* sp. strain rk5 (ANI-99.54%) and MG014241.1: *Halothiobacillus* sp. strain rk6 (ANI-99.54%)]. Similarity in ANI values indicates that the genomes of all six sulphur oxidizing bacterial strains are almost identical in size and no significant variance were observed in the ANI values. In conclusion, the present investigation revealed that sulphur oxidizing bacteria can oxidize sulphur and utilize Na_2S in *in-vitro* conditions. Therefore, these bacteria may be used as bio-inoculants in the shrimp culture environment to enhance sulphur oxidation in soil. However, further studies need to be carried out along with *in situ* trials with shrimps to develop proper understanding on the roles played by these bacteria in sulphur oxidation process.

Acknowledgments

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