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EFFECTIVE SULFUR OXIDIZING BACTERIAL ISOLATION FROM PROCESS WASTE WATER OF FOOD INDUSTRY

Kalyani Borse[1,2], Vikash Agnihotri[1], Hemant Bhandarkar[1] and Suddhanshu Bhardwaj[1]*
[1]Microbiology Laboratory, Jain R&D, Jain Irrigation Systems Ltd, Agri-park, Jain Hills,
PO Box no. 72, Shirsholi Road, Jalgaon, 425001, Maharashtra, India.
[2]North Maharashtra University, Jalgaon, Maharashtra, India.
*Author for correspondence: E-mail: bhardwaj.sudhanshu@jains.com

ABSTRACT

The alternative energy sources are more important due to increasing energy requirement. Biogas production is a prominent renewable energy source which use sewage sludge, agricultural and industrial wastes. The food processing plant waste contains high concentration of sulfur like sulphide and sulphate. Sulfur oxidizing bacteria are capable to oxidizing or reducing sulfur compounds. Utilization of sulfur oxidizing bacteria has done in sewage bioremediation agent and promotes soil fertility. The isolation and characterization of SOB were carried out by growing bacteria in the *Thiobacillus* agar selective medium. Also include pH reduction test, sulphate concentration, morphology and cultural characterization. It has found that out of 10 isolates from different source of samples, only one isolate (KSB 7) was selected for molecular characterization on the basis of high pH reduction and Sulfur concentration. KSB 7 isolates showing rapid drop in pH up to 1.2 within 7 days and producing high sulphate concentration (410 mg/L). 16SrRNA gene sequences were used to generate phylogenetic tree and SOB KSB 7 isolate culture identified was *Acidithiobacillus thiooxidans*.

***Keywords*:** sulfur oxidizing bacteria, sulphate concentration, 16SrRNA, *Acidithiobacillus thiooxidans*.

Introduction

Renewable and alternative energy sources have gained more importance to overcome day by day increasing energy requirements. Biogas produced by anaerobic digestion of sewage sludge, agricultural and industrial wastes has been emerged as a prominent renewable energy source (Namgung *et al.,* 2012; Lin *et al.,* 2013). The biogas product typical present of CH_4 (53–70 %), CO_2 (30–47 %), N_2 (0–3 %), H_2O (5–10 %), O_2 (0–1 %), H_2S (0–10,000 ppmv), NH_3 (0–100 ppmv), hydrocarbons (0–200 mg m^{-3}) and siloxanes (0–41 mg m^{-3}) constituents (Promuan and Thong, 2017). The amount of H_2S varies according to composition of substrate used for biogas production (Hou *et al.,* 2018). Hydrogen sulphide from biogas should be removed by pre-treatment to clean biogas and prevent corrosion of gas engine. In liquid form sulfide caused corrosion of water transport systems. It is inhibiting aerobic respiration to humans and anaerobic processes like methanogenesis at concentrations above 50 mg/L (Promuan and Thong, 2017). Among available processes for removal of hydrogen sulfide from biogas, biological processes have been widely accepted because of relatively low operating costs, effectiveness and environment friendly operation. Also it does not require chemicals except oxygen and have simple nutritional requirements. Bio-filtration removes H2S from biogas using chemoautotrophic sulfur oxidizing bacteria (SOB) that can obtain their energy from the oxidation of reduced sulfur compounds such as hydrogen sulfide, sulfite, sulfur, thiosulfate, and polythionates. Phylogenetically, sulfur-oxidizing prokaryotes show diversity. The one group of *Archaea* has aerobic sulfur oxidizers that are members of *Sulfolobales*, while in the other group of *Bacteria* include sulfur oxidizers aerobic lithotrophs or anaerobic phototrophs. For example members of the genus *Bacillus, Beggiatoa, Thiothrix, Thermothix, Thiovolum, Acidianus, Sulfolobus, Thioalcalimicrobium, Thioalkallividrio,* and *Thiobacillus* can be classified as *Acidithiobacillus, Thermithiobacillus,* and *Halothiobacillus* (Ravichandra *et al.,* 2007; Rojas-Avelizapa *et al.,* 2013; Hou *et al.,* 2018; Behera *et al.,* 2014).

They are ubiquitously distributed especially in the environment where oxidizable sulfur is abundant which includes sulfur springs, sulfide minerals, sewage-treatment areas and sources of sulfur gases, such as H_2S from sediments or anaerobic soils and can be isolated from marine, fresh water and soil environments (Kelly and Wood 2000). In last few decades, members of genus *Thiobacillus*, have been studied and described as inhabitants of wastewater environments. However, these sulphur oxidizing bacteria can be isolated and enriched from wastewater habitats using either of reduced sulphur compounds like thiosulfate or sulfide as an electron donor (Ito *et al.,* 2004).

Kelly and Harrison in 1989 suggested that *Thiobacillus* sp. can be used for removing sulphide from biogas systems and also from wastewater. The idea was supported and studied using reduced inorganic sulfur compounds as an energy source for their isolation. It is interesting that some species of *Thiobacillus* are able to grow obligately chemolithotrophicaly, while some of them are also able to grow chemoorganotrophicaly (Ito *et al.,* 2004; Kantachote and Innuwat, 2004).

Acidithiobacillus is a group of SOB that are widely distributed in soil and water environments that are acidic in nature and rich in sulphur content. Their general habitats contain hot springs, iron-sulfur mineral deposits and acid mine drainage. Taken together, these SOB have been widely studied for their application in microbial desulfurization of coal and gas, heavy metal leaching from mineral ores. Sulfur oxidation being the essential physiological property of *Acidithiobacillus spp*. has gained importance in biological desulphurization (Wang *et al.,* 2019).

Suzuki, 1974 proposed that there are two forms of sulfide viz. free sulfides (H_2S) and dissolved sulfides (HS^-). Further, Visser *et al.,* 1997 extended the idea of bioconversions of sulfide oxidation system as:

$$2HS^- + O_2 \text{ ---------- } \square \; 2S_o + 2OH^- \quad ...(1)$$

$$2HS^- + 4O_2 \text{ ---------- } \square \; 2SO_4^{2-} + 2H^+ \quad ...(2)$$

Oxygen concentration plays very important role in biological oxidation of sulphide and end product formation is function of not only the sulfide concentration but also the concentration of oxygen supply to the reactor. If O_2 concentration is below 0.1 mg/L, sulfur is the end product of the sulfide oxidation, while sulfate is formed under conditions of oxygen limitation (Ravichandra *et al.,* 2007). Isamu in 1999, explained general scheme of biological sulfide oxidation system:

$$S_2 \text{ -------} S_0 \text{--------} SO_3^{2-} \text{ ---------} SO^{2-} \quad ...(3)$$

Phylogenetic studies revealed that, *Acidithiobacillus* belong to class Acidithiobacillia of Proteobateria. In 2000, Kelly and Wood, suggested that formerly they were members of the genus "*Thiobacillus*", however along with higher acid-tolerance they share close evolutionary relationships with each other in comparison with other *Thiobacillus* spp., so they were reclassified as a new genus "*Acidithiobacillus*" (Wang *et al.,* 2019). Members of this genus can be further categorized depending on differences in their ability of oxidizing inorganic sulphur and ferrous iron in their reduced form. 16S rRNA gene sequence comparisons associated with physiological characterization have divided Acidithiobacillus in seven species; only sulphur oxidizing *Acidithiobacillus thiooxidans*, *Acidithiobacillus caldus* and *Acidithiobacillus albertensis* in one group while the other having both sulphur and ferrous iron oxidizing includes *Acidithiobacillus ferrooxidans*, *Acidithiobacillus ferrivorans*, *Acidithiobacillus ferriphilus*, and *Acidithiobacillus ferridurans* (Wang *et al.,* 2019).

Acidithiobacillus as obligatory acidophilic aerobic gram negative rod shaped bacteria. The ultimate end product of these sulphur compounds oxidation and bacteria are sulfate (Kelly and Wood 2000). Some species oxidize reduced organo sulfur compounds or ferrous iron to ferric iron. Indeed, the reason to group all the species under one genus was that all of them are morphologically similar, autotrophic in nutrition, can obtain energy by oxidizing inorganic sulphur substrates. Mesophilic species grow well in temperature range of 30-35 °C and optimum growth temperature for moderately thermophillic species is 45 °C. They contain ubiquinone Q-8 and 52-64 mol% of G+C content in the DNA. They share other general characteristics with members of the *c*-subclass of the *Proteobacteria*. *Acidithiobacillus thiooxidans* (formerly *Thiobacillust hiooxidans*) is the type species. As per description of *Acidithiobacillus thiooxidans* provided by Waksman & Joffe in 1922, Kelly & Harrison in 1989 and Kelly &Wood in 2000 ; It is small rod let, Gram negative , motile by of a polar flagellum, acid loving can sustain even at lower pH 0.5 and able to grow in liquid medium on elemental sulfur, thiosulfate or tetrathionate. Sulfur may be produced transiently during growth on thiosulfate under oxygen limiting conditions (Konishi *et al.,* 1995; Kelly and Wood ,2000).

For isolation and cultivation of *Thiobacillus* species on solid medium a *Thiobacillus* agar is recommended (Starosvetsky *et al.,* 2013). When grown on thiosulphate agar they produce minute colonies (0.5–1.0 mm) that appear transparent or whitish yellow and clear on prolonged incubation; with complete edges. They are strictly aerobic ad possess remarkable ability to oxidize elemental sulphur and reduce pH of media to values of 0.5–0.8. Medium supplemented with Ammonium sulfate served as nitrogen source. Optimum temperature for growth is 28–30 ºC (temperature range: 10–37 ºC). Optimum pH is 2.0–3.0 (pH range is 0.5– 5.5). These are likely to inhabit acidic inorganic reduced sulphur environment and can be isolated from various habitat as soil, sulfur springs, acid mine drainage waters, corroding concrete and steel environments (Hou *et al.,* 2018). Thus, *Acidithiobacillus* have gained much attention for treatment of sulphur containing wastes because of their ability to grow at wide range of temperature and even at extremely acidic pH (Starosvetsky *et al.,* 2013).

The present study was intended to obtain and select chemoautotrophic sulfur-oxidizing bacteria (SOB) from selected sulfur rich environmental samples and propose them as effective candidates for their application in development of processes to treat inorganic sulfur containing wastes and effluent wastewater or inorganic sulphur containing gases. Also their ability to grow at different sulfur concentrations and pH was evaluated.

Materials and Methods

Sample collection

For the isolation of effective sulphur oxidizing bacteria that could be utilized for the treatment of hydrogen sulphide (H_2S) from biogas the process waste water samples were collected from ten different locations from Jain Irrigation Systems, Ltd. Jalgaon, Maharashtra, India. In sterile 200 ml air-tight plastic containers, 150 ml of water samples were collected in triplicate and stored at room temperature till further analysis. Average monthly pH and sulphate concentration analysis were considered to decide appropriate timing for sample collection from different sulfate rich environment.

The OWW, OAT and OCO process waste water samples collected from onion processing effluent treatment plant and from fruit processing effluent treatment plant FWW and FC were collected. From biogas power generation plant Hydrolysis tank-4 (H4) and from biogas scrubbers: HDPE scrubber and SS Scrubber recirculation water (S1, S2, S3, and S4) water samples were collected.

Preparation of samples

The collected samples were continuously aerated using aeration assembly for 7 days at temperature of 30±2°C to restrict the growth of anaerobic microorganisms. In 900 ml of

liquid enrichment broth 100 ml of each aerated sample was mixed and incubated with continuous aeration for next 7 days. For the growth of acidophilic SOB the pH of the mixture should be maintained in between 4.0 to 5.0. After 7 days medium was replaced by a fresh medium and the same process was repeated for five successive transformations. Such acclimatized samples were centrifuged at 10000 rpm for 10 minutes for removal of large particles.

Selective enrichment of chemolithotrophic sulfur oxidizing bacterial consortium

Ten ml of centrifuged broth of aerated and acclimatized samples were separately inoculated in 100 ml of liquid enrichment medium (pH 5.0±0.2) in 250 ml sterile glass bottles and incubated in dark (to avoid photosynthetic contaminants) on rotary shaker at 150 rpm at temperature 30±2°C for 8 days. These enrichments were referred as SOB1, SOB2, SOB3, SOB4, SOB5, SOB6, SOB7, SOB8, SOB9 and SOB10.

Screening of effective sulfur oxidizing bacterial enrichments

TBCG broth and TBCG agar were used as screening medium for selection of effective sulphur oxidizing bacterial enrichments. 5ml each of SOB1, SOB2, SOB3, SOB4, SOB5, SOB6, SOB7, SOB8, SOB9 and SOB10 were transferred to 50 ml TBCG broth in 65ml glass bottles and kept for incubation on shaker (150rpm) at 30±2°C for 7-8 days and observed for change in color of the medium. Sulphur oxidizing bacterial growth monitored by analyzing parameters like reduction in pH and concentration of sulfate after 8 days. When pH drops to 1.0±0.2 enrichments were transferred to fresh medium (Khan *et al.,* 2012).

Isolation of acidophilic *Thiobacillus* spp. on TBCG agar medium

After screening in liquid medium, selected SOB enrichments (SOB1, SOB2, SOB4, SOB6, SOB7, SOB 8, SOB9, and SOB10) were streaked on Thiobacillus Agar (TA) plates and Thiobacillus Bromocresol Green Agar (TBCGA) plates for isolation of acidophilic Thiobacillus spp. and pH of medium was maintained at acidic pH range (4.0-5.0). All inoculated agar plates were incubated in invert position at temperature 30±2°C for 7 days wrapped in aluminum foil for avoiding dehydration of agar medium due to loss of moisture (Khan *et al.,* 2012).

Isolated colonies on both media were observed and compared with morphological and cultural characteristics of members of *Acidithiobacillus* (acidophilic Thiobacillus), as mentioned in Bergey's Manual of Systematic Bacteriology 2nd edition (Kelly and Harrison, 1989). Nineteen distinct colonies showing cultural similarities with *Acidithiobacillus* on TA agar and showing colour change of TBCG agar were marked and labeled (Starosvetsky *et al.,* 2013). All nineteen colonies dissolved to make a uniform suspension. Due to a very small size of colonies, care should be taken during preparation of suspension from each colony by aseptically picking a single isolated colony with sterile loop in 1ml of Thiobacillus broth (pH 4.0± 2) inside sterile micro centrifuge tubes of 2ml capacity.

Microscopic observation using Phase Contrast Microscope

A large drop of suspension was placed over a clean glass slide and observed for morphology and motility under 40X magnification and Gram staining performed using Gram staining kit (HIMEDIA K001-1KT) as per instruction provided and observed under 100X oil immersion objective. Microscopic characters were compared with those of type species of genus *Acidithiobacillus* taken from literature (Kelly and Harrison, 1989; Boone *et al.,* 2001). Eight isolates (referred as KSB 1-8) showing Gram negative, short rod shaped and slightly or highly motile bacteria were selected (Table 3) and purified by successive sub culturing. 1ml of each selected isolate then inoculated in 10 ml TBCG broth in 25 ml serum bottles and mixed well and incubated on shaker (150rpm) at 30± 2°C for 7-8 days. After every 24 hrs aliquots were analyzed for decrease in pH and formation of sulphate.

Effect of different pH and sulphur concentrations on sulphur oxidation ability of KSB1 to KSB8:

As per aim of this study isolated SOB should withstand and oxidize higher sulphur concentration at acidic pH. So effect of different pH and sulphur concentration on sulphur oxidizing ability of selected SOB isolates KSB1 to KSB8 was studied. For each isolate a test set was prepared each containing 30 ml liquid enrichment medium (pH 5.0) supplemented with different elemental sulphur concentrations (2, 4, 6, 8, and 10%) in 100 ml Erlenmeyer flasks. An inoculum of 10% of each one of selected SOB was inoculated in respective medium. Erlenmeyer flasks were incubated at 30°C and 150 rpm for 7 days. Control flasks contained liquid enrichment medium with different sulphur concentration but no inoculum was added. After the incubation period, the oxidation of elemental sulphur by eight SOB was evaluated by sulphate formation and pH decrease of medium.

Effect of pH on sulphur oxidizing activity was evaluated for KSB1 to KSB8 (Fig.) which were prepared similarly in test and control sets as was mentioned above. But initial pH of liquid enrichment medium was adjusted to pH values of 2, 4, 6 and 8 in both sets at sulphur concentration of 1 %, inoculum was added to test set but not in control flasks. After incubation of 8 days bacterial oxidation of sulphur was estimated by sulphate production and pH decrease of medium. KSB 7 withstands higher sulphur concentration at acidic pH range retaining maximum sulphur oxidation ability and selected for further studies.

Molecular identification of KSB 7 using 16 S rRNA

Using centrifuge at 5000 rpm for 15 min the bacteria were harvested from pure culture of KSB 7 isolate.DNA extraction was done using DNeasy Plant Mini Kit (Qiagen, Germany) as per manufacturer's guidelines. The bacterial gDNA were eluted in 50 µl of elution buffer and the quality of eluted DNA checked on 0.8 % agarose gel electrophoresis and Spectrophotometeric analysis by NanoDrop ND 1000 (Thermo Scientific, USA). Fragment of 16S rRNA gene was amplified by 16SrRNA-F and 16SrRNA-R primers. A single discrete PCR amplicon band of 1500 bps was observed when resolved on agarose gel. The PCR amplicon was purified to remove contaminants. Forward and reverse DNA sequencing reaction of PCR amplicon was carried out with 16SrRNA-F and 16SrRNA-R primers form APS Life-tech commercial laboratory, Pune (M.H.), India. The sequence encoded by this fragment was compared with database sequences using the BLAST program.Once the sequencing wasdone, the resultant nucleotide sequence subjected to n-BLAST analysis (http://blast.ncbi.nlm.nih.gov) in National Center of

Biotechnology Information (NCBI) and deposited in gene bank database for universal accession.

Phylogenetic analysis

By using query and control sequence a phylogenetic tree was constructed using Molecular Evolutionary Genetics Analysis Tool (MEGA 6) (Tamura *et al.*, 2013). Based on maximum identity score sequences were selected and aligned using multiple alignment software program Clustal W.

Statistical and Bioinformatics Analysis

The all experiments carried out in triplicates and preparation of graphs were done using Microsoft excel 2010. Tukey's comparison test and One-Way Analysis of Variables (ANOVA) were done to determine the significance, if the significant differences ($P< 0.05$) between the before and after treatment mean values then the difference become significant (Tukey, 1949). Different gene sequences of *16S rRNA* genes were obtained through blast searches in GenBank (http://www.ncbi.nlm.nih.gov/BLAST/) and multiple sequence alignments were done by ClustalW2 programme (http://www.ebi.ac.uk/Tools/clustalw2/index.html) (Thompson *et al.*, 1994). The phylogeny based on the partial DNA sequences was analyzed using the neighbor- joining (NJ) method in MEGA6 software (http://www.megasoftware.net/) (Tamura *et al.*, 2013).

Results and Discussion

Initial pH and sulphate concentration of collected water samples

Sulphur oxidizing bacteria were isolated from the ten locations from four different processes of industrial waste treatment plants of Jain Irrigation Systems, Ltd. Jalgaon, Maharashtra. On basis of previous six months studies the average values of monthly sulphate concentration analysis of samples taken from these systems were considered. The presence in a high concentration of SOB were isolated and reported earlier by different researchers from fresh water, canal water and uranium mines (Berthelot *et al.,* 1993; Kelly and Harrison, 1989; Kubo *et al*., 1995; Takano *et al*., 1997). Also from mud soil, sewage water, biogas slurry, thermogenic composts and tannery effluent sulphur oxidizing bacteria were isolated (Vidyalakshmi and Sridar 2006, Vimala 2008, Vidhyashri and Sridar 2011) .

Table 1: Initial Analysis of pH and sulphate concentration of samples immediately after transportation to laboratory

Sr.no.	Samples	Initial pH	Initial SO_4^{2-} concentration (mg/L)
1	SOB 1	5.6	65.18
2	SOB 2	6.8	70.11
3	SOB 3	6.9	63.81
4	SOB 4	6.8	63.39
5	SOB 5	6.6	57.93
6	SOB 6	4.9	428.23
7	SOB 7	6.3	755.18
8	SOB 8	6.6	395.13
9	SOB 9	6.4	790.00
10	SOB 10	6.2	615.18

Selective enrichment of effective SOB consortium

From initial sample analysis of collected liquid samples it was evident that selected sources were suitable for isolation of SOB as they were rich in sulphate content (Table 1, Figure 1). Continuous aeration of samples restricted growth of anaerobic microorganisms. The maximum isolates were preferred Sulphur as Sulphur source and show good growth in thiobacillus medium. Thiobacillus broth (pH 4.0 -5.0) selectively enriched acidophilic SOB and growth was promoted by providing trace element solution (Ravichandra *et al.,* 2006)

Fig. 1: Selective enrichment (SOB 1-SOB 10) of sulphur oxidizing bacteria. Selection and Screening of effective sulfur oxidizing bacterial enrichments

During the growth of SOB utilizing reduced sulphur compounds in medium as sole energy source acid is produced as intermediate product of sulphur oxidation that reduced pH of medium. Ultimately pH reduction is primary indication for active growth of SOB. Table-2 indicated initial and final pH of all selective enrichments after 8 days incubation. pH of eight SOB consortia were reduced in order of SOB 8 > SOB 10>SOB 2> SOB 9>SOB 7> SOB 6> SOB1> SOB 4. Sulphate concentration were changed in order of SOB 8 > SOB 10>SOB 2> SOB 7>SOB 9 > SOB 6> SOB1> SOB 4. More was sulphate produced more was rate of oxidation of sulphur compounds. Based upon the pH reduction 10 isolates were screened and selected 8 best isolates for further studies (Table 2). The SOB 3 and SOB 5 pH reduced slightly, so these two were excluded and remaining were selected for isolation of effective SOB. The isolate SOB8 (Scrubber recirculation water S2) recorded the highest sulphate production of 1895 mg/L broth followed by SOB10 and SOB2 of 1815 and 1775 mg/L respectively. Comparatively SOB3 recorded lowest sulphate production of 835 mg/L where control sulphate concentration 97 mg/L (Table 2).

Table 2: Selection of effective SOB enrichments on basis of pH reduction and sulphate formation test.

SOB enrichments	pH		SO_4 concentration (mg/L)	
	Initial	Final	Initial	Final
	t=0th day	t= 8th day	t=0th day	t= 8th day
SOB 1	5	4.3	360	1595
SOB 2	5	3.6	470	1775
SOB 3	5	4.9	285	835
SOB 4	5	4.4	345	1260
SOB 5	5	4.8	305	1065
SOB 6	5	4.1	385	1605
SOB 7	5	3.9	435	1710
SOB 8	5	2.3	495	1895
SOB 9	5	3.8	410	1650
SOB 10	5	3.2	480	1815
Control	5	5	100	97

Screening and Isolation of *Acidithiobacillus* spp. on solid medium

From the ten isolates eight effective SOB enrichments (SOB 1, 2, 4, 6, 7, 8, 9, 10) were selected and screened for isolation of *Acidithiobacillus* sp. by using TBCG agar and *Thiobacillus* agar medium on the basis of pH reduction test and sulphate formation test. TBCG agar served as best screening medium as it contained bromocresol green (BCG) solution as pH indicator dye that imparted blue colour at pH 5.0 to Thiobacillus agar that gradually lost its colour at more acidic pH as growth proceeds during incubation at 30± 2°C. Reduction of pH associated with loss of colour and visible growth of sulphur oxidizing colonies were observed on TBCG agar plates (Starosvetsky *et al.,* 2013).

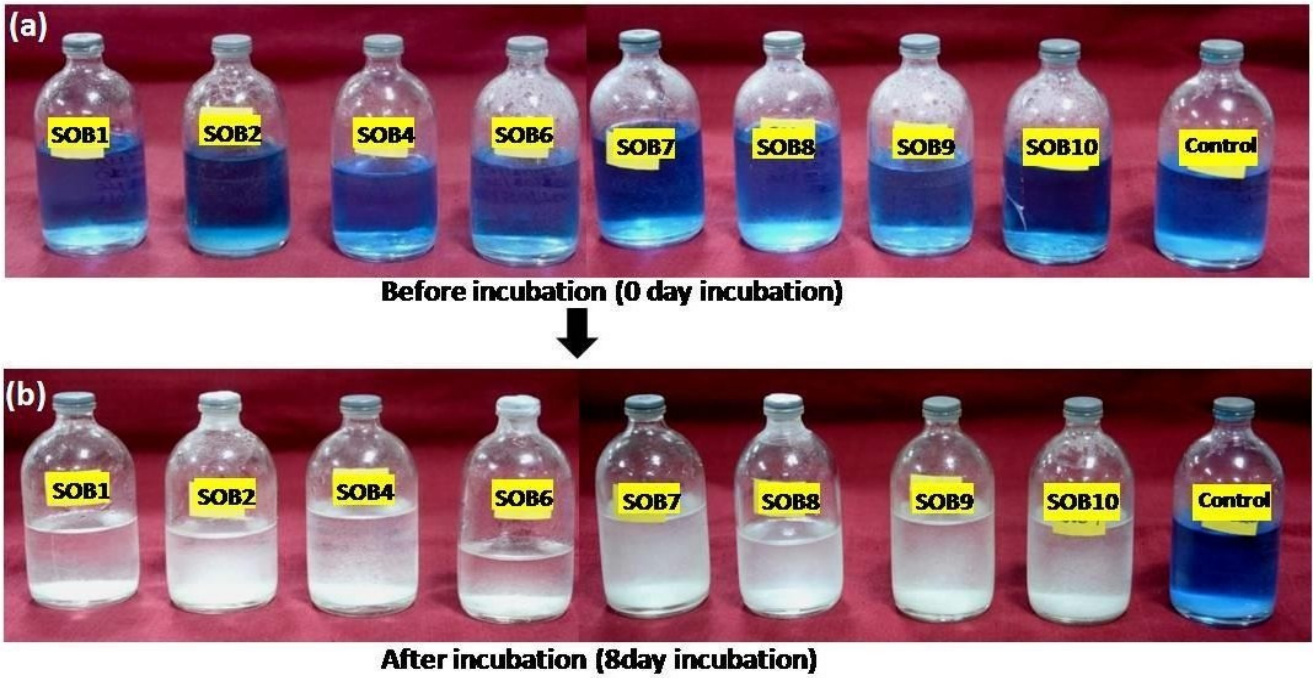

Fig. 2: Screening of effective SOB in TBCG broth

Colonies appeared after 7 days on both medium but as compared to TA plates colonies growing on TBCG agar were more prominent. The reduction of pH ranges from 2.2 to 2.7 by the autotrophic SOB isolates observed by Vimala and Sridar 2009. For isolation and cultivation of Thiobacillus species on solid medium a Thiobacillus agar is recommended. Colonies appear in 7-8 days but very minute in size and transparent not easily visualized due to poor resolution on Thiobacillus agar. Addition of bromocresol green (BCG) pH indicator dye to Thiobacillus agar resolved the problem by providing a clear-cut resolution and visual emergence of the growing bacterial colonies on the 3rd and 4th days, from the initial plating. Actively growing colonies were associated with sulfuric acid production that changed colour of medium from blue to pale yellow (Starosvetsky *et al.,* 2013).

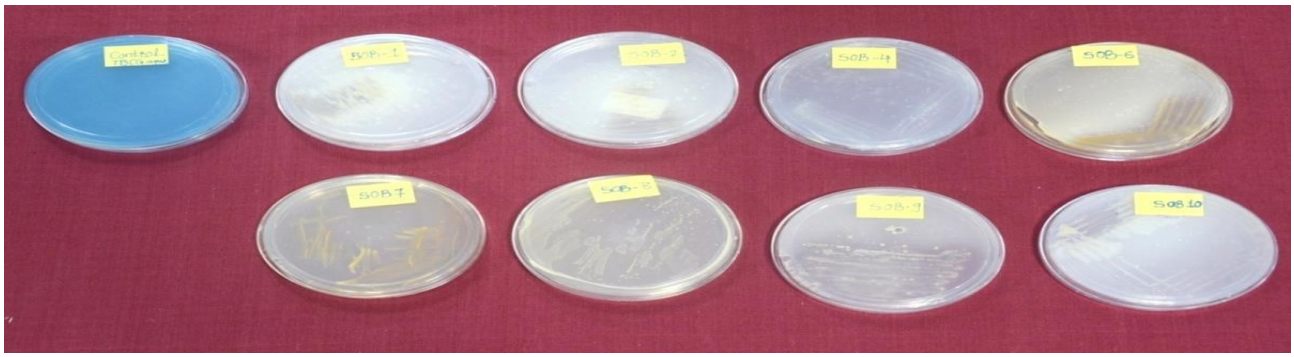

Fig. 3: Screening and Isolation of *Acidithiobacillus* spp. on TBCG agar medium

Among the well isolated colonies obtained on all TBCG agar plates inoculated with selected SOB enrichments, only 19 isolated colonies were marked (Supplementary Table 1) and selected on basis of their colony characteristics similar to Acidithiobacillus sp. on TBCG agar plates (Starosvetsky et al 2013). As these isolates were able to utilize thiosulphate incorporated in medium as a sole energy source at acidic pH and associated with drop in pH of medium that has indicated presence of Acidithiobacillus sp.

Table 4 : Microscopic observation of selected SOB isolates on TBCG agar plates :

Name of isolated colony	Morphology (40 X)	Motility (40 X)	Gram character (100 X)
KSB1	Short rods	Motile	Gram negative
KSB2	Short rods	Motile	Gram negative
KSB3	Short rods	Motile	Gram negative
KSB4	Short rods	Motile	Gram negative
KSB5	Short rods	Motile	Gram negative
KSB6	Short rods	Motile	Gram negative
KSB7	Short rods	Motile	Gram negative
KSB8	Short rods	Motile	Gram negative

The screened isolates were characterized morphologically and results were presented in the Table 4. All the SOB were gram negative, motile, short rod shaped cells and off white to yellowish colonies on TBCG agar plates. All the 19 isolates were identified through conventional biochemical tests and microscopically examined for morphology, motility and Gram character. Characterization of 8 best sulphate producing isolates out of 19 similar to Acidithiobacillus characteristics shown in Table 4 and Table 5 were selected. Kelly and Wood in 2000 described *Acidithiobacillus* as obligatory acidophilic, aerobic, small Gram negative rods (0.4-2.0 µm), some of them are motile; non spore formers. Possess chemolithotrophic mode of nutrition supported by the oxidation of range of reduced sulfur compounds (thiosulphate, sulfur, sulphides, polythionates and thiocyanate).

pH and sulphate concentration of isolates

Monitoring pH and sulphate concentration of eight isolates named KSB 1, KSB2, KSB3, KSB4, KSB5, KSB6, KSB7 and KSB8during incubation was studied (Figure 4, Table 5).

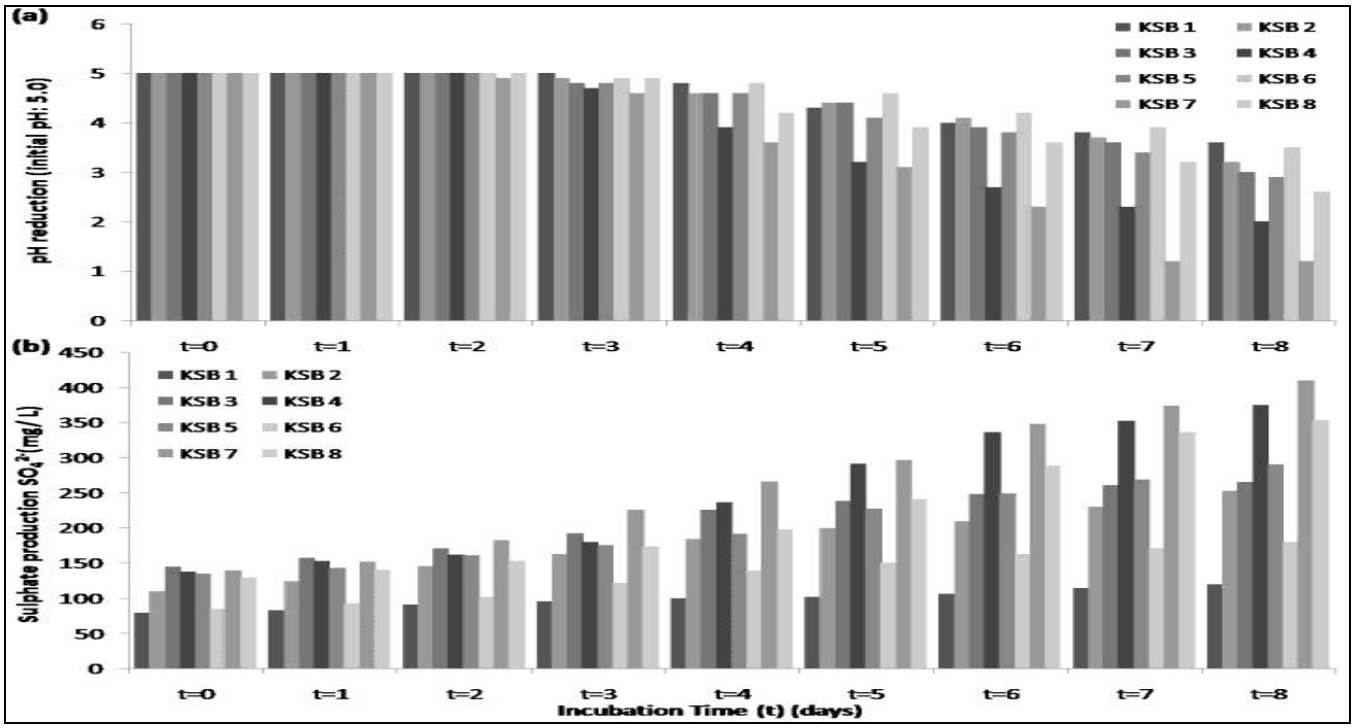

Fig. 4: Monitoring pH and sulphate concentration of 8 isolates during 8 days incubation.
(a) pH reduction of selected isolates from initial pH: 5.0, (b) Sul[phate reduction SO^{2-}(mg/ L).

Table 5 : The pH and sulphate concentration of 8 isolates during 8 days incubation.

Isolate	Incubation Time (t) (days)			
	t=0		t=8	
	pH	SO_4^{2-} (mg/ L)	pH	SO_4^{2-} (mg/ L)
KSB 1	5.0	80± 4.312	3.6 ± 0.198 ***	120± 3.458 ***
KSB 2	5.0	110± 3.284	3.2 ± 0.136 ***	253± 4.782 ***
KSB 3	5.0	145± 4.860	3.0 ± 0.321 ***	265± 5.213 ***
KSB 4	5.0	138± 3.614	2.0 ± 0.209 ***	375± 3.125 ***
KSB 5	5.0	135± 2.698	2.9 ± 0.254 ***	290± 6.236 ***
KSB 6	5.0	85± 3.665	3.5 ± 0.326 ***	180± 1.958 ***
KSB 7	5.0	140± 6.254	1.2 ± 0.187 ***	410± 5.329 ***
KSB 8	5.0	130± 4.946	2.6 ± 0.337 ***	353± 4.358 ***

* represent the p value of significance, $p \leq 0.0001$= *** with respect to day 0.

Effect of different sulfur concentration and pH on KSB 1 to KSB 8 isolates

Effect of different sulphur concentrations on sulphur oxidizing activity of SOB isolates (KSB 1 to KSB 8) was studied. From figure 5, it was evident that all of them can grow at increasing sulphur concentrations in the range of 2% to 10% (w/v). But, KSB 7 produced highest sulphate 990mg/L at 8% sulphur concentration followed by KSB 4 produced 810 mg/L sulphate. When sulphur concentration increased to 10%, production of sulphate by KSB 7 was decreased to 785 mg/L, whereas KSB 4 till produced 895 mg/L of sulphate. Among remaining SOB isolates, KSB 2, KSB 3 and KSB 8 withstands and showed highest sulphate formation at 6% sulphur concentration. KSB 1, KSB 5 and KSB 6 decreased their sulphate formation activity as initial sulphur concentration increased beyond 4%. It is worthwhile to notice that SOB isolates have a different behavior with respect to various sulphur concentrations. KSB 7 decreased its sulphate formation ability when sulphur concentration exceeds 8%. Whereas, KSB 4 improves its sulphur oxidation ability within range of 8% to 10% sulphur concentration. pH of medium broth after incubation varies according to initial sulphur concentration. In general it decreases as sulphate formation increased. KSB 7 showed lowest pH value 0.6 at its highest sulphur oxidation ability. Whereas KSB 4 reduced its initial pH at 0.7 when the maximum sulphur concentration was 10% (data not shown).

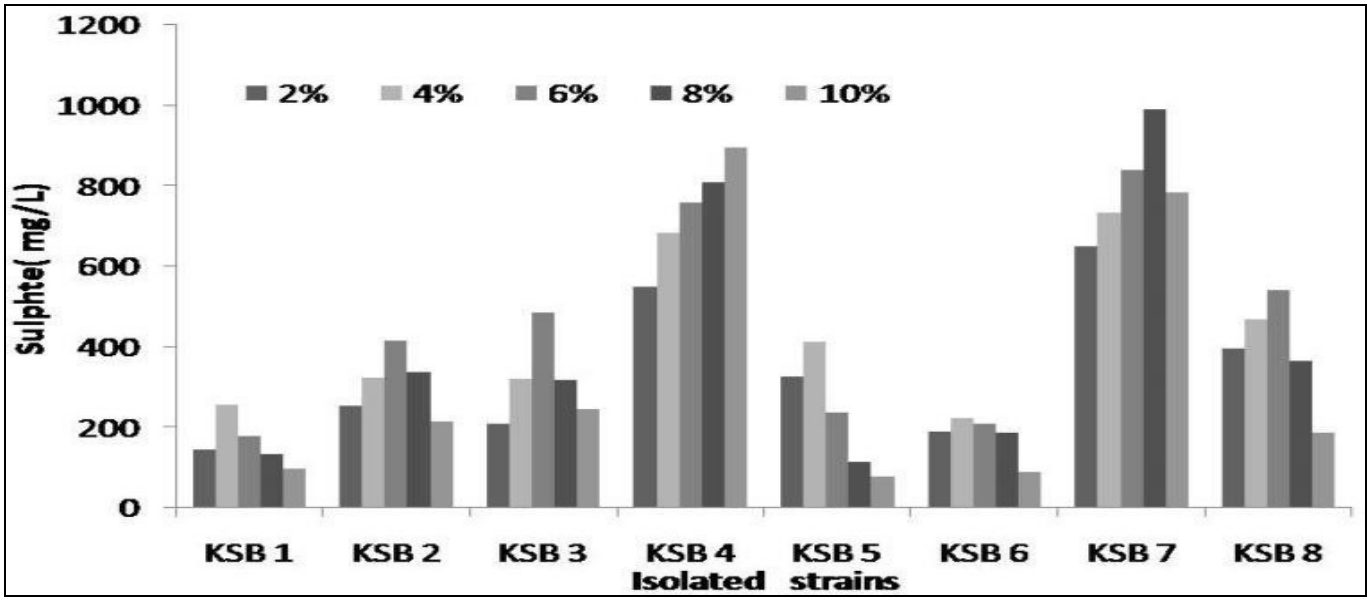

Fig 5: Effect of different sulfur concentration on isolated strains.

To determine the effect of different pH on sulfur oxidizing activity, growth medium was prepared with four different pH (3, 5, 7 and 9). As shown in figure 6, the eight SOB isolates were preferred to grow in the range of pH 3.0 to 9.0 accompanied by the production of sulphate in the medium. Based on the results, KSB1-8 isolates produced sulphate in various concentrations depending on different initial pH of growth medium. On the basis of figure 6 it was observed that the optimal pH of SOB for sulphur oxidation activity was 5.0. Near about all isolates were able to produce highest sulphur oxidizing activity at pH 5.0 in that KSB 7 produced 225 mg/L sulphate followed by KSB 4 that produced 185 mg/L. KSB5 and KSB1 produced 77 and 78 mg/L lowest sulphur oxidizing activity at pH 5.0. As mentioned earlier from figure 6 and 5; KSB 7 and KSB 4 were showing higher sulphate production at various range of sulphur concentration and pH hence both selected as most promising isolates.

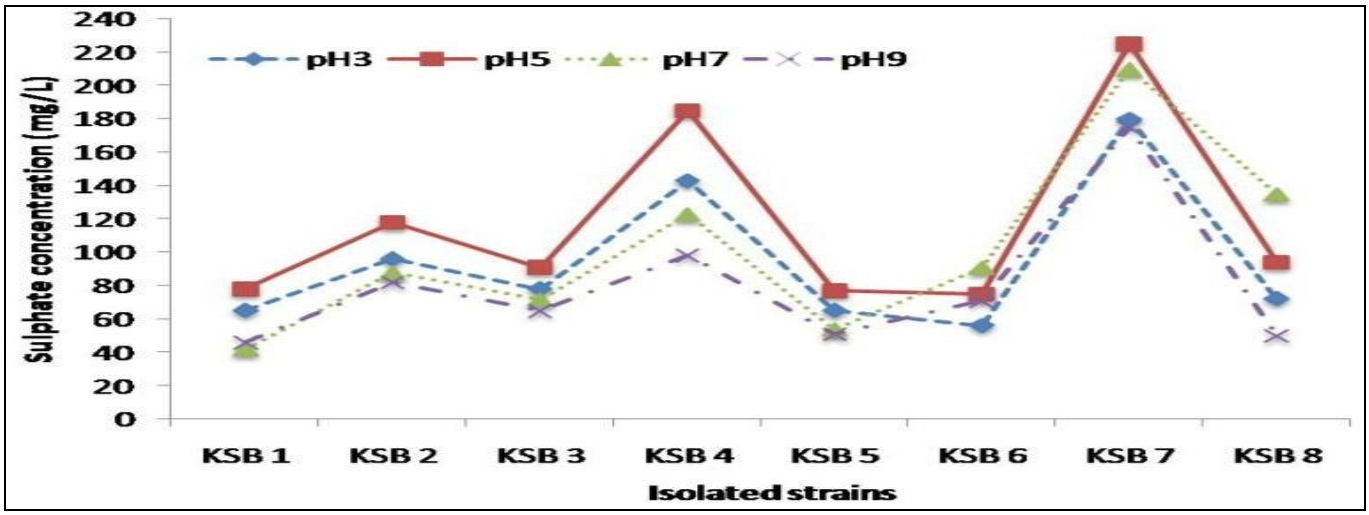

Fig 6: Effect of different pH on sulfur concentration of isolated strains. Identification of KSB7 isolate

The KSB 7 that produced highest amount of sulphate and pH reduction was selected for phylogenetic analysis through 16S rRNA gene sequencing. The band derived from a colony was compared to a band representing cluster bacteria and also band obtained from other colonies observed that the colonies migrated from same position as of KSB7. The 16S rRNA sequences were similar of all the band of KSB 7 and affiliated with the same cluster (Figure 7). The preliminary test showed that their physiological characteristics were identical. The data base search with 16S rRNA sequences showed *Acidithiobacillus thiooxidans* to be the closest relative of KSB 7. The sequences KSB 7 shared 99.67% identity with Acidithiobacillus thiooxidans strain AAU (DQ834372.1) and Acidithiobacillus thiooxidans strain zmb (JQ820325.1) also with (FJ998186.1) it shared 99.47 % identity (Figure 7) . The sequence of KSB 7 reported in this research was deposited in GenBank with accession number MN382350. Sulfur-oxidizing bacteria have also been isolated from soil of Bhitarakanika, Odisha, India were identified as *Pseudomonas, Stenotrophomonas, Alcaligenes, Bordetella*spp and *Thiobacillus*spp. Sulfur oxidizing *Pseudomonas* spp. (Thatoi *et al*., 2012) and mangrove soil of Mahanadi River Delta (Behera *et al*., 2014).

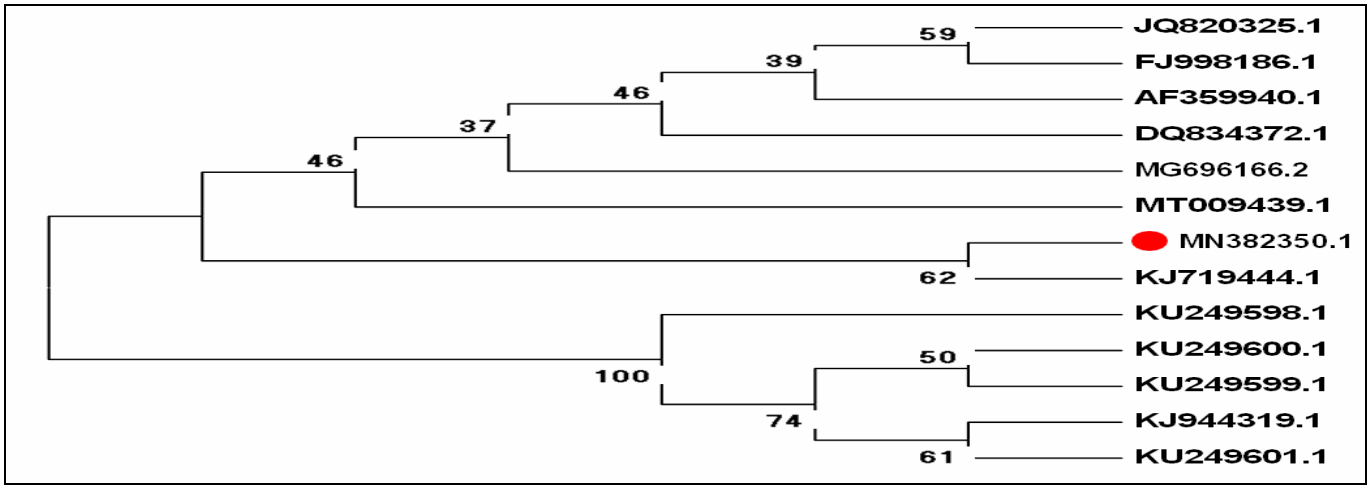

Fig. 7 : Molecular Phylogenetic analysis of KSB7 isolate of SOB by Maximum Likelihood method based on analysis of 16S rRNA gene sequences.

The KBS7 16SrRNA sequence phylogeny constructed using reference sequences from the GeneBank database. The phylogenetic analysis involved 13 amino acid sequences. The coding data was translated assuming a Standard genetic code table. All positions containing gaps and missing data were eliminated. Evolutionary analyses were conducted in MEGA6. Bootstrap values of 1000 are shown.

All the eight isolates were performed the growth in the pH 3.0 to 9.0 and show optimum pH of the isolates from 7.0-7.5 (Fig. 6). The KSB 7 isolates show maximum efficiency on pH 7and produced 225 mg/L sulphate. All of them can grow at increasing sulphur concentrations inthe range of 2% to 10% (w/v). But, KSB 7 produced highest sulphate 990 mg/L at 8% sulphur concentration. The present study emphasizes the importance and the role of sulphur oxidizing bacteria in the oxidation of sulphur in process waste water samples. Also, the pH reducing property of sulphur oxidizing bacteria by the production of sulphuric acid can be utilized for further studies.

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Author's contributions

Dr S.S. Bhardwaj conceived of the presented idea and

supervised the findings of this work.

K.P. Borse, V.A. Agnihotri, H.K. Bhandarkar writing of the manuscript.

Conflict of Interests

The authors declare no conflict of interests.

Abbreviations

SOB: Sulfur oxidizing bacteria, mg/L: milligram per liter, rRNA: ribosomal ribonucleic acid, CH_4: methane, CO_2: carbon dioxide, N_2: nitrogen, O_2: oxygen, H_2S: hydrogen sulfide, NH_3: ammonia, TBCG agar: Thiobacillus bromocresol green agar, BCG: bromocresol green.

References

Avelizapa, N.G.R.; Gómez-Ramírez, M.; Hernández-Gama, R.; Aburto, J. and García de León, R. (2013). Isolation and Selection of Sulfur-oxidizing Bacteria for the Treatment of Sulfur- containing Hazardous Wastes. Chem. Biochem. Eng.; 27 (1): 109-117.

Beffa, T.; Blanc, M. and Aragno, M. (1996). Obligately and facultatively autotrophic, sulfur- and hydrogen-oxidizing thermophilic bacteria isolated from hot composts. Arch. Microbiol.; 165: 34-40.

Behera, B.C.; Patra M.; Dutta, S.K. and Thatoi, H.N. (2014). Isolation and Characterisation of Sulphur Oxidising Bacteria from Mangrove Soil of Mahanadi River Delta and Their Sulphur Oxidising Ability. Jour. Of App. And Enviro. Mcro.; 2(1): 1-5.

Berthelot, D.; Leduc, L. and Ferroni, G. (1993). Temperature studies of iron-oxidizing autotrophs and acidophilic heterotrophs isolated from uranium mines. Can. J. Microbiol.; 39: 384-388.

Boone, D.R.; Castenholz, R.W. and Garrity, G.M. (2001). Bergey's manual of systematic bacteriology. Springer, New York, 60-63.

Hou, N.; Yongzhen, X.; Wang, X.; Liu, H.; Liu, H. and Xun, L. (2018). H2S biotreatment with sulfide-oxidizing heterotrophic bacteria. Biodegradation, 29(6): 511–524.

Isamu and Suzuki (1999). Oxidation of inorganic sulfur compounds: Chemical and enzymatic reactions. Can. J. Microbiol., 45: 97-105.

Ito, T.; Sugita, K. and Okabe, S. (2004) Isolation, Characterization, and In Situ Detection of a Novel Chemolithoautotrophic Sulfur-Oxidizing Bacterium in Wastewater Biofilms Growing under Microaerophilic Conditions. Appl. And Enviro. Micro.; 70(5): 3122-3129.

Kantachote, D. and Innuwat, W. (2004). Isolation of Thiobacillus sp. for use in treatment of rubber sheet wastewater Songklanakarin J. Sci. Technol.; 26(5): 649-657.

Kelly, D. and Harrison, A. (1989). Genus Thiobacillus. Bergey's manual of systematic bacteriology. 1: 1842-1858.

Kelly, D.P. and Harrison, A.H. (1989) Genus Thiobacillus. In Bergey's Manual of Systematic Bacteriology, 1st edn, vol. 3, Edited by J. T. Staley, M. P. Bryant, N. Pfennig & J. G. Holt. Baltimore: Williams & Wilkins. pp. 1842±1858.

Kelly, D.P. and Wood, A.P. (2000). Reclassification of some species of Thiobacillus to the newly designated genera Acidithiobacillus gen. nov.; Halothiobacillus gen. nov. and Thermithiobacillus gen. nov. Int. Jour. of Syst. and Evolu. Micro.; 50: 511-516.

Khan, S.; Haq, F.; Hasan, F.; Saeed, K. and Ullah, R. (2012). Growth and Biochemical Activities of Acidithiobacillus thiooxidans Collected from Black Shale. Jour. Of Microb. Resea.; 2(4): 78-83.

Konishi, Y.; Asai, S. and Yoshida, N. (1995). Growth Kinetics of Thiobacillus thiooxidans on the Surface of Elemental Sulfur. Appl. and Envir. Micro.; 61(10): 3617-3622.

Kubo, I.; Karube, I.; Takeuchi, T.; Furusawa, M.; Arikawa, Y. and Kanagawa, T. (1995). A biosensor based on Thiobacillus thioparus for measuring thiosulfate and methanethiol. Can. J. Microbiol.; 41: 366-371.

Lin, W.C.; Chen, Y.P. and Tseng, C.P. (2013). Pilot-scale chemical–biological system for efficient H2S removal from biogas. Bioresource Technology 135: 283-291.

Namgung H.K.; Ahn H.Y. and Song J.H. (2012). Development of a two-phase bioreactor for the biological removal of hydrogen sulfide from biogas. Energy Procedia., 14: 1143-1148.

Promuan, K. and Thong, S. (2017). Efficiency Evaluation of Biofilter for Hydrogen Sulfide Removal from Palm Oil Mill Biogas. Energy Procedia., 138: 564-568.

Ravichandra, P.; Mugeraya G.; Rao A.G.; Ramakrishna, M. and Jetty, A. (2007). Isolation of Thiobacillus sp from aerobic sludge of distillery and dairy effluent treatment plants and its sulfide oxidation activity at different concentrations. Journal of Environmental Biology, 28(4): 819-823.

Starosvetsky, J.; Zukerman, U. and Armon, R.H. (2013). A simple medium modification for isolation, growth and enumeration of Acidithiobacillus thiooxidans (syn. *Thiobacillus thiooxidans*) from water samples. Jour. of Micro. Metho., 92: 178-182.

Tamura, K.; Stecher, G.; Peterson, D.; Filipski, A. and Kumar, S. (2013). MEGA6: Molecular Evolutionary Genetics Analysis Version 6.0. Mol. Bio. and Evol.; 30: 2725–2729.

Takano, B.; Koshida, M.; Fujiwara, Y.; Sugimori, K. and Takayanagi, S. (1997). Influence of sulfur-oxidizing bacteria on the budget of sulfate in Yugamacrater lake, Kusatsu-Shirane volcano, Japan. Biogeochem. 38: 227-253.

Thatoi, H.N.; Behera, B.C.; Dangar, T.K. and Mishra, R.R. (2012). Microbial biodiversity in mangrove soil of Bhitarakanika, Odisha, India, International Journal of Environmental Biology, 2(2): 50-58.

Thompson, J.D.; Higgins, D.G. and Gibson, T.J. (1994). Clustal W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Research 22: 4673–4680.

Tukey, J.W. (1949). Comparing Individual Means in the Analysis of Variance. Biometrics 5:99.

Vidhyasri, S. and Sridar, R. (2011). Development of a carrier based formulation of sulphur oxidizing bacterium for enhancing the productivity of crops requiring sulphur nutrition. J. Basic. Appl. Sci. Res.; 1: 345-353.

Vidyalakshmi, R. and Sridar, R. (2006). Isolation and characterization of sulphur oxidizing bacteria. J. Culture Collections.; 5: 73-77.

Vimala, P. (2008). Studies on the use of Sulphur oxidizing

bacteria (SOB) to enhace Sulphur nutrition, productivity and oil content in Groundnut (*Arachis hypoges* L.). M.Sc. (Ag.) Thesis submitted to Tamil Nadu Agricultural University, Coimbatore.

Vimala, P. and Sridar, R. (2009). Studies on the use of Sulphur oxidizing bacteria (SOB) to enhace Sulphur nutrition, productivity and oil content in Groundnut (*Arachis hypoges* L.). J. Soil Biol. Ecol.; 29:30-41.

Visser, J.M.; Roberstson, L.A.; Verseveld, H.W.V. and Kuenen, J.G. (1997). Sulfur production by obligately chemolithoautotrophic Thiobacillus species. Appl. Environ. Microbiol.; 63: 2300- 2305.

Waksman, S.A. and Joffe, J.S. (1921). Microorganisms concerned in the oxidation of sulfur in the soil. Techni. Seri.; 33: 239-256.

Wang, R.; Lin, J.; Liu, X.M.; Pang, X.; Zhang, C.J.; Yang, C.L.; Gao, X.Y.; Lin, C.M.; Li, Y.Q.; Li, Y.L.; Lin, J.Q. and Chen, L.X. (2019) Sulfur Oxidation in the Acidophilic Autotrophic Acidithiobacillus spp. Front. Microbiol. 9: 3290-3296.