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Isolation of *Thiobacillus* Species from Distillery Spentwash and Its Sulfide Oxidation Activity

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Abstract
In the present study a total of 14 microorganisms were isolated from distillery spentwash samples using standard methods of isolation and enrichment. Three of them had the ability to oxidize sulfur in liquid culture and able to grow autotrophically using elemental sulfur. These microbial cultures were able to produce sulfates from sulfides within pH range of 3.6 to 8.0. Based on the morphological as well as physiological studies and on comparison with the reference strain of *Thiobacillus novellus* (NCIM 2858), all the isolates were confirmed to belong to the genus *Thiobacillus*. The effect of initial sulfide concentration on the activity of isolated *Thiobacillus* species was studied. Experiments on shake flask level (1L scale) were conducted using three selected isolates based on maximum pH reduction (SOM 05, SOM 06 and SOM 12) against reference strain with initial sulfide concentration of 200 mg/l. Out of three isolates, SOM 05 oxidized 200 mg/l of sulfide within 120 hours. SOM 06 and SOM 11 required 168 hours for oxidation of sulfide. Utilization of sulfide and formation of sulphate was estimated periodically. Presence of sulfides in raw spentwash results in H_2S formation (0.1-2.0 %, v/v) in biogas which is not desirable. The results from the study indicate that it is possible to isolate *Thiobacillus* species from distillery spentwash for further use in sulfide oxidation in sulfide rich effluents.

Keywords
Sulfide oxidizing, sulphate, spentwash, *Thiobacillus*

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INTRODUCTION:
Sulfur compounds are among the chief pollutants in the environment since they cause unfavorable impacts in the ecosystem. It is widely known that the discharge of hydrogen sulfide (H_2S) and reduced sulfur compounds from anthropogenic sources produces corrosion to metallic materials, terrible odour and under particular conditions, they are toxic to human health [1]. To reduce the harm caused by sulfur contaminants, several physicochemical processes are being employed which in most cases are costly and are not ecofriendly [2]. Biological techniques have appeared as probable alternatives to solve this problem since there are microorganisms which are

able to transform and /or capture sulfur compounds, such as chemoautotrophic sulfur-oxidizing microorganisms (SOM). SOM use carbon dioxide as carbon source and gain the energy from oxidation of reduced sulfur compounds such as sulfite, sulfur, thiosulfate, hydrogen sulfide and several polythionates [3].

The SOM are phylogenetically dissimilar. Aerobic sulfur oxidation is constrained to *Sulfolobales* members in the *Archaea* domain. Sulfur is oxidized by anaerobic phototrophs or aerobic lithotrophs in *Bacteria* domain [4]. Members of the genus *Beggiatoa, Bacillus, Acidianus, Sulfolobus, Thiothrix, Thermothrix, Thiovolum, Thioalcalimicrobium, Thioalkallivibrio* and *Thiobacillus* can be classified as *Acidithiobacillus, Thermithiobacillus & Halothiobacillus* [5]. In 1922 the *Thiobacillus* species was first time isolated from soil sample [6]. There are several potential applications of SOM found in literature. It includes oxidation of sulfide used for desulfurization of biogas using *Thiobacillus* and other strains [7]. Biological oxidation using *Thiobacillus* is suitable for the treatment of coal containing finely distributed pyrite [8]. Use of isolated SOM to oxidize sulfur in sulfate rich rubber industry waste is also reported [9]. There are several reports available for isolation and screening of SOM from soil, industrial wastes and activated sludge samples [10,11]. Reports in the literature mention species belonging to the genera *Thiobacillus, Thiomicrospira, Thiosphaera*, *Paracoccus, Pseudomonas Alcaligenes* and *Xanthobacter* that have a chemolithotrophic growth in the presence of inorganic sulfur [8, 12].

The aim of this study was to isolate SOM from distillery spentwash and select suitable strains for pretreatment of sulfide rich industrial effluents including distillery spentwash. It was also important to estimate their sulfur oxidizing ability. Autotrophic SOM such as *Thiobacillus* species has shown high affinity for H_2S. H_2S is produced after the degradation of proteins as well as other sulfur containing compounds available in feed stock of the biodigester and its concentration varies from 0.1.-2.0%, v/v [13]. Certain commercial technologies are available for the removal of H_2S which are chemically based and too expensive [14, 15]. A biological H_2S removal by way of use of potential SOM is one of the attractive options to overcome the disadvantages of H_2S removal by chemical way. The H_2S formed can be easily degraded by use of isolated SOM and can help the industry in future.

MATERIALS AND METHODS:

Reference strain and culture media

Thiobacillus novellus (NCIM 2858) was used as reference strain due to its ability to oxidize sulfur. The media used for enrichment and isolation of SOM was Thiosulfate broth that consisted (g/L) of: $Na_2S_2O_3.5H_2O$ (5.0), K_2HPO_4 (0.1), $NaHCO_3$ (0.2), NH_4Cl (0.1), pH was adjusted to 8.0 using 1N KOH [16]. Starkey broth consisted (g/l) of: KH_2PO_4 (3.0), $MgSO_4.7H_2O$ (0.2), $CaCl_2.2H_2O$ (0.2), $(NH_4)_2SO_4$ (0.5), $FeSO_4$ (traces) with pH 8.0 [17].

Sample collection

A total of 8 liquid samples were collected from aerobic treatment plant of distillery spentwash. Sterile plastic bottles were used for sample collection and stored in ice box during transportation.

Isolation of SOM

For the isolation of SOM, 5 ml of spentwash samples was placed in 125 ml Erlenmeyer flasks containing 30 ml of Thiosulfate medium and Starkey medium. Inoculated flasks were incubated at 30°C on shaker at140 rpm till development of turbidity. Afterwards, serial dilutions were prepared from 10^{-2} to 10^{-6} and 0.1 ml of each dilution was spread on Thiosulfate agar petriplates.

Thiosulfate agar petriplates were incubated at 30°C till appearance of growth [18]. Based on different colonial morphology, the colonies were selected and were plated on fresh medium for further purification and to obtain pure cultures. Purified colonies were retransferred on Thiosulfate medium. Colony characteristics of well isolated colonies were studied. Cultures were maintained and preserved on Thiosulfate slants. The isolated pure cultures were labeled and used.

Screening of isolated SOM by pH reduction test

The obtained isolates were inoculated in Thiosulfate medium with initial pH 8.0 and incubated at 30°C at 140 rpm using rotary incubator shaker (Inkarp make) for 10 days. The drop in pH was measured using pH meter (ESE make). The SOM were screened based on their efficacy to reduce the pH from 8.0 to 4.0 or less than 4.0. The selected SOM were further studied for their morphology, Gram nature, motility, colony characters and nutritional type. Negative control was carried out using pure culture of *Acetobcter aceti* (NCIM 2251). The pure culture was streaked on the Thiosulfate medium plate and incubated at 30°C.

Cell and colony characteristics of isolated SOM

Isolated SOM were negatively stained for morphology observation under microscope (Lawerence & Mayo make). Gram staining and motility was also performed of the same [19]. For colony characterization Thiosulfate agar and Starkey agar with initial pH of 8.0 were prepared. The isolated SOM were plated in sterile petriplates by the streak plate method and plates incubated at 30°C for 5 days. Colony characters of well isolated colony were studied.

Evaluation of sulfur oxidizing activity by isolates using sulfur sources

Out of total 25 microorganisms, 11 isolates were eliminated because of poor microbial growth (Table 1).

Several isolates were obtained but only those microorganisms which were able to grow faster and show higher sulfur-oxidizing activity were selected for further studies. The isolated SOM were studied for the utilization of thiosulfate and elemental sulfur using Starkey and thiosulfate broth. Inoculum of 14 isolates and reference strain were prepared in 50 ml antibiotic type bottles containing 10 ml of Thiosulfate medium. The inoculated flasks were left for 5 days to achieve appropriate growth, then an inoculum of 3 ml was transferred to fresh medium to evaluate their sulfur-oxidizing ability in batch culture, using 30 ml Thiosulfate medium in 125 ml Erlenmeyer flasks. Flasks were incubated at 30°C at 140 rpm for 5 days on shaker. The production of sulfuric acid was evaluated in the supernatant by pH measurement and microscopic observation.

Experiments were conducted for sulfide oxidation using three selected isolates (SOM 06, SOM 06, SOM 11) and reference strain using 200 mg/l of initial sulfide concentration on shake flask level. Each 2 l flask was filled with sterile 800 ml of Thiosulfate medium consisting of maintenance medium components defined above except $Na_2S_2O_3.5H_2O$. The $Na_2S_2O_3.5H_2O$ was substituted with 200 mg/l sodium sulfide. The flasks were inoculated with 20% (v/v) of 5 days old respective SOM as mentioned above. The flasks were incubated at 30°C at 240 rpm on shaker till the depletion of sulfide concentration. Samples were drawn at interval of 24 hours and analyzed for various parameters mentioned below.

Influence of various biochemicals on elemental sulfur oxidation using SOM

Filter sterile 0.1 % solution each of biotin, yeast extract, peptone, fructose, glucose, sucrose, lactose, maltose, starch and sodium acetate were added to the Starkey broth. The tubes along with control were incubated at 30°C for 15 days and observed for reduction in pH. Simultaneously, a control was maintained.

Analytical methods

Shake flask samples were analyzed for drop in pH, sulfide and sulfate content using Standard methods, APHA [20]. The sulfate can be estimated by dilution of sample at several times, added 20 ml of buffer solution, mixed in stirrer apparatus, added barium chloride crystal and stir for 60±2 seconds at constant speed. Turbidity was estimated within 5 minutes at 420 nm using spectrophotometer. Standard calibration curve was performed to estimate sulfate content of experimental samples. Using iodometric method sulfide content was estimated [20].

RESULTS AND DISCUSSION:

Isolation and screening of SOM

It is reported in the literature that the SOM reduces the pH of the growth medium [21]. The pH reduction of the medium was due to the sulfuric acid formed by SOM. Based on reduction of pH, 14 isolates were selected. It can be observed from Table 1 that total 14 isolates were selected based on reduction of pH. The isolates were named as SOM 01, SOM 03, SOM 07, SOM 09, SOM 13, SOM 17, SOM 22, SOM 28, SOM 31, SOM 42, SOM 45, SOM 47, SOM 51 and SOM 54. All the SOM except SOM 03 reduced the pH of Starkey broth from 8.0 to less than 3.84 within 10 days of incubation at 30°C. The remarkable fall in pH was observed in the Starkey broth by 12 isolates out of 14. This indicated that the isolated SOM have rapidly utilized the elemental sulfur after degradation of ammonium sulfate. All the SOM shows positive growth in Thiosulfate broth. This indicates that the thiosulfate was oxidized into sulfate very slowly by all the heterotrophic SOM. Negative control plate of Thiosulfate medium using *Acetobacter aceti* (NCIM 2251) does not shows growth.

Characterization of selected SOM

Table 2 refers the results of the characterization of the selected isolates. The predominant microbial group was Gram negative, short rod & non-spore forming long rod-shaped bacteria. The colonies obtained by SOM 01, SOM 03, SOM17 and SOM 31 were smooth, round straw yellow coloured probably due to the deposition of sulfur [22]. Rest of the isolated SOM showed smooth, raised and white to yellowish colonies. All SOM except SOM 17 and reference strain are motile. The SOM 01, SOM 03, SOM17 and SOM 31 have utilized the yeast extract and peptone. Hence these are chemoautotroph in nature. All the isolates have utilized the sucrose, glucose, fructose, lactose, maltose, starch and sodium acetate. All the isolates were also positively influenced by biotin, which was in conformity with the findings in literture that the cultures belong to *Thiobacillus* species [23].

Sulfur oxidation and microbial production of sulfate by selected isolates

It was mentioned previously; 14 SOM were isolated using different culture media. All the isolated SOM reduced the pH from 8.0 to below 5.0 and utilized both sulfur and thiosulfate. Depending on utilization of sulfur source, heterotrophic sulfur oxidizers may be categorized into three major groups [24]. Firstly, those oxidize elemental sulfur producing thiosulfate as their predominant end product. Secondly those capable of oxidizing elemental sulfur & producing sulfate, and third group involves those capable of oxidizing thiosulfate to sulfate [24].

The isolated SOM showed autotrophic growth using elemental sulfur as energy source and drop in pH was

observed (Table 1). As mentioned in table 1, the initial pH for Starkey media was 8, isolates which produce lower amounts of sulfate, decrease the final pH within 4.74-5.62 while those isolates which were able to produce higher amounts of sulfate, decrease the final pH to 3.59-3.80.

Figure 1a and 1b shows the oxidation of sulfide and sulfate formation with time using SOM 05, SOM 06, SOM 11 and reference strain at initial sulfide concentration of 200 mg/l on shake flask level. From the figure 1a, it is evident that, at initial sulfide concentration of 200 mg/l, sulfide was oxidized to 0 at the end of 120 hours by SOM 05. During initial 24 hours slow sulfate formation rate was observed in all the flasks. During first 24 hours, sulfide oxidation rate was slow as compared to sulfate formation in all the SOM flasks except reference strain flask. The reference strain shows more sulfide oxidation rate as compared to sulfate formation. The difference in sulfur balance during this phase might be because of the sulfide was rapidly converted into elemental sulfur by reference strain. SOM 05 shows very fast oxidation of sulfide as compared to SOM 06 and SOM 11. During 96 hours 180 mg sulfide was oxidized. It is reported that sulfate production from elemental sulfur in sulfur media ranged from 31.7 mg/100 cc to 118.9 mg/100 cc [25]. However, there was no co-relation observed in drop in pH, oxidation of sulfide and rate of sulfate formation. Out of three selected isolates, SOM 06 shows maximum of 1000 mg of sulfate formation within 168 hours.

Table 1: Reduction of pH in the growth media by isolated SOM and reference strain

Sr. No.	Name of SOM	pH of Starkey broth	pH of Thiosulfate broth
1	SOM 01	3.72	5.45
2	SOM 03	5.00	4.48
3	SOM 07	3.80	6.93
4	SOM 09	3.61	6.35
5	SOM 13	3.69	4.74
6	SOM 17	3.59	5.62
7	SOM 22	3.66	6.05
8	SOM 28	3.84	6.15
9	SOM 31	3.65	6.35
10	SOM 41	3.70	6.31
11	SOM 45	3.67	7.24
12	SOM 47	3.71	5.28
13	SOM 51	3.76	6.79
14	SOM 54	3.78	5.02
15	*Thiobacillus novellus* NCIM 2858	3.70	6.78

a. Initial pH of Starkey &Thiosulfate broth was adjusted to 8.0

Table 2: Characterization of the isolated SOM and reference strain

Characteristics	SOB 01	SOB 03	SOB 07	SOB 09	SOB 13	SOB 17	SOB 22	SOB 28	SOB 31	SOB 41	SOB 45	SOB 47	SOB 51	SOB 54	Reference strain
Cell shape	Rod	Rod	Rod	Rod	Rod	Rod	Rod	Rod	Rod	Rod	Rod	Rod	Rod	Rod	Rod
Cell arrangement	single	Single	Single	single	Single	single	single	single	Single	single	single	Single	Single	Single	Single
Motility	+	+	+	+	+	-	+	+	+	+	+	+	+	+	-
Catalase	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Oxidase	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Chemolithotroph (thiosulphate)	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Influence of biotin	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+

Chemoorganotroph (YE + Peptone)	+	+	-	-	-	+	-	-	+	-	-	-	-	-	-
Fructose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Glucose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Sucrose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Lactose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Maltose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Hydrolysis of starch	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Sodium acetate	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+

a. Legend: utilized (+), unutilized (-)

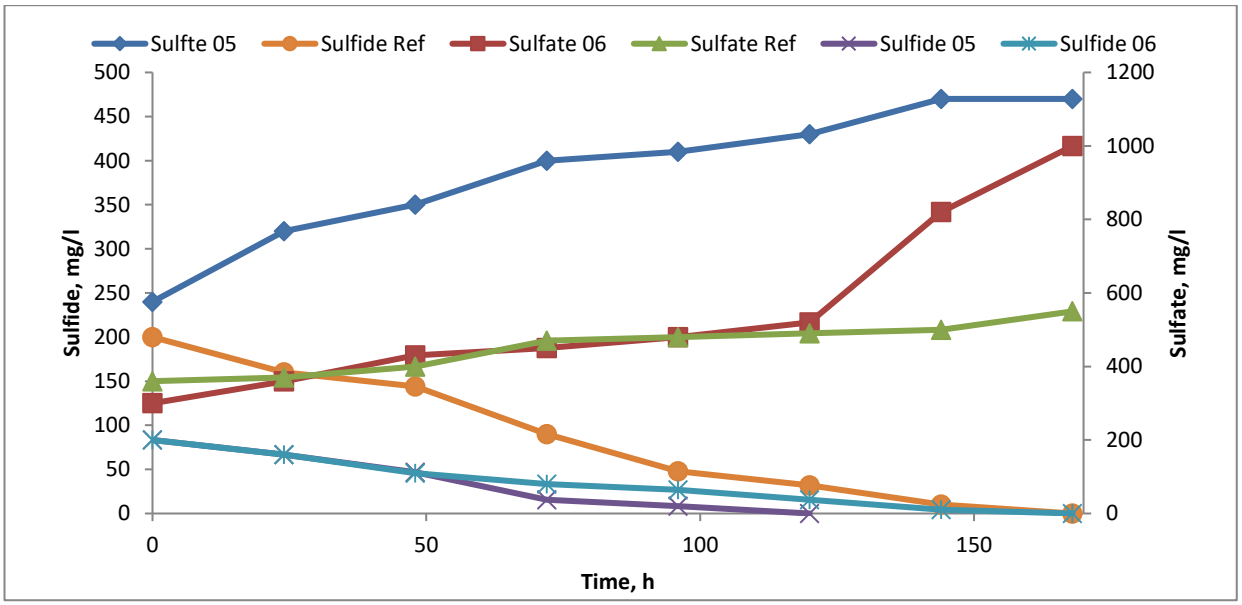

Figure 1a: Trend of sulfide oxidation and sulfate formation with time of SOM 05, SOM 06 against reference strain

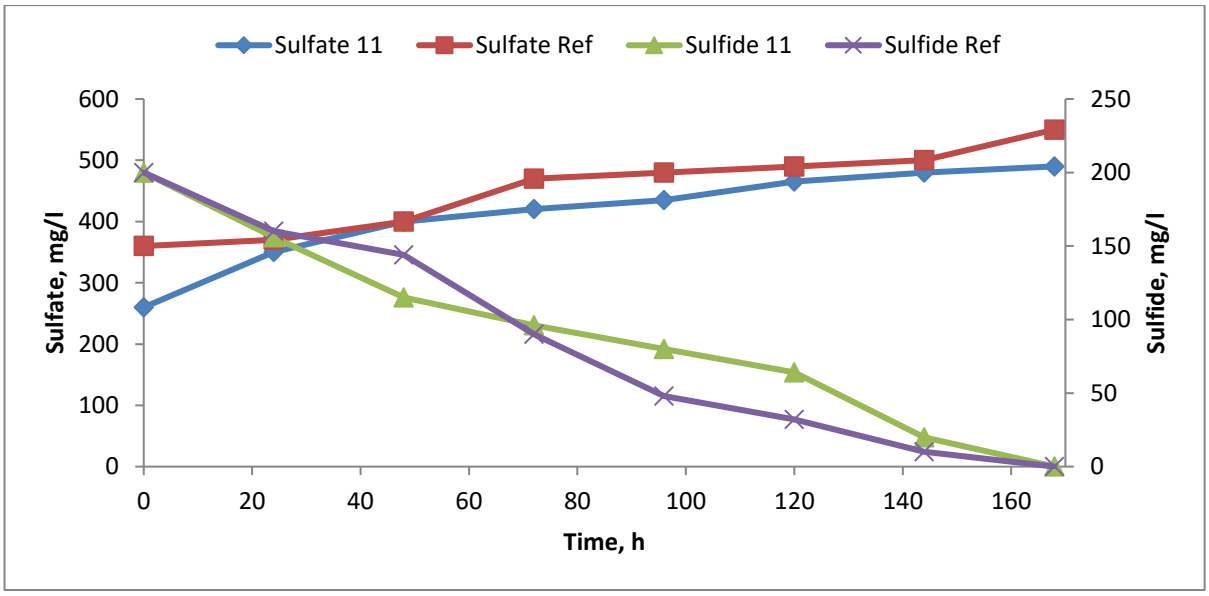

Figure 1b: Trend of sulfide oxidation and sulfate formation with time of SOM 11 against reference strain

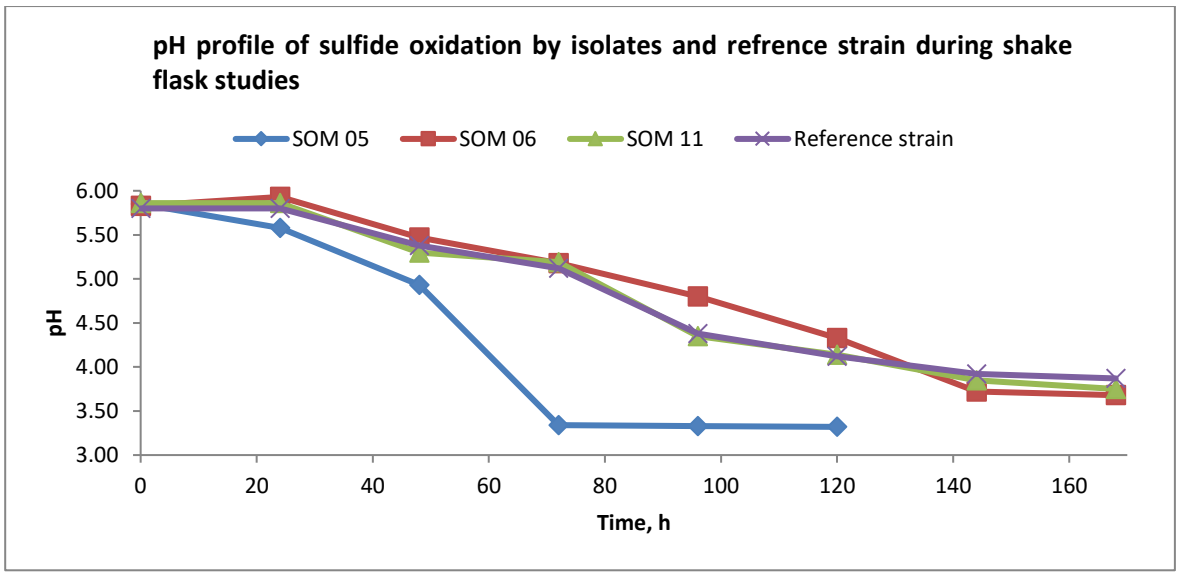

Figure 2: pH profile of sulfide oxidation by isolates and reference strain during shake flask studies

Trend of variation in pH during shake flask studies

Figure 2 shows the trend of pH during shake flask studies. After addition of inoculum, pH in all flasks was 5.80 to 5.86. As the sulfide oxidation rate increased, pH fall was observed. After 120 hours, pH of 3.32 was observed in SOM 05 flask. At the end of 168 hours, SOM 06 and SOM 11 show pH of 3.68 and 3.75 respectively. The reference strain shows pH of 3.87 at the end of experiment. During the shake flask studies, initially the sulfide was oxidized to sulfur and as the concentrations of sulfide decreased, SOM started using the elemental sulfur as source of energy for metabolism. After formation of sulfate and once the sulfur depletes in the flask, the SOM shift to sulphate for their survival and resulted into formation of sulfuric acid. This is evidenced by the variation in pH during the shake flask studies.

CONCLUSIONS:

It was possible to isolate a good number of sulfur oxidizing microorganisms from environmental samples using different sulfur sources. Only three of them showed an outstanding ability to grow autotrophically and utilize sulfur with formation of sulfate within pH range of 3.30 to 3.75. The pH reduction test, morphological, biochemical tests and sulfur oxidation studies shows that all the isolates are belonging to species of *Thiobacillus.* Data suggest them as representatives for the pre-treatment of sulfur containing industrial wastes including distillery spentwash. Further experiments can be conducted for use of screened isolates to breakdown of H_2S formed during biomethanation process in future.

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