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Screening, identification, and optimization of sulfur-oxidizing bacteria from post-coal mining ponds of different ages in Samarinda, East Kalimantan, Indonesia

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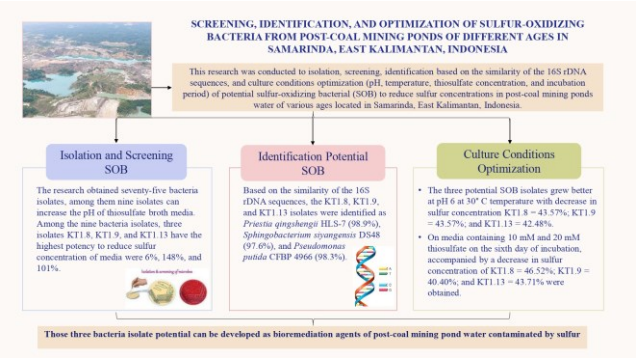
Graphical abstract



Abstract

This research was conducted to isolation, screening, identification, and culture conditions optimization (pH, temperature, thiosulfate concentration, and incubation period) of potential sulfur-oxidizing bacterial (SOB) to reduce sulfur concentrations in post-coal mining ponds water of various ages located in Samarinda, East Kalimantan, Indonesia. The ponds used in this study were ponds $\leq$ 5 years old and $\geq$ 20 years old. The research obtained seventy-five bacteria isolates, among them nine isolates can increase the pH of thiosulfate broth media. Among the nine bacteria isolates, three isolates KT1.8, KT1.9, and KT1.13 have the highest potency to reduce sulfur concentration of media were 6%, 148%, and 101%. Based on the similarity of the 16S rDNA sequences, the KT1.8, KT1.9, and KT1.13 isolates were identified as *Priestia qingshengii* HLS-7 (98.9%), *Sphingobacterium siyangensis* DS48 (97.6%), and *Pseudomonas putida* CFBP 4966 (98.3%). The three potential SOB isolates grew better at pH 6 at 30° C temperature with the growth of KT1.8 = $146x10^{14}$ cell/mL; KT1.9 = $81x10^{7}$ cell/mL; and KT1.13 = $33x10^{7}$ cell/mL; decrease in sulfur concentration KT1.8 = 43.57%; KT1.9 = 43.57%; and KT1.13 = 42.48%. On media containing 10 mM and 20 mM thiosulfate on the sixth day of incubation, cell density isolates KT1.8 = $98x10^{12}$ cell/mL; KT1.9 = $25x10^{7}$ cell/mL; and KT1.13 = $85x10^{6}$ cell/mL; accompanied by a decrease in sulfur concentration of KT1.8 = 46.52%; KT1.9 = 40.40%; and KT1.13 = 43.71% were obtained. Those three bacteria isolate potential can be developed as bioremediation agents of post-coal mining pond water contaminated by sulfur.

Keywords: Bioremediation, coal mining, pond water, sulfur-oxidizing bacteria

1. Introduction

The most mining methods with open pit mining are dominates mining activities in the world, causing the formation of large openings which are then filled with water (Koščová *et al.* 2018; Tuheteru *et al.* 2021). In companies that have a mining permit, these dug holes are backfilled with soil taken from the surrounding land or from the previous land cover (Ardiansyah & Fajri, 2016; Zhang *et al.* 2019). The problem with this open pit mining system is usually that small mines do not carry out reclamation and backfilling (backfilling method) which causes ex-mining holes. Ex-mining pits or also called voids left behind in post-mining without proper utilization planning have the potential to cause ecological damage to mining areas (Zhang *et al.* 2019; Absori *et al.* 2021). The post-coal mining area can experience changes due to the exposure of rock layers composed of naturally oxidized sulfur supported also by high rainfall which accelerates sulfur oxidation. Sulfur contained in post-coal mining pool water comes from coal material containing 2-11% sulfur. Sulfur in post-mining ponds also comes from the coal washing process. During washing, the elements present in the coal will dissolve or undergo several chemical reactions and physical reactions and cause the washing results to dissolve into the washing solution (Dutta *et al.* 2018). These post-coal mining open ponds remove vegetation cover, disrupt the ecological

balance of the land surface, and reduce productivity and environmental quality.

Biological oxidation of inorganic sulfur compounds is an important process in the global sulfur cycle. In this process, the energy generated from sulfur oxidation is used by aerobic chemoautotrophs for carbon dioxide fixation, while the electrons derived from the reduced sulfur compounds are used by anoxygenic photoautotrophs for carbon dioxide reduction (Luo *et al.* 2018). Sulfur-oxidation bacteria are the most active and ancient metabolic processes in sulfur cycle that operate in diverse ecosystems and considered as key phenomenon in sulfur biogeochemical cycling (González & Pérez, 2021; Kumar *et al.* 2018). Usually, on the basis of nutrition, SOB is categorized as lithoautotrophs. The SOB oxidize the reduced sulfur compounds such as hydrogen sulfide (H_2S), elemental sulfur (S^0), sulfite (SO_3^{-2}), thiosulfate ($S2O_3^{2-}$), and various polythionates ($S_nO_6^{2-}$ or $-S_nO_{6-}$) into sulfate (SO_4^{-2}) (Kumar *et al.* 2018). Sulfur oxidizing bacteria (SOB) control the main processes of organic matter degradation and the biogeochemical cycles of sulfur. Sulfur oxidizing bacteria enhances the rate of natural oxidation of Sulfur. In the metabolism of SOB, sulfur can be synthesis into amino acid are cysteine and methionine of the bacteria cell (Chen *et al.* 2023). Studying the community structure and diversity of SOB is important to reveal the role of these bacteria in the sulfur biogeochemical cycle (Konrad *et al.* 2023). However, the activity of these sulfur-oxidizing bacteria is influenced by environmental factors and the age of the post-coal mining pond that drive the sulfur cycle (Méndez-García *et al.* 2015; Zhang *et al.* 2017). The increase of the pond age caused increase the diversity of plankton, fish, and other aquatic organisms in natural stagnant waters. Post-mining ponds generally consist of young post-mining ponds were less than 5 years old and old post-mining were more than 20 years old (Prasetiyono, 2015). Phylogenetic and potency assay of sulfur-oxidizing bacteria from water post-coal mining ponds of different ages in Samarinda, East Kalimantan, Indonesia has not been explored yet. This study will help us in understanding the characteristics of sulfur oxidizing bacteria in post-coal mining ponds water. Various sulfur oxidizing bacteria will isolate, evaluate of their potency to reduce of sulfur concentration, and it identify.

2. Materials and methods

2.1. Description of the location and sampling of post-coal mining pond water

The research was carried out in July-September 2022 from three locations of post-coal mining ponds with an age of $\leq$ 5 years and three locations of post-coal mining ponds with an age of $\geq$ 20 years in Samarinda City, East Kalimantan and at the Water Quality Laboratory of the Faculty of Fisheries and Marine Sciences, Mulawarman University, Samarinda, Indonesia, and Animal Ecology and Diversity Laboratory, Faculty of Mathematics and Natural Sciences, Universitas Brawijaya, Malang, Indonesia. Details of the sampling locations were presented in Figure 1. Each water sample from the post-coal mining pond was taken in three replicates and each replicate was taken from five random points with a depth of 5 cm from the surface of pond water using a water sampler. Samples from each point were mixed in a container to obtain a composite sample. This procedure was repeated with samples from every three replicates and five different points. Water samples were transported in closed bottles and stored at 4°C to be brought to the Microbiology Laboratory, Faculty of Mathematics and Natural Sciences, Universitas Brawijaya to prepare for further analysis.

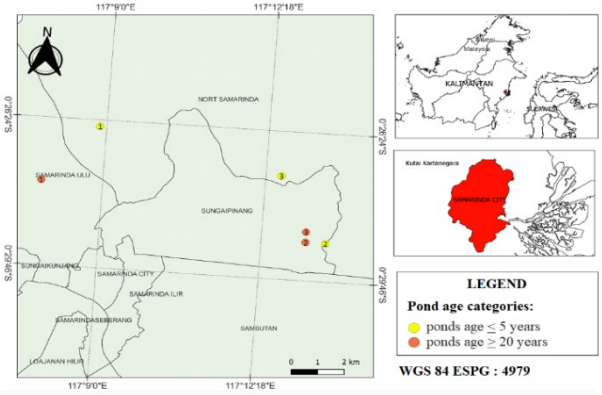


Figure 1. Details of the sampling locations

2.2. Culture media and growth conditions

Sulfur-oxidizing bacteria were isolated from post-coal mining pond water samples using the direct screening method (Pokorna & Zabranska, 2015; Yousef *et al.* 2019). The medium used to isolate of SOB was thiosulfate broth medium with the composition of 1 g NH_4Cl, 0.1 g $CaCl_2.2H_2O$, 10 g NaCl, 0.5 g KH_2PO_4, 2 g K_2HPO_4, 0.8 g $MgSO_4.7H_2O$, 3 g $Na_2S_2O_3.5H_2O$ (Mustafa *et al.* 2015), 2 mL of 0.5% phenol red as a color indicator for changes of pH (Hidayat *et al.* 2017; Reddy *et al.* 2018) and dissolved in 1000 mL of distilled water. The pH of the media was adjusted with 1 M NaOH or HCl until pH 7.3 (Mustafa *et al.* 2015). Sulfur oxidizing bacteria were incubated at 30°C for 7 days under aerobic conditions (Mustafa *et al.* 2015).

2.3. Isolation of sulfur-oxidizing bacteria

Sulfur oxidizing bacteria were isolated by inoculating 25 mL of water sample into 225 mL of 0.85% NaCl solution as a 10^{-1} dilution (Khotimah *et al.* 2020), continued until the 10^{-5} dilution. Sample suspension 0.1 mL was inoculated by pour plate into thiosulfate agar media containing 1.5% bacteriological agar and 2 mL of 0.5% phenol red. The culture incubated at 30°C for 7 days under aerobic conditions (Mustafa *et al.* 2015). The SOB isolates were purified based on streak plate method on thiosulphate agar medium. The pure cultures of SOB were preserved on thiosulphate agar slants at 4° C.

2.4. Screening of SOB isolates base on pH increasing and decreasing of sulfur concentration

The pure culture of each SOB isolate as much as 10 mL (Malviya *et al.* 2022) at logarithmic growth phase with 10^7 cell/mL density (Khotimah *et al.* 2020) was inoculated in the 90 mL thiosulfate broth medium with initial pH 6.0. The culture of SOB was incubated at 30°C for 7 days. The final pH of the growth media was measured using a pH meter. The isolates were screened based on their efficacy to

increase the pH of culture medium. The selected isolates were assayed based on the potency to decrease sulfur concentration of the culture medium.

The experiment to assay of the SOB isolate to decreased of sulfur concentration in the medium was carried out according Cha et al. (1999) and Hindersah et al. (2018). The SOB isolate at logarithmic growth phase as much as 10 mL with 10^7 cell/mL density was inoculated in the 90 mL thiosulfate broth medium. The cultures were incubated at 30°C for 7 days. The sulfur concentration at initial and final incubation were measured. The culture suspension was centrifuged at 10.000 rpm for 5 min, the supernatant was added by barium chloride ($BaCl_2$) (10% w/v) with 1:1 ratio. The suspension was shake and the absorbance was measured with spectrophotometer at 480 nm wavelength. The absorbance values were integrated in the sulfur standard curve to determined sulfur concentration of the SOB culture medium (Kolmert *et al.* 2000). The standard sulfur solution was made by dissolving potassium sulfate (K_2SO_4) in deionized water to obtained sulfur concentration 0 to 40 mM. The sulfur concentration data was variance analysis to determine the potential of SOB. The potential of SOB will identify molecularly based on 16S rDNA sequence similarity. The decreasing data of sulfur concentration (%) was calculated using the below formula.

$$\text{Decreased sulfur concentration}(\%) = \frac{\text{initial sulfur concentration} - \text{final sulfur concentration}}{\text{initial sulfur concentration}} \times 100\%$$

2.5. Bacteria identification base on 16S rDNA sequencing similarity

The chromosomal DNA of SOB potential isolates was extracted by Zymo DNA Extraction Kit. The region of 16S rDNA was amplified using primers 27f 5'-GAGTTTGCTCCTGGCTCAG-3' and 1492r 5'-GGTTACCTTGTTACGACTT-3' (Huber *et al.* 2016; Hassanshahian & Ghoebani, 2018; Nadella *et al.* 2019). The 16S rDNA was amplified in the 25 µL PCR mix witch 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 of distilled water. The amplification of 16S rDNA was carried out in the PCR machine that programmed with 94° C (2 min) for initial denaturation, followed by 35 cycles consist of denaturation, annealing, and extension at 94° C (30 sec), 50° C (1 min), and 68° C (90 sec) respectively, and final extension at 68° C (7 min). The 16S rDNA amplicons were analyzed by nanodrop spectrophotometer. The Amplicons of 16S rDNA were purified and sequenced at First Base, Malaysia. Amplicons were sequenced using an ABI 3130 Automatic Sequencer Analyzer. The 16S rDNA sequences were edited using the Sequencer Scanner V.1 program and combined using CAP Contig Assembly in the BioEdit V.7.2.5 program. The 16S rDNA sequence was BLAST using the NCBI BLASTN program and the nucleotide sequence was determined based on GenBank (http://www.ncbi.nlm.nih.gov). The 16S rDNA sequences of the SOB and reference strains were aligned with the MEGA program's Clustal W Multiple Alignment. Phylogenic trees were constructed and inferred using the Neighbor Joining algorithm with a bootstrap of 1000 times (Alang *et al.* 2019; Rohmah *et al.* 2020).

2.6. Optimization of SOB culture conditions

The optimum growth of SOB isolate potential was determined in the thiosulfate broth medium. The experiment was carried out with three replications based on complete randomized design according Hidayat et al. (2017). The potential of SOB isolate at logarithmic growth phase was 10 mL with 10^7 cell/mL density inoculated in the 90 mL thiosulfate broth medium pH 6, and it incubated 120 rpm at 30° C for 7 days. The culture suspension at 24 h incubation interval was measured cell density of SOB according Khotimah et al. (2020) and sulfur concentration according Cha et al. (1999) and Kolmert et al. (2000). The optical density of culture suspension was measured with a spectrophotometer at 600 nm (UV-1700-Shimadzu, Kyoto, Japan). The sulfur concentration was measured with a spectrophotometer at 480 nm. The culture media containing thiosulfate (3 mM) as electron donor was adjusted at varies conditions:

2.6.1. Optimization of pH

The isolates were incubated in thiosulfate broth media with different pH at 3, 4, 5, 6, 7, 8 and a temperature at 30° C.

2.6.2. Temperature optimization

In the same way, the thiosulfate medium was adjusted to the optimal pH 6 at different temperatures at 25° C, 30° C and 35° C, respectively.

2.6.3. Optimization of thiosulfate concentration

In the same way, thiosulfate media with pH 6, temperature 30°C were treated with different concentrations of thiosulfate at 5 mM, 10 mM, 20 mM, and 40 mM.

2.6.4. Incubation period

Bacterial cultures were incubated at different time intervals of 0, 1, 2, 3, 4, 5, 6, and 7 days with the observed parameters being cell density and decreasing of sulfur concentration.

2.7. Statistical analysis

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

3. Results and discussion

3.1. Sulfur-oxidizing bacteria isolate from post-mining pond water in Samarinda

The current research was to obtained SOB isolate from post-coal mining pond water with $\leq$ 5 years and $\geq$ 20 years old. The research obtained 75 bacteria isolates that able to use thiosulfate for growth (Table 1). However, only 40 bacteria isolates were able to raise the pH of the media to become non-acidic, so only 40 SOB isolates were further tested.

Tabel 1. SOB isolates from post-coal mining pond water with ≤ 5 years and ≥ 20 years old

Isolates		Media Thiosulfat Mineral	Isolates		Media Thiosulfat Mineral	Isolates		Media Thiosulfat Mineral	Isolates		Media Thiosulfat Mineral	Isolates		Media Thiosulfat Mineral
KM2	1	-	KM3	8	-	KT2	1	+	KT3	2	+	KT3	17	+
KM2	2	-	KM3	9	-	KT2	2	+	KT3	3	-	KT3	18	+
KM2	3	-	KT1	1	-	KT2	3	+	KT3	4	+	KT3	19	-
KM2	4	-	KT1	2	+	KT2	4	+	KT3	5	+	KT3	20	+
KM2	5	-	KT1	3	-	KT2	5	+	KT3	6	+	KT3	21	+
KM2	6	-	KT1	4	-	KT2	6	+	KT3	7	+	KT3	22	+
KM2	7	-	KT1	5	-	KT2	7	+	KT3	8	+	KT3	23	-
KM2	8	-	KT1	6	-	KT2	8	-	KT3	9	+	KT3	24	+
KM3	1	-	KT1	7	+	KT2	9	-	KT3	10	+	KT3	25	-
KM3	2	-	KT1	8	+	KT2	10	-	KT3	11	+	KT3	26	+
KM3	3	-	KT1	9	+	KT2	11	+	KT3	12	+	KT3	27	+
KM3	4	-	KT1	10	+	KT2	12	-	KT3	13	+	KT3	28	+
KM3	5	-	KT1	11	+	KT2	13	-	KT3	14	+	KT3	29	+
KM3	6	-	KT1	12	-	KT2	14	-	KT3	15	-	KT3	30	+
KM3	7	-	KT1	13	+	KT3	1	-	KT3	16	+	KT3	31	+

Note = + (increase the pH of the medium);
- (decreased the pH of the medium);
KM (post-coal mining pond water with ≤ 5 years old);
KT (post-coal mining pond water with ≥ 20 years old)

3.2. The potency of SOB isolates from water of post-coal mining ponds

The 40 isolates were screened based on the potency as SOBs and 9 isolates have higher ability to increase the pH and decreasing sulfur concentration in the medium. Among 9 SOB isolates, three isolates were KT1.8, KT1.9 and KT1.13 showed highest potency to decrease sulfur concentration in the medium (Figure 2).

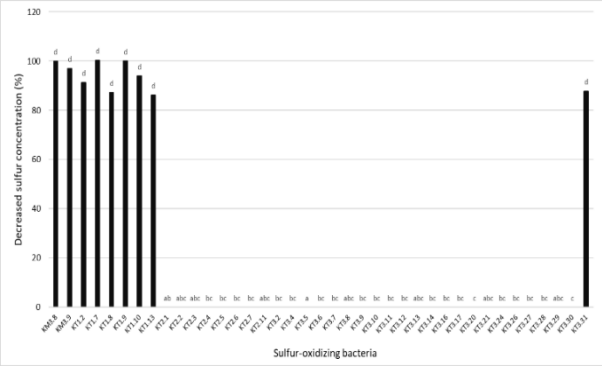


Figure 2. The potency of SOB for decreased sulfur concentration in TSM medium

Those three bacterial isolates were able to change the color of phenol red on thiosulfate agar media by increasing the pH of the medium from initial pH of 6.0 within 7 days of incubation. The ability of sulfur-oxidizing bacteria to increase the pH of the medium was also reported by Mason & Kelly (1988). The SOB isolate of KT1.9, KT1.13, and KT1.8 were able to decrease of sulfur concentration in the medium were 148 %, 101 %, and 6 % respectively (Figure 3). The current results reveal that not all SOBs isolated from water of post-coal mining ponds are able to reduce sulfur concentrations in the media by adding $Na_2S_2O_3$ as a source of sulfur.

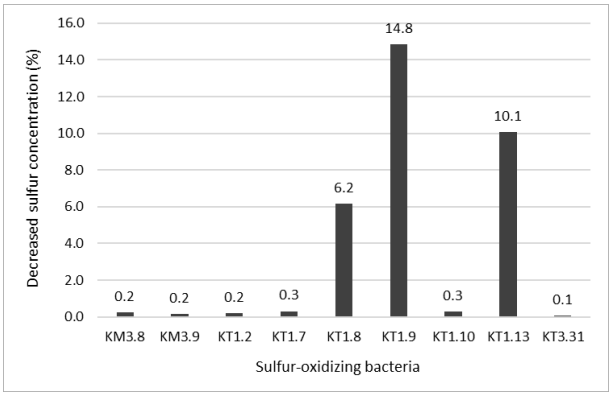


Figure 3. The potency of SOB isolates to decrease of sulfur concentration in the medium

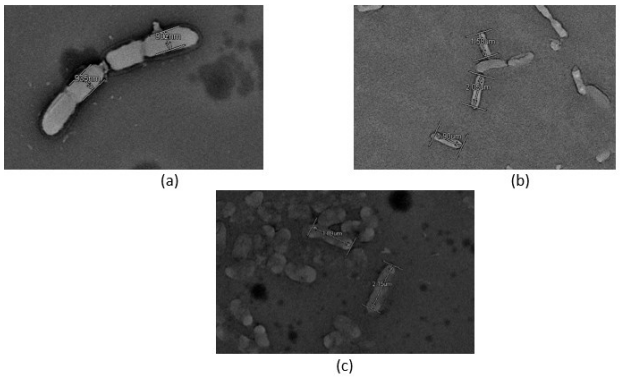


Figure 4. SEM results of potential sulfur-oxidizing bacteria (a) KT1.8, (b) KT1.9 and (c) KT1.13 from post-coal mining pond in Samarinda

In sulfur-rich environments, sulfur cycling is an important driver of microbial growth and element conversion in many ecosystems. Biological sulfur oxidation is a vital part of the sulfur cycle, and in natural environments the oxidation of sulfur species is mediated by chemolithotrophic and

phototrophic bacteria. A variety of sulfur oxidizing bacteria use sulfur as an energy source, and this group encompasses bacteria with little or no taxonomic relationship to each other. Aerobic sulfur-oxidizing bacteria, so-called colorless sulfur-oxidizing bacteria, are mainly mesophilic (Nosalova *et al.* 2023). Sulfur-oxidizing bacteria from post-coal mining pond water were isolated using enrichment culture technique in the thiosulfate mineral media. In general, the thiosulfate used in this study is usually used to isolate pure cultures of obligate chemolithoautotrophic sulfide-oxidizing bacteria. Thiosulfate is stable at neutral pH, easily soluble and non-toxic at high concentrations (Kantachote *et al.* 2008). Sulfur-oxidizing bacteria can use sulfur as a basic substrate during the oxidation process and oxidize it to sulphate (Pokorna & Zabranska, 2015). These oxidized sulfur compounds are used as electron donors for anaerobic phototrophic and aerobic chemotrophic growth, and are mostly oxidized to sulphate (Friedrich *et al.* 2001).

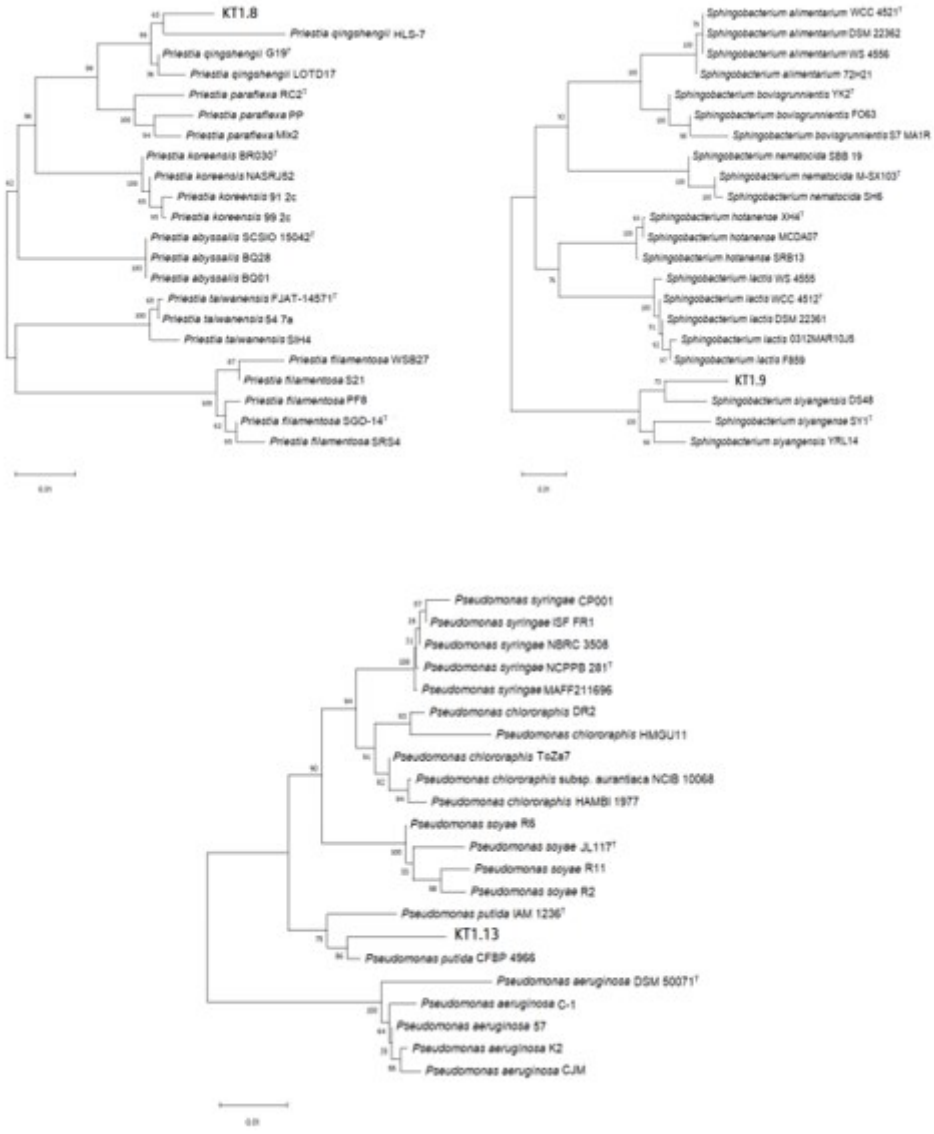


Figure 5. Phylogeny tree of potential sulfur-oxidizing bacteria and reference strains base on 16S rDNA sequence similarity according Neighbor joining algorithm

3.3. Species Identity of the Potential SOB isolate

The SOB potential isolates (KT1.8, KT1.9 and KT1.13) were identified based on SEM (scanning electron microscope) and 16S rDNA sequence similarity. The results SEM showed that Figure 4 and the results 16S rDNA sequence similarity that KT1.8 isolate had highest 98.9% similarity with *Priestia qingshengii* HLS-7, KT1.9 isolate had highest 97.6% similarity with *Sphingobacterium siyangensis* DS48, and KT1.13 isolate had highest 98.3% similarity with *Pseudomonas putida* CFBP 4966 (Figure 5).

Sulfur-oxidizing bacteria are one of the main microorganisms that influence the sulfur cycle; they use this inorganic element to form organic compounds (assimilation, energy-dependent process) and to produce energy (dissimilar sulfur metabolism). They are essential processes for the global sulfur cycle (González & Pérez,

2021). Most of the known Sulfur oxidising bacteria (SOB) belongs to the genera *Thiobacillus*, *Thiothrix*, *Thiomicrospira*, *Achromatium*, and *Desulfuromonas* (Behera *et al.* 2014). As shown in Figure 5, sulfur oxidation is not limited only to true sulfur bacteria but also other heterotrophic bacteria isolated from soil and marine environment carry the same process. The heterotrophic bacteria belong to genera *Pseudomonas* involve in sulfur oxidation with 98% sequence similarity with *Pseudomonas putida* NR113651 (Sajjad *et al.* 2016). Sulfur oxidizing *Pseudomonas* spp. have also been isolated from soil of Bhitarakanika, Odisha, India (Thatoi *et al.* 2012). Sulfur oxidising activity of *Pseudomonas* spp. and *Bacillus* spp. (*Priestia* spp.) were also well reported (Behera *et al.* 2014). This study was conducted to isolate Sulfur oxidising bacteria from mangrove soil of Mahanadi river delta, Odisha, India and evaluate their Sulfur oxidation ability. Figure 5 also shows other heterotrophic microorganisms not commonly associated with sulfur oxidation and thus termed non-SOB, e.g., *Sphingobacterium* sp. was detected in this study as well. The identification of heterotrophic non-SOB in the enrichment cultures was probably due to their presence in the original biofilm. A contaminated with organic residues from the biofilm sample may have enabled the growth of heterotrophic non-SOB in the culture media. Furthermore, many obligate chemolithotrophic sulfur oxidizers produce organic substances that could be subsequently utilized by heterotrophs leading to the growth of non-SOB species (Huber *et al.* 2016).

3.4. The optimum of culture medium for potential SOB growth

3.4.1. The potency of potential SOB isolates in the various of pH medium

Hydrogen ion concentration is the most important factor influencing enzyme activity to reduce sulfur concentration in the bacterial media. To determine the effect of pH on SOB growth rate and sulfur oxidation activity, TSM media was prepared at six different pHs (3, 4, 5, 6, 7, and 8). As shown in Figure 6, The three potential SOB isolates grew better at pH 6 with optimal growth of KT1.8 = $124x10^{14}$ cell/mL; KT1.9 = $101x10^{7}$ cell/mL; and KT1.13 = $111x10^{7}$ cell/mL; accompanied by a decrease in sulfur concentration of KT1.8 = 43.57%; KT1.9 = 45.85% and KT1.13 = 43.24% in the culture medium. On the other hand, at high acidic pH, enzyme induction and reduction of sulfur concentrations are very low. However, the isolate KT1.13 showed growth activity at pH 4 media (Figure 6).

Most microorganism better growth at a certain pH range, where changes in pH culture medium can affect their growth and activity. Thus, it is important to investigate the optimal pH range of microorganisms. In this study, microbial growth and sulfur oxidation activity of all potential SOB isolates were observed to occur under neutral and slightly alkaline conditions with a pH range of 6-8. Generally, the optimum pH for SOB varies depending on the microbial habitat and species. These results reinforce the fact that the post-coal mining ponds that are the habitat for isolated bacteria have a pH value ranging from 6.0 to 8.0. Thus, the preference for the pH of the isolates in this study is not surprising because the enrichment conditions used were pH 6 and the pH of the isolation environment. In addition, some representatives of *Thiobacillus* sp. has shown neutrophilic characters such as *Thiobacillus novellus* and *Thiobacillus thioparus* with optimum growth at pH 7.0 and 7.5 respectively (Pokorna & Zabranska, 2015). The quantitative survey indicated that soxB was more abundant than sqr and dsrA in the Pearl River water, which suggested that Sox was probably the key enzyme that catalyzed sulfur oxidation in this environment. The Sox enzyme complex not only converts thiosulfate to sulfate without the formation of any free intermediate, but also oxidizes sulfide and sulfur by feeding HS− and S0 into the Sox pathway as appropriate intermediates via enzymatic or non-enzymatic conjugation to a carrier protein SoxY (Ghosh & Dam, 2009). The Sox system commonly controls sulfur oxidation in aerobic chemotrophic and anaerobic phototrophic Alphaproteobacteria and a shortened *sox* gene cluster was identifed in the genomes of other chemotrophic or phototrophic bacteria (including colorless sulfur bacteria, green sulfur bacteria, purple sulfur bacteria, purple non-sulfur bacteria); this has given rise to the hypothesis about emergence of a common mechanism in SOB (Friedrich et al. 2001, Ghosh & Dam, 2009). From the above analysis, Luo et al. (2018) infer that the sulfur oxidation in the Pearl River water was mainly catalyzed by the Sox enzyme system.

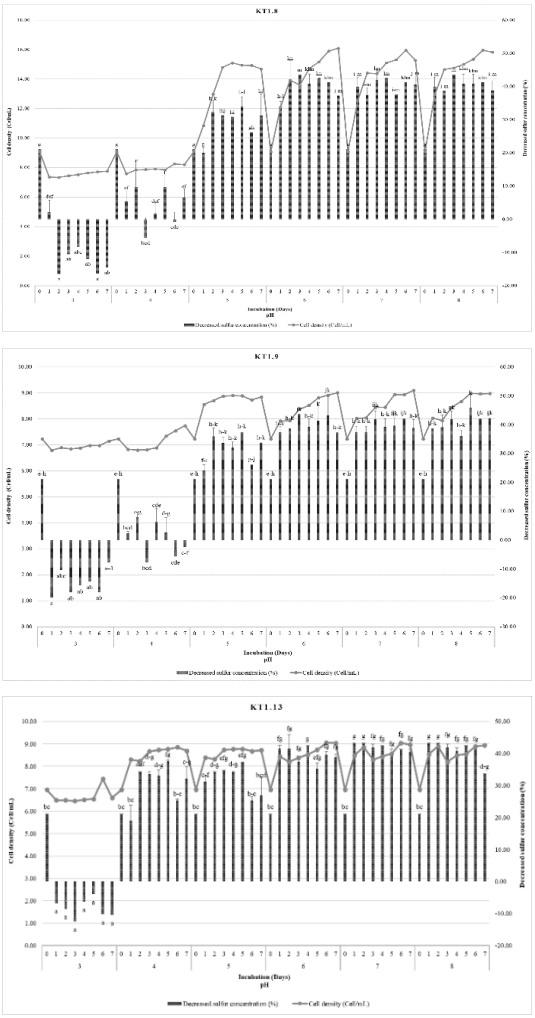


Figure 6. Growth activity of three SOB isolates in the various pH of TSM medium

Figure 7. Growth activity of three SOB isolates in the various temperature of TSM medium

3.4.2. The potency of potential SOB isolates in the various of medium temperatures

The rate of bacterial growth and metabolism depends on the composition of the media and the prevailing environmental conditions. Various bacterial strains differ in nutritional requirements and culture conditions for optimal growth. The effect of temperature on the growth rate and sulfur oxidation activity of isolates KT1.8, KT1.9 and KT1.13 were shown at Figure 7. The results showed that the three potential SOB isolates were growth optimally at 30° C. The KT1.8 isolate with cell density is $146x10^{14}$ cell/mL; able decrease sulfur concentration is 43.57%, KT1.9 cell density is $81x10^{7}$ cell/mL; decrease sulfur concentration is 43.57%, and KT1.13 cell density is $33x10^{7}$ cell/mL; decrease sulfur concentration is 42.48%; they had highest potency to decrease sulfur concentration ($P<0.05$) in media thiosulfate. The growth of the three bacterial isolates and their sulfur oxidation activity were different from among temperature tested. Bacterial growth and the highest decrease in sulfur concentration occurred at 30° C (Figure 7) and it was classified as mesophilic SOB.

3.4.3. The potency of potential SOB isolates in the various of thiosulfate concentrations

Thiosulfate concentration plays an important role in the activity of sulfur-oxidizing bacteria. This may be due to thiosulfate is a stimulator of the enzyme sulfide oxidase. The thiosulfate concentrations that tested in the TSM media were 5 mM, 10 mM, 20 mM, and 40 mM. As shown in Figure 8, KT1.8 isolate (cell density is $98x10^{12}$ cell/mL; decrease sulfur concentration is 46.52%), KT1.9 (cell density is $25x10^{7}$ cell/mL; decrease sulfur concentration is 40.40%) and KT1.13 (cell density is $85x10^{6}$ cell/mL; decrease sulfur concentration is 43.71%), they had highest potency to decrease sulfur concentration ($P<0.05$) in media containing 10 mM and 20 mM thiosulfate on the sixth days of incubation. However, no significant differences in terms of cell growth were observed for all the isolates tested. The research showed that all potential isolates were able to grow at thiosulfate concentrations up to 40 mM where the microbial growth of isolates was affected by the amount of thiosulfate concentration added to the growth medium. This result can be explained by the availability of sufficient substrate (thiosulfate) to supply energy for the bacteria for significant growth.

All isolates that were optimized in culture media showed the ability to oxidize inorganic sulfur compounds which were added as a source of energy for growth. In chemotrophic and phototropic sulfur oxidizers that do not form sulfur precipitates, a periplasmic thiosulfate oxidizing multienzyme complex (Sox complex) has been described as responsible for sulfate formation from thiosulfate. Thiosulfate is thought to fulfill a key role in the biogeochemical sulfur cycle as it is the energy source of most sulfur-oxidizing bacteria. In addition to the sulfur dissimilation pathways of representatives of the Archaea domain, there are currently at least three well-described pathways postulated for use by representatives of the Bacteria domain. These include the complete oxidation of reduced sulfur compounds to sulfate end products, known as the Sox metabolic pathway, the tetrathionate (S4I) pathway involving polythionate intermediates typical for acidophilic bacteria, and the branched thiosulfate oxidation pathway (Konrad *et al.* 2023; Nosalova *et al.* 2023). Green sulfur bacteria use various reduced sulfur compounds such as sulfide, elemental sulfur, and thiosulfate as electron donors for photoautotrophic growth. This article briefly summarizes what is known about the inorganic sulfur oxidizing systems of these bacteria with emphasis on the biochemical aspects. Enzymes that oxidize sulfide in green sulfur bacteria are membrane-bound sulfide-quinone oxidoreductase, periplasmic (sometimes membrane-bound) flavocytochrome c sulfide dehydrogenase, and monomeric flavocytochrome c (SoxF). Some green sulfur bacteria oxidize thiosulfate by the multienzyme system called either the TOMES (thiosulfate oxidizing multi-enzyme system) or Sox (sulfur oxidizing system) composed of the three periplasmic proteins: SoxB, SoxYZ, and SoxAXK with a soluble small molecule cytochrome c as the electron acceptor. The oxidation of sulfide and thiosulfate by these enzymes in vitro is assumed to yield two electrons and result in the transfer of a sulfur atom to persulfides, which are subsequently transformed to elemental sulfur. The elemental sulfur is temporarily stored in the form of globules attached to the extracellular surface of the outer membranes. The oxidation pathway of elemental sulfur to sulfate is currently unclear, although the participation of several proteins including those of the dissimilatory sulfite

reductase system etc. is suggested from comparative genomic analyses.

Figure 8. Growth activity three isolates in the various thiosulfate concentration in TSM medium

4. Conclusion

From the results of the research, it can be concluded that three of SOB isolates KT1.8 had 98.9% similarity with *Priestia qingshengii* HLS-7, KT1.9 had 97.6% similarity with *Sphingobacterium siyangensis* DS48, and KT1.13 had 98.3% similarity with *Pseudomonas putida* CFBP 4966 were potential to reduce sulfur concentrations in the culture medium. Those SOB isolates had optimum growth and oxidizing of sulfur at pH 6, 30^{o} C temperature, and 10 mM and 20 mM concentration in sixth days incubation of the culture thiosulphate mineral media. The SOB potential isolates in this study can be developed as bioremediation agent in the reclamation of post-coal mining ponds water which polluted by sulfur.

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