Exploring the ability of sulfur-oxidizing bacteria in biodesulfurization

A Dissertation Submitted in partial fulfillment of the requirements for the degree of

Masters of Science in Marine Biotechnology

by

HIMANSHI UPADHYAY

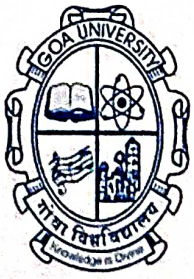
Roll Number: 21P050011

Under the Supervision of

PROF. SAVITA KERKAR

Dean

School of Biological Sciences and Biotechnology



GOA UNIVERSITY

APRIL 2023

DECLARATION BY STUDENT

I hereby declare that the data presented in this Dissertation entitled, **"EXPLORING THE ABILITY OF SULFUR-OXIDIZING BACTERIA IN BIODESULFURIZATION"** is based on the results of investigations carried out by me in the Discipline of Marine Biotechnology at the School of Biological Sciences and Biotechnology, Goa University under the supervision of Prof. Savita Kerkar and the same has not been submitted elsewhere for the award of a degree or diploma by me. Further, I understand that Goa University or its authorities will not be responsible for the correctness of observations / experimental or other findings given the dissertation.

I hereby authorize the University authorities to upload this dissertation to the dissertation repository or anywhere else as the UGC regulations demand and make it available to anyone as needed.

Himanshi upadhyay

Himanshi Upadhyay

Roll Number: 21P050011

M.Sc. Marine Biotechnology

School of Biological Sciences and Biotechnology

Date: 04/05/23

Place: Goa University

Examined by:

Seal of the School

COMPLETION CERTIFICATE

This is to certify that the dissertation report **"EXPLORING THE ABILITY OF SULFUR-OXIDIZING BACTERIA IN BIODESULFURIZATION"** is a bonafide work carried out by **Ms. Himanshi Upadhyay** under my supervision in partial fulfillment of the requirements for the award of the degree of Masters of Science in Marine Biotechnology in the Discipline of Biotechnology at the School of Biological Sciences and Biotechnology, Goa University.

sanitakerkar

Prof. Savita Kerkar
Dean
School of Biological Sciences
and Biotechnology

Date: 4/5/23

SBSB: Biotechnology
Taleigao Plateau, Goa 403206
GOA UNIVERSITY

School stamp

sanitakerkar

Prof. Savita Kerkar

Dean of School of Biological Sciences & Biotechnology
Goa University, Goa-403206
Office Ph. 8669609246

Name of Discipline : Biotechnology

School of Biological Sciences and Biotechnology

Date: 4/5/23

Place: Goa University

ACKNOWLEDGMENT

I would like to express my deepest appreciation to everyone who played a part in the successful completion of this project. I am incredibly grateful to my dissertation guide, Prof. Savita Kerkar, Dean, School of Biological Sciences and Biotechnology for her guidance and support throughout my dissertation. She provided me with clear direction and advice and was always available to answer my questions and provide valuable feedback.

I would also like to express my gratitude to Ph.D. scholar, Priti Gawas for her invaluable guidance during my dissertation. It was a pleasure to work with her. She was always willing to share her knowledge and expertise and was patient in answering my questions and helping me to understand the project. Her experience and expertise helped me to better understand the work and allowed me to make the most of my dissertation. With great pleasure, I would like to extend my gratitude and thanks to the entire faculty of the Department of Biotechnology, Goa University, SBSB for giving me the opportunity to do this project. I am deeply indebted to the entire non-teaching staff, Mr. Sameer and Mr. Ashish for their assistance throughout the course of my work. I would also like to extend my sincere thanks to all the research scholars, my classmates, and my friends for always giving helpful advice whenever I needed it.

Lastly, Special thanks to the Department of Biotechnology, Ministry of Science and Technology, Government of India, for funding this project.

ABBREVIATIONS

1. SOB- Sulfur oxidizing bacteria
2. SQR- Sulfide: quinone oxidoreductase
3. PDO- Persulfide dioxygenase
4. Sox- Sulfur oxidation
5. TsdA- Thiosulfate dehydrogenase
6. FAD- Flavin adenine dinucleotide
7. Apr- APS reductase
8. APS- adenosine-5'-phosphosulfate
9. ATP- adenosine triphosphate
10. Pi- inorganic phosphate
11. AtsB-. ATP sulfurylase
12. AMP- adenosine monophosphate
13. Sat- Sulfate adenylyltransferase
14. PSO- *Paracoccus* sulfur oxidation
15. nm- Nanometer
16. MR- Methyl red
17. VP- Voges Proskauer
18. SIM- Sulfide indole motility
19. AST- Antimicrobial susceptibility test
20. SEM- Scanning electron microscopy
21. DMPD- N, N-dimethyl-p-phenylenediamine

LIST OF TABLES

LIST OF FIGURES

TABLE OF CONTENTS

INTRODUCTION

1. INTRODUCTION

Sulfur-oxidizing bacteria (SOB) are important in the bioremediation of contaminated environments (Shinde et al. 2022). These bacteria oxidize reduced sulfur compounds like sulfur (S^0), hydrogen sulfide (H_2S), thiosulfate ($S_20_3^2$), thiocyanate (^-SCN), or sulfite as electron donors in energy conversion, typically with O_2 as electron acceptor.

By oxidizing elemental sulfur, sulfite, and thiosulfate into sulfate (SO_4^{2-}), sulfur-oxidizing bacteria play a crucial part in the sulfur cycle. Sulfate is a form of sulfur readily used by plants and other organisms, making it an essential ecosystem component. These bacteria are found in various environments, such as hot springs, deep-sea hydrothermal vents, and terrestrial sulfur springs.

The sulfur cycle begins with the weathering of rocks, which releases elemental sulfur. Sulfur oxidizing bacteria then convert this sulfur into sulfite, which can be further oxidized to sulfate. Sulfate can be used by plants to produce organic molecules containing sulfur, which are consumed by animals. When plants and animals die, sulfur compounds are released back into the environment, and the cycle begins anew.

The gas H_2S (hydrogen sulfide) is colorless, combustible, and has a pungent smell akin to rotten eggs (Jin and Pei, 2015). H_2S is produced naturally in the environment by decomposing organic matter, but it can also be found in a variety of industrial settings. H_2S is harmful because it can be toxic at high concentrations. When inhaled, H_2S can irritate the eyes, nose, throat, and respiratory system, leading to symptoms such as coughing, shortness of breath, and chest pain. At higher concentrations, H_2S can cause a loss of consciousness, respiratory failure, and even death. Long-term exposure to low amounts of H_2S can also have negative health effects, such as chronic headaches, eye irritation, and symptoms of the nervous system and the respiratory system (Lim, et al. 2016). It is a strong greenhouse gas that also damages ecosystems and adds

to the acidification of the atmosphere. Therefore, it is important to take appropriate measures to prevent exposure to H_2S and ensure safe handling and disposal of this gas.

Many industrial activities such as wastewater treatment, food processing, petroleum refining, and methane-containing biogas production, produce H_2S and other sulfur compounds which are toxic at high levels and produce an unpleasant smell (Saeedi et al. 2015).

Sulfur compounds present in fossil fuels, such as natural gas, can have a number of negative effects on both the environment and infrastructure. Hydrogen sulfide and mercaptans are highly corrosive and can cause significant damage to transmission pipelines (Zhang, et al. 2008). This can lead to leaks and other safety hazards. Additionally, the combustion of sulfur compounds produces sulfur oxides (SOx) which contribute to air pollution and the formation of acid rain. These pollutants can have serious health effects on humans and animals, as well as cause damage to buildings and vegetation. The high volumetric mass of these compounds also means that they tend to deposit on the earth's surface, leading to further environmental damage (Rieks de Rink et al. 2020).

There are several methods used in wastewater treatment plants to remove H_2S gas, including:

1. Chemical treatment: Chemicals such as chlorine or hydrogen peroxide can be added to wastewater to oxidize H_2S gas and convert it into sulfuric acid, which can then be neutralized with lime. This method is effective, but it can be expensive and may produce additional waste products.

2. Biological treatment: Some wastewater treatment plants use biological processes, such as aerobic or anaerobic digestion, to remove H_2S gas. These processes use bacteria to break down organic matter and produce sulfur, which can be removed from the wastewater.

3. Physical treatment: Physical methods such as air stripping and adsorption can be used to remove H_2S gas from wastewater. Air stripping involves bubbling air through the wastewater to remove H_2S gas, while adsorption uses materials such as activated carbon to adsorb H_2S gas.

4. Gas-phase treatment: This method uses chemicals such as iron or zinc oxide to adsorb H_2S gas in a separate reactor. The chemicals can then be regenerated and reused.

The demerits of these methods can vary depending on the specific method used. For example, chemical treatment can be expensive and produce additional waste products, while biological treatment may require longer treatment times and can be sensitive to changes in temperature and pH. Physical treatment methods may require additional equipment and energy to operate, while gas-phase treatment may require more frequent regeneration of the adsorbent material. Additionally, all these methods may have limitations in terms of the amount of H_2S gas they can remove and the effectiveness of the treatment over time. (Pudi et al. 2022).

Research on microbiological solutions has been prompted by the ongoing hunt for more affordable H_2S removal techniques. Based on chemolithotrophic or photoautotrophic bacterial processes, H_2S can be biologically removed. Biotreatment or biodesulfurization is used to remove these compounds because they are effective, low-energy consuming, and generate minimal by-products compared to physical and chemical methods (Sorokin et al. 1994). Biological processes take place at ambient temperature and atmospheric pressure, therefore the need for heating, cooling, and high pressure can be avoided, thus reducing energy costs.

Sulfur oxidizing bacteria (SOBs) are used to perform these processes. These bacteria use sulfide: quinone oxidoreductase (SQR) and persulfide dioxygenase (PDO) and rhodanese for detoxification of sulfide (Liu et al. 2014; Xin et al. 2016). Bioinformatic analysis showed that the sequenced genome contains *sqr* and *pdo* genes.

SOBs conserve energy from the oxidation of sulfur compounds by the Sox (for sulfur oxidation) system. This system contains over 15 genes encoding various cytochromes and proteins required for the oxidation of reduced sulfur compounds to sulfate.

The key proteins present in the Sox system are: SoxXA, SoxYZ, SoxB, and SoxCD, and all of them are located in the periplasm. The enzyme SoxXA forms a heterodisulfide bond between the sulfur compound and the carrier protein, SoxYZ, this marks the beginning of the pathway. The sulfur compound remains bound to the carrier protein and is ultimately released as sulfate through the activity of SoxB. The enzyme SoxCD (sulfur dehydrogenase) mediates the removal of 6 electrons from the sulfur compound that is bound to the carrier. These electrons are used in the electron transport chain while the protons generated in the periplasm are released in the external environment.

Thiosulfate oxidation, a reduced form of sulfur is also a common pathway used by sulfur-oxidizing bacteria (SOB) to generate energy. The conversion of thiosulfate to sulfate involves several enzymatic steps, which are summarized below:

1. Thiosulfate dehydrogenase (TsdA): This enzyme catalyzes the initial step in thiosulfate oxidation, which involves the conversion of thiosulfate to sulfite. TsdA is a flavoprotein that uses flavin adenine dinucleotide (FAD) as a cofactor.

2. Sulfite oxidase (Sox): Sulfite is further oxidized to sulfate by the enzyme sulfite oxidase (Sox). This enzyme is a molybdopterin-containing protein that uses oxygen as an electron acceptor.

3. APS reductase (Apr): Alternatively, sulfite can be converted to adenosine-5'-phosphosulfate (APS) by the enzyme APS reductase (Apr). This reaction also generates ATP from adenosine triphosphate (ATP) and inorganic phosphate (Pi).

4. ATP sulfurylase (AtsB): APS is then converted to sulfate by the enzyme ATP sulfurylase (AtsB), which uses ATP to transfer a sulfate group to adenosine monophosphate (AMP), generating adenosine-5'-phosphosulfate (APS).

5. Sulfate adenylyltransferase (Sat): The final step in the process involves the conversion of APS to sulfate by the enzyme sulfate adenylyltransferase (Sat). This enzyme transfers an adenosine monophosphate (AMP) group from ATP to APS, generating sulfate and adenosine diphosphate (ADP).

Overall, the conversion of thiosulfate to sulfate by SOB involves a complex set of enzymatic reactions that allow these bacteria to use sulfur compounds as an energy source. (Rameez et al. 2020)

SOB also plays an important role in the removal of sulfur from industrial waste streams. In these settings, SOBs are used to catalyze the conversion of hydrogen sulfide and other sulfur compounds into less toxic forms, such as sulfate, that can be safely discharged into the environment. (Cheng Y et al. 2018)

In addition to their role in the sulfur cycle, SOB can be used in bioremediation, as they can degrade organic pollutants containing sulfur compounds. This process, known as sulfur dependent biodegradation, is particularly effective in removing organic sulfur from contaminated soils and water.

Overall, sulfur-oxidizing bacteria play a critical role in many ecological and industrial processes, and their study has important implications for environmental management and sustainability.

In this study, we have analyzed three different strains of SOBs (SKPH1, SKPH11, and SKPH19) isolated from salt pans of Goa with an aim to investigate their thiosulfate and sulfide oxidation efficiency with the following objectives:

1) To subculture bacteria that show thiosulfate and sulfide oxidation.
2) To determine the biochemical and morphological characteristics of the subcultured bacterial isolates.
3) Assessment of their thiosulfate and sulfide oxidation efficiency.
4) Identification of the functional genes involved in thiosulfate and sulfide oxidation.

LITERATURE REVIEW

2. LITERATURE REVIEW

Understanding the potential for extreme life in extreme environments is important as these organisms have adapted to survive in harsh conditions like high temperature, high pressure, low nutrition, and high salinity. Their metabolic processes and growth are supported by the energy produced by the oxidation of reduced inorganic compounds.

Deep-sea hydrothermal vent-dwelling microorganisms get their energy from oxidizing reduced inorganic substances, such as hydrogen sulfide, sulfur (S^0), thiosulfate ($S_2O_3^{2-}$), ammonium (NH_4^+), nitrite (NO_2^-), and ferrous iron (Fe^{2+}). This process is known as chemosynthesis, and it is similar to the role of light in photosynthesis, which is used by plants to convert sunlight into energy (Jannasch et al. 1983)

Ying-Chien-Chung, et al. (2000) studied the biological elimination of various mixtures of H_2S and NH_3 from waste gases with the help of a biofilter packed with co-immobilized heterotrophic bacteria (*Pseudomonas putida* CH11 for H_2S and *Arthrobacter oxydans* for NH_3). The reported results suggest that 95% of the NH_3 and 90% of the H_2S were removed. Additionally, it was discovered that the heterotrophic bacteria *Pseudomonas putida* outperforms the autotrophic bacteria *Thiobacillus thioparus* for the long-term removal of H_2S (Chung et al. 1996). Similar findings were made for the removal of NH_3, where it was discovered that the heterotrophic bacteria *Arthrobacter oxydans* CH8 isolated from piggery wastewater was superior to the autotrophic bacteria *Nitrosomonas europaea*, especially at high concentrations of NH3 (Chung et al. 1997; Chung et al. 1998).

Acidithiobacillus thiooxidans TAS, a sulfur-oxidizing bacterium with tolerance to high concentrations of $(NH4)_2SO_4$ was isolated from activated sludge (Lee et al. 2005). In the soils surrounding plant roots (the rhizosphere), Anandham et al. (2008) identified and characterized seven distinct groups of novel thiosulfate-oxidizing bacterial strains. This includes both Beta

and Gamma subclasses of Proteobacteria, as well as Actinobacteria. Out of the seven different groups of thiosulfate-oxidizing bacteria that were identified in the rhizosphere soils, five of them (*Dyella, Burkholderia, Alcaligenes, Microbacterium, and Leifsonia*) were actually previously unknown as sulfur oxidizers in this environment. Furthermore, these bacteria were shown to grow using thiosulfate as an energy source and exhibited growth coupled with thiosulfate oxidation. This suggests that thiosulfate may be an important energy source for these bacteria in the rhizosphere and that they play an important role in nutrient cycling in this environment.

Their research concentrated on the S-oxidation and *soxB* genes found in other bacterial strains, as well as the *Paracoccus* sulfur oxidation (PSO) pathway of thiosulfate oxidation used by *Paracoccus versutus* and *Paracoccus pantotrophus*. The study noted that *Halothiobacillus spp.* accumulate intermediate products of thiosulfate oxidation, while *Pandoraea sp.* uses the S4I pathway and Sox enzyme system for thiosulfate oxidation. The study suggests that the development of a bio-inoculant containing thiosulfate-oxidizing bacteria may enhance sulfur oxidation in the rhizosphere, and further research is needed to elucidate the role of these bacteria in plant S nutrition.

In sulfide-oxidizing bacteria and archaea, cytochromes and iron-sulfur proteins involved in electron transport were examined by Barton, et al. (2014). Additionally, these bacteria might be utilized in H_2S biotreatment.

Halothiobacillus neapolitanus DSM 581, a bacteria isolated from sulfur-compound contaminated soil, was used by Fatemeh Nazari, et al (2017) in their description of the use of microbiological solutions for the elimination of hydrogen sulfide. According to the authors, the Postgate medium with 0.7% thiosulfate is the best medium for the bacterium's growth and sulfur oxidation activities. Because it dissolves more easily in water than sodium sulfide and

elemental sulfur, thiosulfate is used as an electron donor in the isolation of SOB. According to reports, the isolated strain of *Halothiobacillus sp.* can create up to 89.14% and 93.14% of sulfate after 24-72 hours, respectively, and can remove up to 100% of thiosulfate after 18-24 hours. In comparison to other obligate SOBs, *Halothiobacillus sp*. grew and eliminated sulfur compounds more quickly, which is significant for the gas and petroleum industries.

Recent findings suggested that heterotrophic bacteria possess the enzyme sulfide: quinone oxidoreductase (SQR) and persulfide dioxygenase (PDO), and rhodanese, which allow them to oxidize sulfide to sulfite and thiosulfate. The enzymes were found in various types of bacteria, including Gram-negative bacteria, and are capable of oxidizing both self-produced and exogenous H_2S. The bacteria with SQR and PDO can collectively oxidize sulfide to sulfate and prevent its accumulation and volatilization as H_2S.

When adjacent genes like *pdo* and *sqr* were searched in the genomes of 4929 GenBank genomes, it was discovered that the majority of bacteria carrying these genes were able to oxidize sulfide. It was found that bacteria with SQR and PDO did not release significant amounts of H_2S when growing in rich media. The authors suggested that this oxidation of sulfide by heterotrophic bacteria may have ecological significance, as abiotic oxidation of sulfide is slow. In marine waters and sequenced marine bacterial genomes, *pdo* genes are more prevalent than *sqr* genes. Both Gram-positive and Gram-negative bacteria as well as archaea have Type III PDOs, which are more distinct from Type I and II PDOs. Overall, the findings suggest that many types of heterotrophic bacteria are capable of oxidizing sulfide and that this process may have ecological significance in preventing the accumulation of H_2S. (Xia et al. 2017)

Various heterotrophic bacteria that were capable of rapidly oxidizing H_2S to zero-valence sulfur and thiosulfate, causing no apparent acidification were reported (Hou et. al. 2018).

According to a 2018 study by Ter Heijne, et al. SOB from a dual-reactor system eliminated more sulfide from the solution than SOB from a single-aerated bioreactor system. H_2S concentration in the treated gas decreased as biomass concentration in the flow to the absorber column increased, demonstrating that SOB boosts the H_2S absorption rate.

The use of sulfur-oxidizing bacteria (SOB) for the removal of sulfide in coastal environments, as well as in wastewater treatment and aquaculture practices was studied by Shinde, et al. (2022). Their main goal was to create a probiotic consortium that improves the habitat for both animals and the surrounding ecosystem by increasing sulfate ion production through the use of a combination design matrix. The probiotic consortium used SOB strains with the highest oxidation efficiency, including *Pseudomonas stutzer*i B6-SOB and *Cytobacillus firmus* C8-SOB. As a bio-augmenting species, the utilization of these native probiotics may be taken into account for integration in aquaculture systems to improve sulfur oxidation. This can reduce the need for imported goods and other pricey physical and chemical clean-up techniques. A promising bio-inoculant for the remediation of sulfide and other reduced sulfur compounds from different environments, such as wastewater treatment plants, may also be thought of as part of the consortium.

Hydrogen sulfide removal from sour gas via a biological desulfurization process was studied by Rieks de Rink, et al. (2022). At a pilot-scale biodesulfurization plant, the impact of the SOB activity on H_2S absorption was investigated by adjusting the temperature of the solution in the absorber column. H_2S absorption efficiency increased with temperature, with the treated gas's H_2S content falling from 715 ± 265 ppmv at 25.4 °C to 69 ± 25 ppmv at 39.4 °C.

Similarly, H_2S can be removed from gas streams and converted to elemental sulfur by chemolithotrophic sulfur-oxidizing bacteria such *T. versutus* and *T. aerophilum*, which can then be recycled as a valuable raw material (Reza Peighami, et al. 2022)

MATERIALS AND METHODS

3. MATERIALS AND METHODS

3.1 Subculturing

Lieske's media containing 2% agar was prepared and autoclaved at 121°C, 15 psi, for 20 minutes. After autoclaving the medium was brought to room temperature and poured in sterile petri plates under sterile conditions. Bacterial isolates SKPH1, SKPH11, and SKPH19 isolated from the salt pans of Goa were subcultured on plates by the streaking method. Petri plates were then incubated at 28°C-30°C for 10-15 days.

3.2 Culturing of bacterial samples in Lieske's broth

SKPH1, SKPH11, and SKPH19 bacterial isolates were inoculated in nutrient broth and incubated at 28 to 30 degrees Celsius for five days. The SKPH1, SKPH11, and SKPH19 cultures grown in the nutrient broth were used to inoculate flasks containing Lieske's broth and they were incubated at 28-30 degrees Celsius for 15-30 days and the absorbance was measured at 600 nanometers (nm) using a UV-spectrophotometer (Shimadzu, Japan).

3.3 Morphological and Biochemical Analysis

3.3.1 Gram Characterization

Bacterial isolates were checked for their Gram character by the Gram staining method. Gram stain was introduced in 1880 by the Danish bacteriologist Christian Gram. Almost all bacteria may be divided into two major types termed Gram-positive and Gram-negative bacteria based on the staining reaction and the characteristics of their cell walls.

The staining procedure:

1. Preparation of the bacterial smear: A small amount of the bacterial isolate was spread onto a glass slide, then left to air dry. It was heat fixed to kill the bacterial cells and adhere them to the slide.

2. Crystal violet staining: The slide was flooded with a basic dye called crystal violet, which stains all the cells on the slide.

3. Iodine treatment: Iodine, which serves as a mordant to fix the crystal violet in the cells, was added to the slide.

4. Decolorization: The slide was briefly washed with a decolorizing agent such as 95% ethanol. This step removes the crystal violet from some bacterial species, leaving only those with thick peptidoglycan cell walls stained purple (Gram-positive).

5. Counterstaining: The slide was then stained with a contrasting dye such as safranin, which stains the remaining bacteria pink or red (Gram-negative).

6. Examination: The slide was examined under a microscope, typically using an oil immersion objective (100X), to observe the color and morphology of the bacterial cells.

3.3.2 KOH test

The KOH test is a simple microbiological technique used to divide bacterial cells into two categories- Gram-positive and Gram-negative bacteria. The basis for this test is that Gram-negative bacteria cell walls are more vulnerable to the effects of potassium hydroxide (KOH) than Gram-positive bacteria. To perform the test, the following procedure was used:

1. Take a clean microscope slide and place one drop of 3% potassium hydroxide solution on it.

2. Obtain a small amount of the test culture using a sterile inoculating loop or needle.

3. Emulsify the test culture in the drop of potassium hydroxide solution to create a dense suspension.

4. Stir the suspension continuously for 60 seconds to ensure the complete mixing of the sample with the KOH solution.

5. After 60 seconds, gently pull the loop away from the suspension.

6. Observe the sample under a microscope.

The KOH will break down the cell walls of Gram-negative bacteria, resulting in the release of chromosomal material. This, in turn, leads to the formation of a thick and slimy suspension.

In contrast, the cell walls of Gram-positive bacteria are not as susceptible to the action of KOH, and therefore, do not disintegrate. As a result, the KOH solution remains clear when added to a culture of Gram-positive bacteria.

3.3.3 Catalase test

The catalase test is a biochemical test used to detect the presence of the catalase enzyme in bacterial cells. Catalase breaks down hydrogen peroxide into water and oxygen.

To perform the catalase test, a small amount of bacterial culture is mixed with 3% hydrogen peroxide. If catalase is present, it will catalyze the breakdown of hydrogen peroxide, producing bubbles of oxygen gas. The presence of bubbles indicates a positive test result, meaning that the bacterium is catalase-positive.

The catalase test is commonly used to differentiate between different types of bacteria, particularly those that are aerobic or facultatively anaerobic, as these bacteria produce catalase to protect themselves from oxidative stress caused by oxygen.

3.3.4 Oxidase test

The oxidase test is a biochemical test used to determine the presence of cytochrome c oxidase; an enzyme involved in the electron transport chain in aerobic organisms. The test is commonly used to differentiate between different types of bacteria, particularly those in the *Enterobacteriaceae* family.

To perform the oxidase test, a small amount of the bacterial culture is transferred to a filter paper that contains a reagent called tetramethyl-p-phenylenediamine dihydrochloride. If the bacteria are positive for cytochrome c oxidase, the reagent will be oxidized and turn blue or purple. If the bacteria are negative for the enzyme, there will be no color change.

The oxidase test is a simple and rapid test that can be performed in the laboratory to aid in bacterial identification. It is often used in conjunction with other tests, such as the Gram stain and biochemical tests, to identify bacterial species

3.3.5 MR-VP (Methyl red and Voges Proskauer test) test

The MR-VP test is a biochemical test used to distinguish between different groups of bacteria based on their metabolic characteristics. The test consists of two parts, the Methyl Red test and the Voges Proskauer test, which are performed together.

Procedure:

1. Inoculate the bacterial culture into the MR-VP broth using an inoculating loop.

2. Incubate the broth for 24-48 hours at 37°C.

3. After incubation, divide the broth into two portions in separate test tubes.

4. To perform the Methyl Red test, add 0.5 ml of MR reagent to one of the test tubes.

5. Mix the contents of the tube by gently swirling it, then observe the color of the broth.

6. If the broth turns red, it indicates that the bacteria have produced acid from glucose fermentation, and the test is positive for the Methyl Red test.

7. To perform the Voges Proskauer test, add 0.2 ml of VP reagents (alpha-naphthol and 40% potassium hydroxide) to the other test tube.

8. Mix the contents of the tube by gently swirling it, then incubate it for 10-30 minutes at room temperature.

9. After incubation, observe the color of the broth. If a pink or red color develops, it indicates that the bacteria have produced acetoin from glucose fermentation, and the test is positive for the Voges Proskauer test.

Interpretation:

- If both the Methyl Red and Voges Proskauer tests are positive, it indicates that the bacteria are in the mixed-acid fermentation group.
- If the Methyl Red test is positive and the Voges Proskauer test is negative, it indicates that the bacteria are in the acid fermentation group.
- If both the Methyl Red and Voges Proskauer tests are negative, it indicates that the bacteria are in the neutral fermentation group.

3.3.6 Citrate test

The citrate test is a biochemical test used to determine whether an organism can use citrate as a carbon source. The test is useful in the identification of members of the *Enterobacteriaceae* family.

Procedure:

1. Pick a well-isolated colony of the test organism and streak it onto the Simmons citrate agar slant using the sterilized inoculating loop.

2.. Incubate the slant at 28-30°C.

3. After incubation, observe the slant for growth and color changes. A positive test is indicated by growth and a change in color of the medium from green to blue, indicating that the organism has used citrate as a carbon source. A negative test is indicated by no growth and no color change.

3.3.7 SIM test

The SIM test (Sulfide Indole Motility test) is a biochemical test used to identify bacteria based on their ability to produce hydrogen sulfide, indole, and exhibit motility.

Procedure:

1. Sterilize the inoculating loop by passing it through the flame of a Bunsen burner until it glows red-hot.

2. Inoculate the SIM medium by lightly stabbing the inoculating loop into the center of the medium, avoiding the surface.

3. Incubate the SIM medium at the appropriate temperature for the organism you are testing. For example, for *Enterobacteriaceae*, incubate at 37°C for 24-48 hours.

4. After incubation, observe the medium for the following characteristics:

Interpretation:

- Hydrogen sulfide production: If the organism is capable of producing hydrogen sulfide, a black precipitate will be visible throughout the medium.
- Indole production: Add 2-3 drops of Kovacs reagent (5% dimethylamino benzaldehyde in amyl alcohol) to the top of the medium. If the organism is capable of producing indole, a red color will appear at the top of the medium.
- Motility: Observe the medium for signs of motility. If the organism is motile, it will appear to radiate outwards from the point of inoculation.

It's important to note that the SIM test is just one of many tests used to identify microorganisms, and should be used in conjunction with other biochemical and morphological tests.

3.3.8 Carbohydrate utilization tests

All 3 isolates were studied for utilization of various substrates using the HiCarbo(™) kit (KB009, HiMedia labs). Each isolate was evaluated for the utilization of 35 carbohydrates through colorimetric tests based on the principle of pH change and substrate utilization.

Procedure:

- The isolates were aseptically inoculated in Nutrient broth and incubated overnight at 28-30°C.
- After incubation, the test kit consisting of 3 parts - A, B, and C (each containing 12 tests with C containing 11 tests + 1 control) were opened aseptically inside a biosafety cabinet and inoculated with 0.1 ml of culture containing broth in each of the 36 test wells. This was repeated for all 3 isolates.
- All the KB009 test strips were incubated at 37°C for 24 hours as required by the protocol and the results were evaluated according to the result interpretation chart supplied with the kit. All

the test strips were further incubated and observed up to the 48-hour mark at RT in order to note any late reactions.

All test results were interpreted with the control strips as a reference to account for color changes due to the nature of the culture media

3.3.9 Antibiotic Susceptibility test (Kirby Bauer method of agar disc diffusion)

Antimicrobial susceptibility test (AST) is frequently carried out using the disc diffusion method in clinical microbiology labs.

Procedure for the disc diffusion method:

1. Obtain the bacterial culture: The first step is to obtain a pure culture of the bacterial strain you want to test. This was done by growing the bacteria in Nutrient broth.

2. Inoculate the agar plate: The inoculum is then streaked onto a Mueller-Hinton agar plate using a sterile swab.

3. Place the antimicrobial discs: After the inoculum has dried on the agar plate, sterile antimicrobial discs are placed on the surface of the agar. The discs contain a specific concentration of the antimicrobial agent being tested.

4. Incubation: The plates are then incubated at 28-30°C temperature for 24 hours.

Interpretation:

After incubation, the plates are examined for zones of inhibition around the antimicrobial discs to determine if the bacterial strain is susceptible, intermediate, or resistant to the antimicrobial agent.

3.3.10 Scanning Electron Microscopy

SEM is a powerful imaging technique used to observe the surface of a sample at high magnification. In order to prepare a sample for SEM imaging, the following steps were taken:

1) Coverslip was cleaned ($1cm^2$) and fixed onto a glass slide using cello tape. This provides a flat surface for the bacteria to adhere to during the sample preparation process.

2) Fresh bacterial culture was spread (10 µl) onto the coverslip and allowed to air dry. The bacteria should be evenly distributed across the coverslip.

3) Bacteria were fixed to the coverslip by treating the sample with 2.5% glutaraldehyde, which is a fixative that preserves the structure of the cells. (Leave the sample overnight to ensure complete fixation)

4) The next day, the coverslip was washed with sterile phosphate buffer saline (PBS) pH 7.4 to remove any remaining fixative.

5) Sample was dehydrated using a series of acetone washes. (Start with 30% acetone and increase the concentration by 20% for each subsequent wash (i.e., 50%, 70%, 80%, and 90%) leave the sample in each concentration of acetone for 10 minutes.)

6) Finally, the sample was treated with 100% acetone for 30 minutes to ensure complete dehydration.

7) Sample was air-dried before imaging with SEM

3.3.11 Hemolysis test

The hemolysis test is a procedure used to determine whether a substance causes the destruction of red blood cells (hemolysis). Here's a general outline of the procedure.

1. Use a sterile loop or wire to streak a blood agar plate with the test organism.

2. Allow the plate to dry for a few minutes.
3. Incubate the plate overnight at 35-37°C.
4. After incubation, examine the plate for hemolysis.

Interpretation:

There are three types of hemolysis that can occur: alpha, beta, and gamma.

- Partial destruction of red blood cells, resulting in a greenish discoloration around the organism is called Alpha hemolysis
- Complete destruction of red blood cells, resulting in a clear zone around the organism is called Beta hemolysis.
- No destruction of red blood cells and no visible change around the organism is called Gamma hemolysis.

3.4 Thiosulfate oxidation by bacterial isolates SKPH1, SKPH11, and SKPH19

Lieske's broth with three different concentrations of $Na_2S_2O_3$ (40mM, 50mM, and 60mM) and positive control was inoculated with an actively grown culture (O.D = 0.5) in duplicates. The end product of thiosulfate oxidation is sulfate. The sulfate estimation was done after 18 days of inoculation at 30˚C using the below-mentioned equation

$$\text{Sulfate (mg/L)} = \frac{\text{Test reading x 0.4 (Concentration of standarad solution)}}{\text{Standard O. D x Amount of sample in ml (1 ml)}}$$

Sulfate estimation was performed by following procedure:

The sulfate estimation test is a quantitative biochemical test used to determine the amount of sulfate present in a sample. This test is often used in environmental and industrial applications, such as in the monitoring of water quality or in the analysis of sulfuric acid production.

We used the colorimetric determination of sulfate using the barium chloride method (Peighami R et al. 2022).

Procedure:

1. Take 1 ml of the sulfate sample in a standard volumetric flask and add MilliQ water to make the volume up to 25 ml.

2. Add 1.25 ml of conditioning reagent to the volumetric flask containing the sample and mix well.

3. Pour the sample and conditioning reagent mixture into a beaker with a magnetic stirrer and start agitating continuously.

4. Add 2 ml of $BaCl_2$ solution slowly to the sample mixture, and continue to stir for 1 minute to ensure thorough mixing.

5. Allow the mixture to stand for 10 minutes for the complete formation of the $BaSO_4$ precipitate.

6. Take the absorbance reading of the mixture at 365nm using a spectrophotometer. Be sure to take the reading immediately after the 10-minute waiting period to avoid interference from further precipitation.

To determine the concentration of sulfate in the sample, compare the absorbance reading to a standard curve generated from known concentrations of sulfate. The concentration of sulfate in the sample can then be calculated using the equation of the standard curve.

3.5 Sulfide oxidation by bacterial isolates SKPH1, SKPH11, and SKPH19

Lieske's media with three different concentrations of Na_2S (100 ppm, 200 ppm, 300 ppm), and negative controls were prepared (without thiosulfate), and in positive controls, thiosulfate was added as a supplement. They were dispensed in serum bottles (50 ml) in duplicates and inoculated with bacterial isolates SKPH1, SKPH11, and SKPH19. Sulfide production was estimated after 22 days of inoculation.

Sulfide estimation was performed by following the procedure (Peighami R et al. 2022)

To estimate sulfide, in the first step 0.5 ml of the test sample was added to 0.5 ml of 5% zinc acetate solution in the test tube. Zinc acetate is used to precipitate the sulfide ions present in the sample as zinc sulfide. The mixture is vortex mixed or shaken to ensure complete mixing and then incubated for 30 minutes to allow the formation of the zinc sulfide precipitate.

In the second step, solution A and solution B are added to the test tube containing the sample and zinc acetate mixture. Solution A contains DMPD (N, N-dimethyl-p-phenylenediamine) and concentrated sulfuric acid, which reacts with the sulfide ions to produce a colored complex. Solution B contains $Fe(NH_4)SO_4)_2.12H_2O$ and sulfuric acid, which serves as a reducing agent and helps to maintain the pH of the solution.

The test tube is then stirred well to ensure thorough mixing of all the components. The mixture is again incubated for 30 minutes at room temperature to allow the reaction between the reagents and the sulfide ions to take place. The formation of a blue color indicates the presence of sulfide in the sample.

To quantify the amount of sulfide present in the sample, a standard curve can be generated using known concentrations of sulfide. The absorbance reading at 660 nm obtained from the test tube using a spectrophotometer can then be compared to the standard curve to determine

the sulfide concentration in the sample. The sulfide concentration can be reported in appropriate units, such as milligrams per liter (mg/L).

3.6 Polymerase chain reaction for *soxB* gene

PCR was carried out for the isolation and amplification of *soxB* marker genes for sulfur oxidation. Colony PCR was performed using *SureCycler 8800 by Agilent Technologies and MJ Mini™ Personal Thermal Cycler by Bio-Rad.* Below listed are the specifications and parameters used for the isolation and amplification of target genes.

soxB gene primer used

soxB 432F - 5' GAY GGN GGN GAY ACN TGG 3'

soxB 1446B 5' CAT GTC NCC NCC RTG YTG 3'

soxB gene PCR profile

COMPONENT	**VOLUME (in µl)**
10x Assay Buffer	2.5
50mM $MgCl_2$	1.25
dNTP	2.5
Template	2.0
1 µM Reverse Primer	2.5
1 µM Forward Primer	2.5
Taq Polymerase	1.25
Molecular Grade Water	10.5
Total Volume	25

PCR parameters for *sox*B gene

	Temperature	Time
Denaturation	95ºC	2 min
30 cycles		
Denaturation	95ºC	30 sec
Annealing	43ºC	30 sec
Elongation	72ºC	1 min
End cycle		
Elongation	72ºC	8 min

- **Electrophoresis**

Agarose gel (1%) was prepared to check the amplification of the target gene using agarose gel electrophoresis. Gel was poured into a tray and combs were inserted. 6 µl (1 µl 6x loading dye + 5 µl PCR product) PCR products were loaded in the well. 500bp DNA ladder and 1000bp ladder were loaded along with *sox*B gene products. Electrophoresis was allowed to run at 100V till the dye front reached ¾ of the gel length.

RESULTS AND DISCUSSION

4. RESULTS AND DISCUSSION

4.1 Subculturing: Bacterial isolates SKPH1, SKPH11, and SKPH19 from the salt pans of Goa were streaked on Lieske's media. The colorless transparent colonies of all three isolates were observed after 10 days of incubation at 28-30°C

4.2 Culturing of bacterial samples in Lieske's broth: The growth in the liquid medium was observed after 15-18 days and the absorbance was found to be 0.08, 0.03, and 0.07 for SKPH1, SKPH11, and SKPH19 respectively at 600nm.

4.3 Morphological and Biochemical Analysis

4.3.1 Gram characterization

Gram staining showed that bacterial isolates SKPH1 and SKPH19 were Gram-negative and SKPH11 was Gram-positive. Gram-negative bacteria showed red-pink color cells due to a thinner peptidoglycan layer and an outer membrane containing lipopolysaccharides that do not allow the crystal violet stain from penetrating the cell wall, resulting in the cells appearing red-pink after counterstaining with safranin. Gram-positive bacteria will appear purple/blue after staining, as the crystal violet-iodine complex is retained in their thick peptidoglycan cell wall. Sulfur-oxidizing bacteria can be both gram-negative or gram-positive. Some examples of gram-negative sulfur-oxidizing bacteria include *Thiomargarita, Beggiatoa, and Thiobacillus*. Gram-positive sulfur-oxidizing bacteria include *Bacillus and Desulfovibrio*.

4.3.2 KOH (potassium hydroxide) test

When a small amount of SKPH1 and SKPH19 bacterial cultures were separately mixed with a drop of 3% KOH solution, they released their lipids and form a sticky, viscous mass due to which these cultures formed a string or thread, indicating they are Gram-negative. On the other hand, SKPH11 did not form a sticky, viscous mass indicating it is a Gram-positive bacterium.

The difference in the thickness of the peptidoglycan layer in the cell wall of Gram-negative and Gram-positive bacteria makes them react differently to KOH treatment. Gram-negative bacteria have a thin peptidoglycan. The presence of KOH causes the lipopolysaccharides to break down, making the cell wall unstable and leading to cell lysis.

In contrast, Gram-positive bacteria have a thicker peptidoglycan layer that is not surrounded by an outer membrane. The thick peptidoglycan layer protects the cell from the effects of KOH, making it resistant to cell lysis. Therefore, when treated with KOH, Gram-positive bacteria do not release their DNA, and no viscosity is observed.

4.3.3 Catalase test:

The release of bubbles was observed for all three cultures SKPH1, SKPH11, and SKPH19 that indicated the presence of catalase enzyme. In aerobic organisms, O_2 acts as an H_2 acceptor and the cell produces H_2O_2. H_2O_2 is poisonous to cells at large concentrations. In order to convert harmful hydrogen peroxide into hydrogen and oxygen, aerobes have catalase. The creation of oxygen as a by-product causes bubbles to be released, which confirms the existence of the catalase enzyme.

Figure 1. Catalase test

4.3.4 Oxidase test

The oxidase test result for all three cultures was found to be positive as the development of blue color was observed on the oxidase disc after applying a loopful of the bacterial cultures SKPH1, SKPH11, and SKPH19.

Cytochrome oxidase, which is present in oxidase-positive bacteria, catalyses the oxidation of cytochrome C necessary for the movement of electrons in the electron transport chain (ETC). In aerobic species, the cytochrome c oxidase enzyme is frequently present. Oxidase enzymes are commonly present in SOBs, as these enzymes are often involved in the oxidation of sulfur compounds to generate energy for the cell. However, not all SOBs produce oxidase enzymes, and the prevalence of these enzymes can vary depending on the species and the specific metabolic pathways used by the bacteria.

For example, some sulfur-oxidizing bacteria, such as *Thiobacillus denitrificans*, are known to produce high levels of oxidase enzymes, which are essential for their metabolism. Other sulfur-oxidizing bacteria, such as *Acidithiobacillus ferrooxidans*, produce lower levels of oxidase enzymes and may rely on alternative pathways for sulfur oxidation.

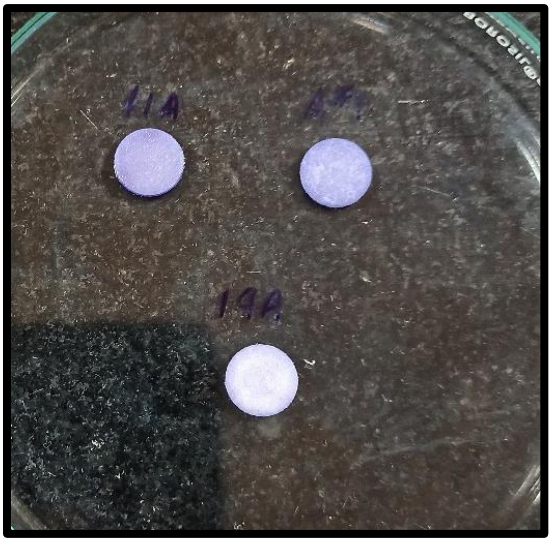


Figure 2a. Positive Oxidase test by SKPH1, SKPH11 and SKPH19

Figure 2b. Negative oxidase test (control)

4.3.5 MR-VP (Methyl red and Voges Proskauer test) test

The MR-VP test is a differential test used to distinguish between organisms that ferment glucose via the mixed acid pathway (MR-positive) and those that ferment glucose via the butylene glycol pathway (VP-positive). A negative MR-VP test indicates that the organism does not produce acidic end products from glucose fermentation (MR-negative) and does not produce acetoin (VP-negative).

Therefore, if all three bacterial isolates (SKPH1, SKPH11, AND SKPH19) showed negative tests for MR-VP, this means that they are not acid fermenters but neutral fermenters. This

indicates that the isolates do not produce significant amounts of acidic end products from glucose fermentation, and they do not produce acetoin.

Neutral fermentation is a type of fermentation that produces neutral end products such as ethanol, lactate, or succinate, rather than acidic or basic end products. This type of fermentation is commonly used by bacteria that inhabit environments where acidic or basic end products would be harmful.

4.3.6 Citrate Test

The observation suggests that all three of the bacterial isolates (SKPH1, SKPH11, and SKPH19) were capable of utilising citrate as a carbon source and produced ammonia as a result. The change in color from green to blue in the citrate agar slants indicated an increase in pH, which suggests that the bacteria have the enzyme citrate lyase, which breaks down citrate into oxaloacetate and acetyl-CoA. The acetyl-CoA is then used in the TCA cycle to generate energy, while the ammonia produced can be used by the bacteria as a source of nitrogen. However, it is important to note that additional tests may be necessary to confirm the identity and characteristics of the bacterial isolates.

4.3.7 Sulfide Indole Motility Test (SIM test)

Based on the results of the tests, it can be concluded that all three isolates were non-motile and did not produce hydrogen sulfide or indole. The absence of black precipitation in the medium used for the sulfide test indicates that the isolates did not produce hydrogen sulfide. The lack of radial outward growth in the motility test indicated that the isolates are non-motile, meaning they were incapable of self-movement via flagella or other means.

The inability to produce indole suggested that the isolates lack the tryptophanase enzyme that is responsible for the breakdown of tryptophan into indole.

Further identification of the isolates may be necessary using other biochemical tests or molecular methods, such as 16S rRNA sequencing, to determine their exact taxonomic classification.

Table 1. Morphological and Biochemical analysis for the Bacterial isolates SKPH1, SKPH11, and SKPH19.

Bacterial isolates	Gram character	Catalase	Oxidase	MR	VP	Citrate	SIM
SKPH1	N	P	P	N	N	P	N
SKPH11	P	P	P	N	N	P	N
SKPH19	N	P	P	N	N	P	N

N= Negative and P= Positive

4.3.8 Carbohydrate utilization profile of bacterial isolates

Table 2. Carbohydrate utilization profile for bacterial isolates SKPH1, SKPH11, SKPH19

Carbohydrate Source		SKPH1	SKPH11	SKPH19
Lactose	Part A	N	N	N
Xylose		P	N	P
Maltose		N	P	N
Fructose		N	P	N
Dextrose		N	P	N
Galactose		P	P	P
Raffinose		N	P	N
Trehalose		N	P	N
Melibiose		N	P	N
Sucrose		N	P	N
L-Arabinose		P	N	P
Mannose		N	N	N
Inulin	Part B	N	P	N
Sodium gluconate		N	N	N
Glycerol		P	N	P
Salicin		N	N	P

Dulcitol		**N**	**N**	**N**
Inositol		**N**	**N**	**N**
Sorbitol		**N**	**N**	**N**
Mannitol		**N**	**N**	**N**
Adonitol		**N**	**N**	**N**
Arabitol		**N**	**N**	**N**
Erythritol		**N**	**N**	**N**
alpha-Methyl-D-glucoside		**N**	**N**	**N**
Rhamnose	**Part C**	**N**	**N**	**N**
Cellobiose		**N**	**N**	**N**
Melezitose		**N**	**N**	**N**
α-Methyl-D-Mannoside		**N**	**N**	**N**
Xylitol		**N**	**N**	**N**
ONPG		**N**	**P**	**N**
Esculin		**P**	**P**	**P**
D-arabinose		**N**	**N**	**N**
Citrate		**P**	**P**	**P**

Malonate		**N**	**N**	**N**
Sorbose		**N**	**N**	**N**

N=Negative, P=Positive

The carbohydrate utilization profile of each isolate varied considerably. Each isolate has a unique profile, indicating that they may have different metabolic capabilities and preferences.

Isolate SKPH1 can utilize xylose, galactose, L-arabinose, glycerol, esculin, and citrate. Xylose, galactose, and L-arabinose are monosaccharides, while glycerol, Esculin, and citrate are not carbohydrates but can still be utilized by some microorganisms.

Isolate SKPH11, on the other hand, has a more extensive profile, including maltose, fructose, dextrose, raffinose, trehalose, melibiose, sucrose, insulin, O.N.P.G, esculin, and citrate. This isolate can utilize various sugars, including disaccharides such as maltose, sucrose, and melibiose, and even some oligosaccharides like raffinose.

Isolate SKPH19 has a more limited profile than SKPH11 but shares some similarities with SKPH1, as it can also utilize xylose, galactose, and L-arabinose, as well as glycerol. Additionally, it can utilize salicin, which is another non-carbohydrate compound that can be metabolized by some microorganisms.

It is interesting to note that all three isolates can utilize galactose, esculin, and citrate, suggesting that these compounds may be important for their growth and survival.

Figure 3. Carbohydrate utilization test strips after 24 hours of incubation.

4.3.9 Antibiotic Susceptibility test (Kirby Bauer method of agar disc diffusion)

An antibiotic susceptibility test was performed to understand the susceptibility of the bacterial cultures used. The plates were examined for the presence of a zone of inhibition after 24 hours of incubation at 30°C. The diameter of the inhibitory zone includes the diameter of the disc. The SKPH1 isolate was susceptible to Levofloxacin, Tetracycline, Tobramycin, Neomycin, Ofloxacin, Amikacin but resistant to Cotrimoxazole, Nitrofurantoin, Amoxyclav Cephalexin, Cephotaxime, Oxallin, and Clindamycin.

Isolate SKPH11 was susceptible to Cotrimoxazole, Levofloxacin, Nitrofurantoin, Tetracycline, Cephalexin, Oxallin, Tobramycin, Neomycin, Ofloxacin, Clindamycin, and Amikacin but resistant to Amoxyclav, Cephotaxime and isolate.

Isolate SKPH19 was susceptible to Levofloxacin, Tetracycline, Tobramycin, Ofloxacin, Neomycin, and Amikacin but resistant to Cotrimoxazole, Nitrofurantoin, Amoxyclav, Cephalexin, Cephotaxime, Oxallin, and Clindamycin.

Table 3. Antibiotic Susceptibility test for bacterial isolates SKPH1, SKPH11, SKPH19. Diameter of zone of inhibition showing antibiotic sensitivity in mm. Antibiotic disc diameter = 6mm

Antibiotic	**SKPH1**	**SKPH11**	**SKPH19**
Cotrimoxazole Co^{25}	R	S (24 mm)	Resistant
Levofloxacin Le^{5}	S (36 mm)	S (22 mm)	S (35 mm)
Nitrofurantoin Nf^{100}	R	S (18 mm)	R
Amoxyclav Ac^{30}	R	R	R

Tetracycline T^{30}	S (24 mm)	S (20 mm)	S (22 mm)
Cephalexin Cp^{30}	R	S (23 mm)	R
Cephotaxime Ce^{30}	R	R	R
Oxallin Ox^{1}	R	S (22 mm)	R
Tobramycin Tb^{10}	S (24 mm)	S (20mm)	S (24 mm)
Neomycin N^{30}	S (22 mm)	S (25 mm)	S (20mm)
Ofloxacin Of^{5}	S (38 mm)	S (24 mm)	S (36 mm)
Clindamycin Cd^{2}	R	S (20 mm)	R
Amikacin Ak^{30}	S (25 mm)	S (20 mm)	S (30)

S- Susceptible, R- Resistant

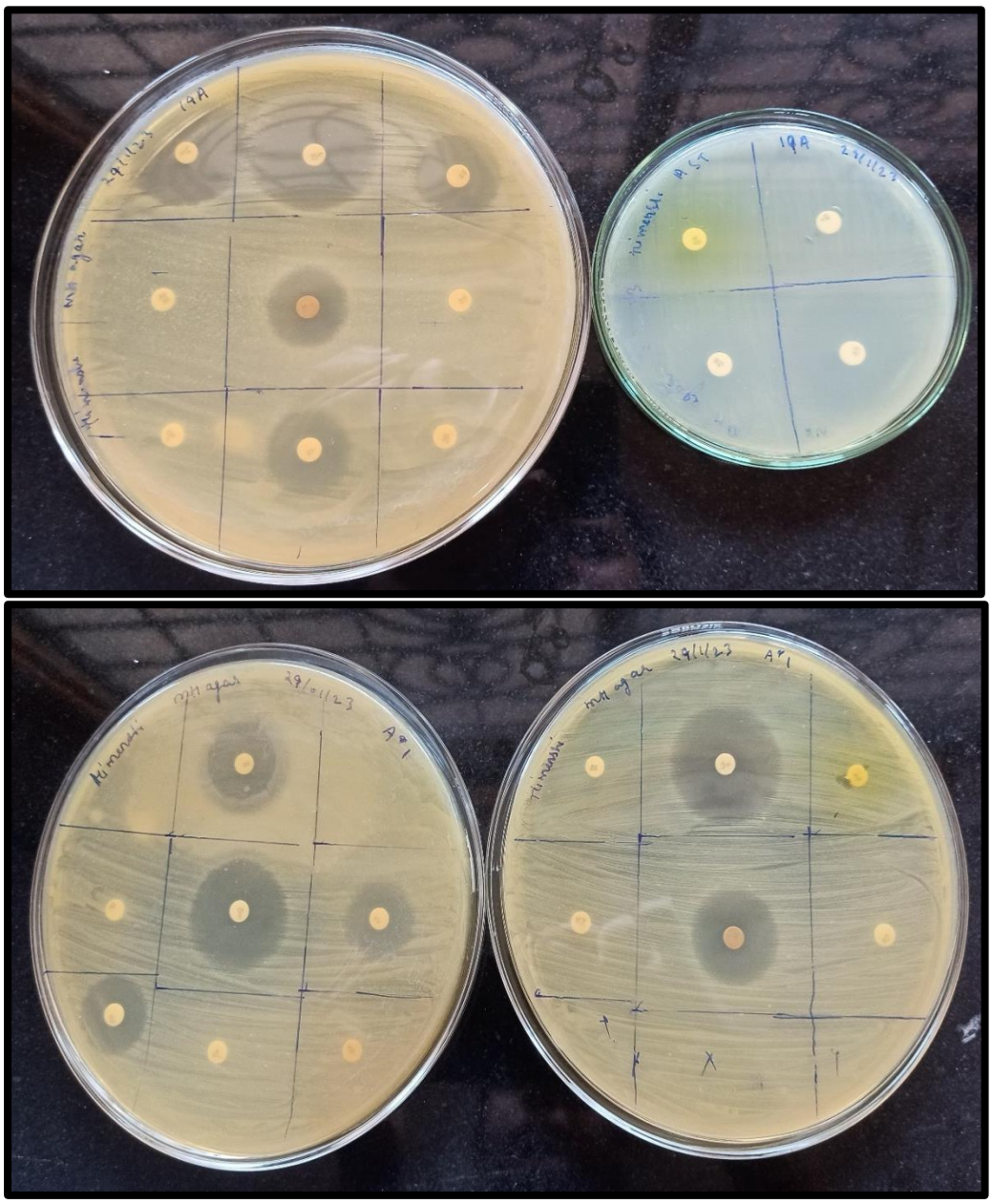


Figure 4. Antibiotic susceptibility test by disc diffusion method.

4.3.10 Scanning Electron Microscopy (SEM)

SEM can provide valuable information about the morphology and size of bacterial cells. Measuring the size of cells is important in understanding their structure and function.

The SKPH1 was found to be coccobacillus with a blunt centre and SKPH11 and SKPH19 were rods shaped.

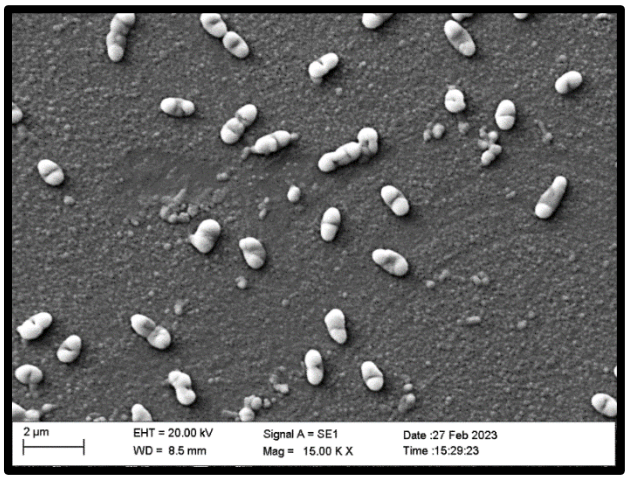


Figure 5a. SEM image of SKPH1

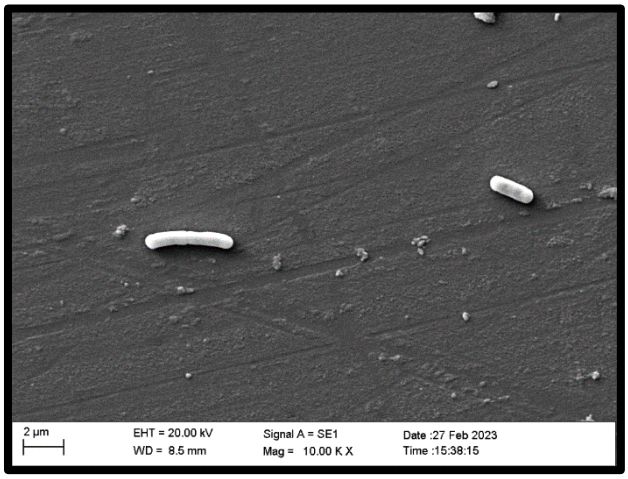


Figure 5b. SEM image of SKPH11

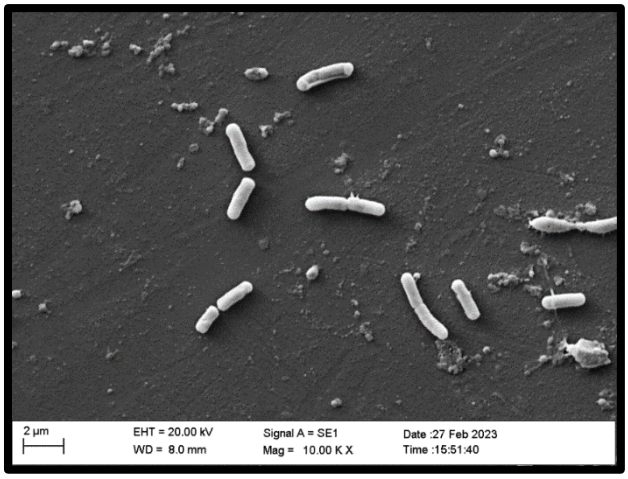


Figure 5c. SEM image of SKPH19

4.3.11 Hemolysis test

The blood agar plates with bacterial isolates (SKPH1, SKPH11, and SKPH19) streaked on them were incubated at 30°C and observed for alpha, beta, and gamma hemolysis after 24 hours of incubation. The absence of any color change or destruction of red blood cells around the bacterial isolate on the blood agar plates indicated that they are gamma-hemolytic, which suggests that they do not produce any hemolysins.

Table 4. Hemolysis activity of bacterial isolates SKPH1, SKPH11, SKPH19

Bacterial strain	**Type of Hemolysis**
SKPH1	Gamma
SKPH11	Gamma
SKPH19	Gamma

Figure 6. Hemolysis test for SKPH1, SKPH11, and SKPH19

4.4 Thiosulfate oxidation by SKPH1, SKPH11, and SKPH19

Thiosulfate is used by most of the sulfur-oxidizing bacteria whether chemo-lithotrophic bacteria or chemo-organotrophic bacteria. Oxidation of thiosulfate to sulfate denotes complete oxidation of reduced sulfur compounds including intermediates and thus equates with oxidation efficiency of the isolates (Petri et al. 2001).

The end product of thiosulfate oxidation is sulfate. The result for sulfate production was observed after 18 days. The results showed that SKPH1 produced the highest amount of sulfate while SKPH11 and SKPH19 produced less (Figure 7). This suggests that SKPH1 is more effective at converting thiosulfate to sulfate. The fact that the amount of sulfate formed in the test cultures was higher than in the negative control indicates that the increase in sulfate production is due to the biological activity of the SOB cultures. Longer incubation at 30˚C could result in an even greater conversion of thiosulfate to sulfate due to the activity of SOBs.

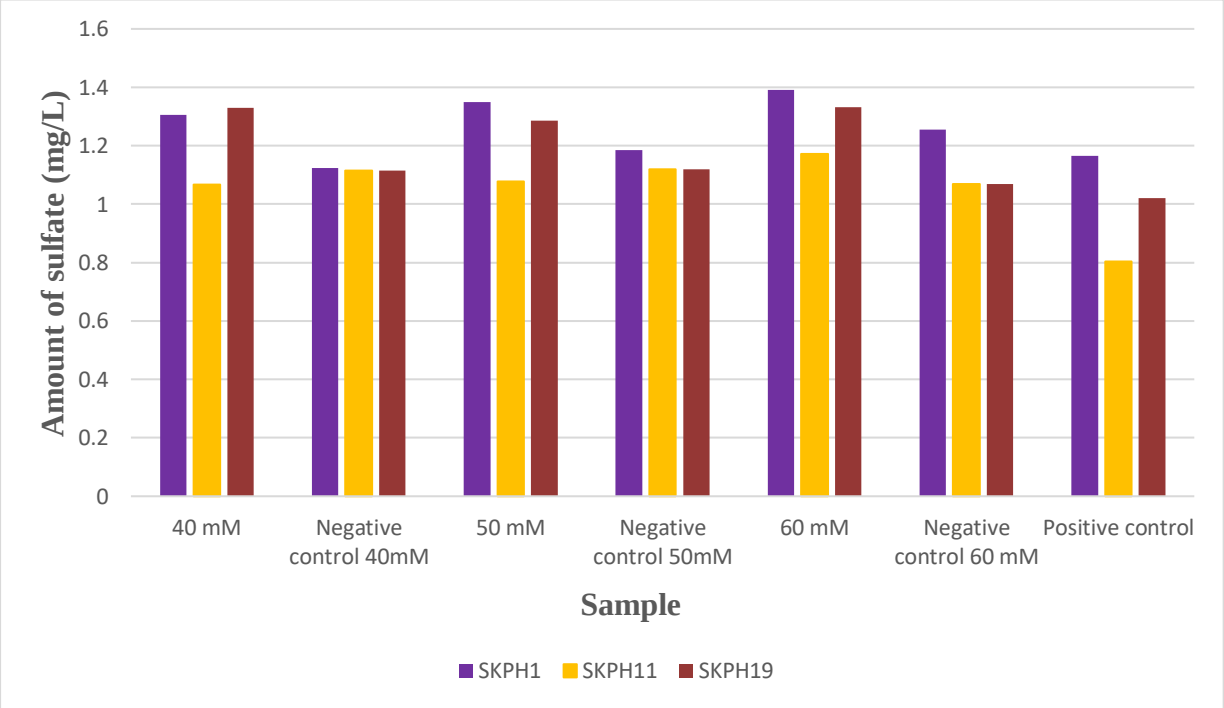


Figure 7. Sulfate produced by bacterial isolates SKPH1, SKPH11, and SKPH19

4.5 Sulfide oxidation by SKPH1, SKPH11, and SKPH19

The bacterial isolates SKPH1 and SKPH19 could oxidize all the sulfide in the medium, while SKPH11 could oxidize small amounts of sulfide in each of the tested concentrations (100 ppm, 200 ppm, and 300 ppm) of sulfide in the medium (Figure 8a).

These results suggested that SKPH1 and SKPH19 can utilize sulfide as a substrate for growth and convert it to sulfate, whereas SKPH11 may not be using sulfide as efficiently compared to the other two bacterial isolates. In positive controls only thiosulfate was used as substrate therefore no sulfide was produced.

Peighamiet al. (2022) screened the strength of seven different SOBs at various sulfide concentrations and reported *T. versutus and T. aerophilum* were the most effective strains since they can completely eliminate sulfide from the environment.

Many aquaculture practices use SOB as a probiotic to remove sulfide, which is widely used in wastewater treatment. Moreover, the use of many commercial probiotic products lacks accuracy owing to their misidentification as per the reports (Huys et al. 2006; Tuan et al. 2013; Amenyogbe et al. 2020). *Enterobacter ludwigii* HS1-SOB, *Pseudomonas stutzeri* B6-SOB, and *Cytobacillus firmus* C8-SOB are potent probiotic isolates with the highest oxidation efficiency. (Shinde, et al. 2022). When bacterial species are used in bioremediation techniques, native bacteria outperform allochthonous species in terms of sustenance and environmental adaptability (Li et al. 2013; Ortiz et al. 2015). Therefore, for sulfide removal in the specific environmental remediation, locally originating sulfur-oxidizing bacteria are preferable (Zhao et al. 2016).

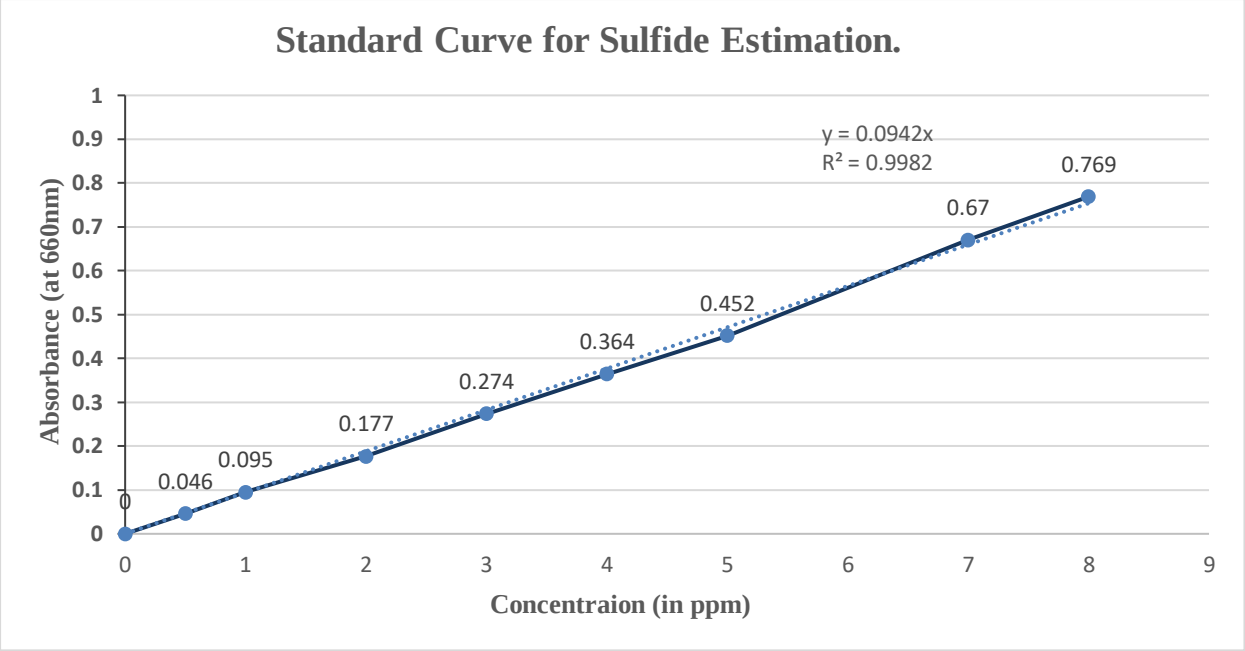


Figure 8b. Standard curve for sulfide.

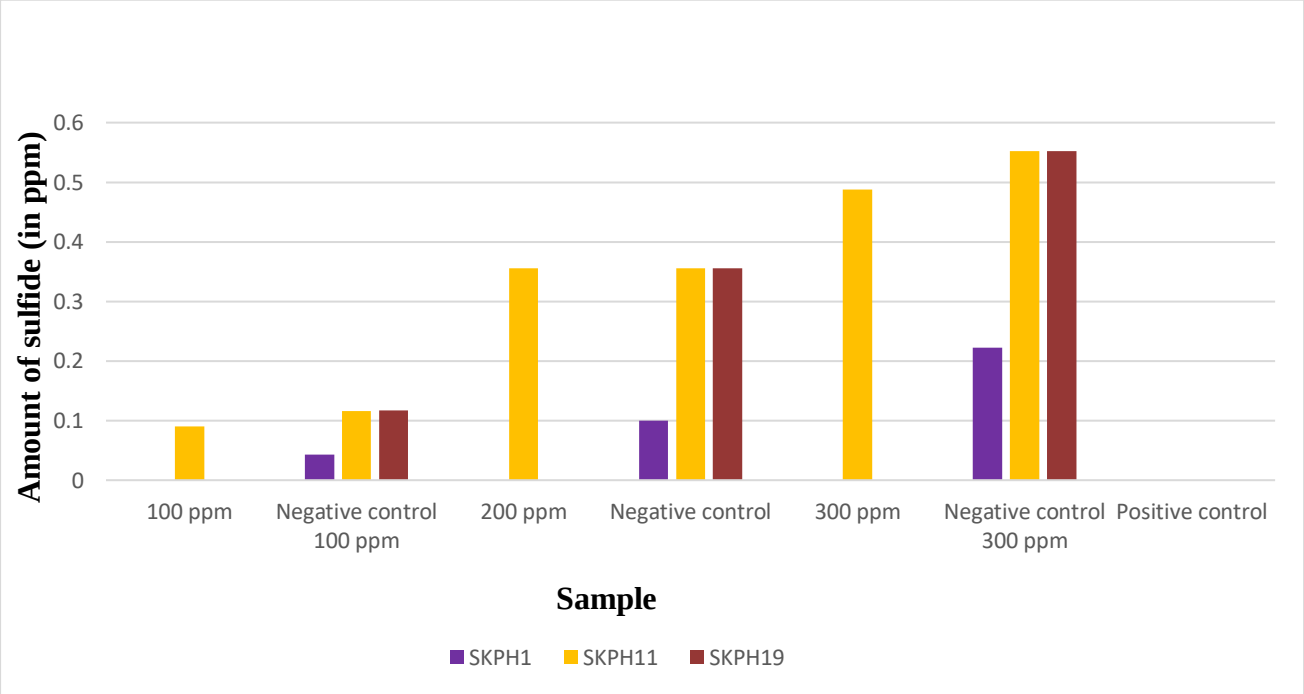


Figure 8a. Residual sulfide after oxidation by bacterial isolates SKPH1, SKPH11, and SKPH19.

4.6 Polymerase chain reaction for *soxB* gene

PCR was done targeting the *soxB* gene in bacterial isolate SKPH1 since it showed the highest efficiency in converting thiosulfate to sulfate. After amplifying the DNA using gel electrophoresis, the PCR products were visualized under UV light. The encircled band (as shown in Figure 9) was identified as the amplified product of the *soxB* gene with an approximate size of 1200 bp. Non-specific amplification of DNA was also observed, which could be due to non-specific primer binding or suboptimal PCR conditions.

The literature suggests that the expected size of the *soxB* PCR product varies between 800-1200 base pairs (bp) for most thiosulfate oxidizing bacteria (Meyer et al. 2007). The size of the encircled band in Lane 1 (Figure 9) was estimated by comparing it to a DNA ladder, which typically contains known DNA fragments of different sizes. Lane 2 contained a 1000 bp DNA ladder and Lane 3 contained a 500 bp DNA ladder (Figure 9).

The oxidative sulfur cycle is a series of chemical reactions that occur in nature and involve the oxidation of reduced sulfur compounds. The availability of reduced sulfur compounds for the oxidative sulfur cycle can come from two main sources: the activity of sulfate-reducing bacteria, which reduce sulfate to sulfide, and geological sources.

Ralf et al. (2000) studied phylogeny and distribution of the *soxB* gene among thiosulfate-oxidizing bacteria and they found that the PCR yielded a *soxB* fragment of approximately 1000 bp from most of the bacteria.

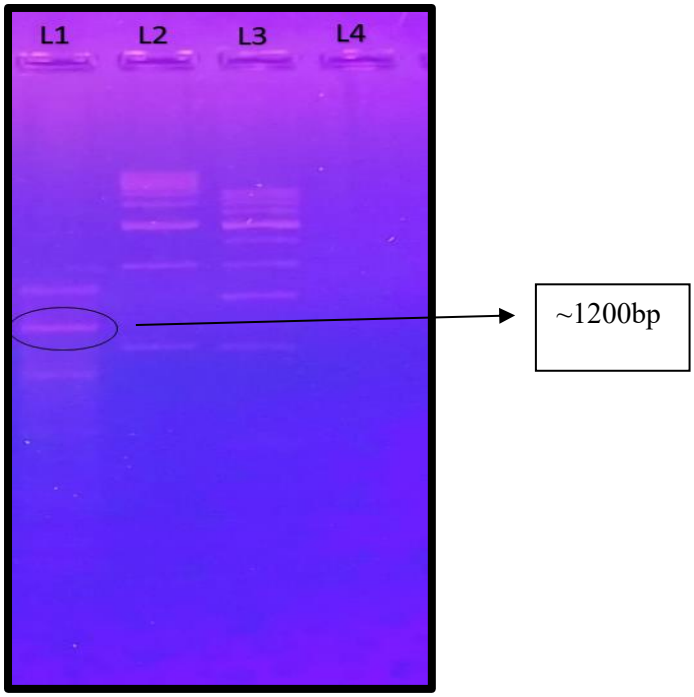


Figure 9. Gel electrophoresis image after PCR amplification of *soxB* gene for bacterial isolate SKPH1.

SUMMARY

5. SUMMARY

- Three bacterial isolates, SKPH1, SKPH11, and SKPH19, were evaluated for their ability to perform biodesulfurization.
- The isolates were grown in nutrient-rich media and then transferred to Lieske's broth supplemented with thiosulfate to determine their efficiency in converting thiosulfate to sulfate.
- The Gram character of the isolates was determined using gram staining and KOH tests and the two isolates (SKPH1 and SKPH19) were found to be Gram-negative and one isolate (SKPH11) was Gram-positive.
- Morphological and biochemical analyses were conducted to characterize the isolates, including catalase, oxidase, MR-VP, SIM, antibiotic susceptibility, and carbohydrate utilization.
- Scanning electron microscopy suggested that SKPH1 is a coccobacillus with a blunt centre and SKPH11, and SKPH19 were rod-shape shaped bacteria.
- All three isolates showed gamma hemolysis with no destruction of red blood cells.
- The isolates' thiosulfate and sulfide oxidation efficiencies were measured, and it was found that SKPH1 was the most efficient in oxidizing thiosulfate and sulfide, followed by SKPH19 and then SKPH11.
- PCR targeting the *soxB* gene was performed on bacterial isolate on bacterial isolate SKPH1 which yielded a *soxB* gene of approximately 1200 bp.

CONCLUSION

6. CONCLUSION

- The bacterial isolates SKPH1 and SKPH19 were gram-negative bacteria and SKPH11 was Gram-positive with unique characteristics that make them potential candidates for use in bioremediation of sulfide from aquatic systems.
- The isolate SKPH1 was more effective in oxidizing thiosulfate and sulfide followed by SKPH19 and SKPH11.

FUTURE PROSPECTS

7. FUTURE PROSPECTS

1. Use of these bacterial isolates in wastewater treatment plants to remove sulfur compounds from wastewater is a promising future prospect. The ability of these bacteria to convert thiosulfate and sulfide to sulfate with no apparent acidification in the medium can help reduce the environmental impact of wastewater.

2. These bacteria can also be used in environmental bioremediation processes to remove sulfur-containing pollutants from soil and water. This can help reduce the impact of pollutants on the environment and promote sustainability.

REFERENCES

8. REFERENCES

1. Anandham, R., Indiragandhi, P., Madhaiyan, M., Ryu, K. Y., Jee, H. J., & Sa, T. M. (2008). Chemolithoautotrophic oxidation of thiosulfate and phylogenetic distribution of sulfur oxidation gene (*sox*B) in rhizobacteria isolated from crop plants. *Research in Microbiology*, *159*(9-10), 579-589.
2. Barton, L. L., Fardeau, M. L., & Fauque, G. D. (2014). Hydrogen sulfide: a toxic gas produced by dissimilatory sulfate and sulfur reduction and consumed by microbial oxidation. *The metal-driven biogeochemistry of gaseous compounds in the environment*, 237-277.
3. Cheng, Y., Yuan, T., Deng, Y., Lin, C., Zhou, J., Lei, Z., ... & Zhang, Z. (2018). Use of sulfur-oxidizing bacteria enriched from sewage sludge to biologically remove H_2S from biogas at an industrial-scale biogas plant. *Bioresource Technology Reports*, *3*, 43-50.
4. Chung, Y. C., Huang, C., & Tseng, C. P. (2001). Biological elimination of H_2S and NH_3 from waste gases by biofilter packed with immobilized heterotrophic bacteria. *Chemosphere*, *43*(8), 1043-1050.
5. Chung, Ying-Chien, Chihpin Huang, and Ching-Ping Tseng. "Operation optimization of Thiobacillus thioparus CH11 biofilter for hydrogen sulfide removal." *Journal of biotechnology* 52.1 (1996): 31-38.
6. De Rink, R., Gupta, S., de Carolis, F. P., Liu, D., ter Heijne, A., Klok, J. B., & Buisman, C. J. (2021). Effect of process conditions on the performance of a dual-reactor biodesulfurization process. *Journal of Environmental Chemical Engineering*, *9*(6), 106450.
7. De Rink, R., Klok, J. B., van Heeringen, G. J., Keesman, K. J., Janssen, A. J., Ter Heijne, A., & Buisman, C. J. (2020). Biologically enhanced hydrogen sulfide

absorption from sour gas under haloalkaline conditions. *Journal of hazardous materials*, *383*, 121104.

8. Dobbins, J. J. (2010). Prescott's microbiology. (Book)
9. El Brahmi, A., & Abderafi, S. (2021). Hydrogen sulfide removal from wastewater using hydrogen peroxide in-situ treatment: Case study of Moroccan urban sewers. *Materials Today: Proceedings*, *45*, 7424-7427.
10. Hou, N., Xia, Y., Wang, X., Liu, H., Liu, H., & Xun, L. (2018). H 2 S biotreatment with sulfide-oxidizing heterotrophic bacteria. *Biodegradation*, *29*, 511-524.
11. Jannasch, H. W. (1983). Microbial processes at deep sea hydrothermal vents. *Hydrothermal processes at seafloor spreading centers*, 677-709.
12. Jin, Z., & Pei, Y. (2015). Physiological implications of hydrogen sulfide in plants: pleasant exploration behind its unpleasant odour. *Oxidative Medicine and Cellular Longevity*, *2015*.
13. Kim, H., Kim, Y. J., Chung, J. S., & Xie, Q. (2002). Long-term operation of a biofilter for simultaneous removal of H_2S and NH_3. *Journal of the Air & Waste Management Association*, *52*(12), 1389-1398.
14. Lee, E. Y., Cho, K. S., & Ryu, H. W. (2005). Simultaneous removal of H_2S and NH_3 in biofilter inoculated with *Acidithiobacillus thiooxidans* TAS. *Journal of bioscience and bioengineering*, *99*(6), 611-615.
15. Lim, E., Mbowe, O., Lee, A. S., & Davis, J. (2016). Effect of environmental exposure to hydrogen sulfide on central nervous system and respiratory function: a systematic review of human studies. *International journal of occupational and environmental health*, *22*(1), 80-90.
16. Madigan, M. T., Clark, D. P., Stahl, D., & Martinko, J. M. (2010). *Brock biology of microorganisms 13th edition*. Benjamin Cummings. (Book)

17. Meyer, B., Imhoff, J. F., & Kuever, J. (2007). Molecular analysis of the distribution and phylogeny of the soxB gene among sulfur-oxidizing bacteria–evolution of the Sox sulfur oxidation enzyme system. *Environmental Microbiology*, *9*(12), 2957-2977.
18. Montebello, A. M., Bezerra, T., Rovira, R., Rago, L., Lafuente, J., Gamisans, X., ... & Gabriel, D. (2013). Operational aspects, pH transition and microbial shifts of a H_2S desulfurizing biotrickling filter with random packing material. *Chemosphere*, *93*(11), 2675-2682.
19. National Research Council. (2002). *Review of submarine escape action levels for selected chemicals*. National Academies Press. (Book)
20. Nazari, F., Kefayati, M., & Raheb, J. (2017). Isolation, identification, and characterization of a novel chemolithoautotrophic bacterium with high potential in biodesulfurization of natural or industrial gasses and biogas. *Energy Sources, Part A: Recovery, Utilization, and Environmental Effects,* 39(10), 971–977.
21. Okabe, S., Odagiri, M., Ito, T., & Satoh, H. (2007). Succession of sulfur-oxidizing bacteria in the microbial community on corroding concrete in sewer systems. *Applied and environmental microbiology*, *73*(3), 971-980.
22. Peighami, R., Rasekh, B., Motamedian, E., Yazdian, F., & Khodaverdi, H. (2022). Investigating the ability of sulfur oxidizing strains in biodesulfurisation of sulfide-containing streams, screening the most capable strain and determining the optimum condition for elemental sulfur recovery from sulfide using response surface method. *Fuel*, *309*, 121985.
23. Petri, R., Podgorsek, L., & Imhoff, J. F. (2001). Phylogeny and distribution of the *sox*B gene among thiosulfate-oxidizing bacteria. *FEMS Microbiology Letters*, *197*(2), 171-178.

24. Promnuan, K., & Sompong, O. (2017). Efficiency evaluation of biofilter for hydrogen sulfide removal from palm Oil mill biogas. *Energy Procedia*, *138*, 564-568.

25. Pudi, A., Rezaei, M., Signorini, V., Andersson, M. P., Baschetti, M. G., & Mansouri, S. S. (2022). Hydrogen sulfide capture and removal technologies: A comprehensive review of recent developments and emerging trends. *Separation and Purification Technology*, 121448.

26. Rameez, M. J., Pyne, P., Mandal, S., Chatterjee, S., Alam, M., Bhattacharya, S., ... & Ghosh, W. (2020). Two pathways for thiosulfate oxidation in the alphaproteobacterial chemolithotroph Paracoccus thiocyanatus SST. *Microbiological research*, *230*, 126345.

27. Saeedi, A., Najibi, A., & Mohammadi-Bardbori, A. (2015). Effects of long-term exposure to hydrogen sulfide on human red blood cells. *The International Journal of Occupational and Environmental Medicine*, *6*(1), 20.

28. Shinde, A. H., Sharma, A., Doshi, S., Kumar, M. A., & Haldar, S. (2022). Isolation and screening of sulfur-oxidizing bacteria from coast of Bhavnagar, India, and formulation of consortium for bioremediation. *Environmental Science and Pollution Research*, *29*(36), 54136-54149.

29. Ter Heijne, A., de Rink, R., Liu, D., Klok, J. B., & Buisman, C. J. (2018). Bacteria as an electron shuttle for sulfide oxidation. *Environmental science & technology letters*, *5*(8), 495-499.

30. Xia, Y., Lü, C., Hou, N., Xin, Y., Liu, J., Liu, H., & Xun, L. (2017). Sulfide production and oxidation by heterotrophic bacteria under aerobic conditions. *The ISME journal*, *11*(12), 2754-2766.

31. Xia, Y., Lü, C., Hou, N., Xin, Y., Liu, J., Liu, H., & Xun, L. (2017). Sulfide production and oxidation by heterotrophic bacteria under aerobic conditions. *The ISME journal*, *11*(12), 2754-2766.
32. Zhang, J., Liu, R., Xi, S., Cai, R., Zhang, X., & Sun, C. (2020). A novel bacterial thiosulfate oxidation pathway provides a new clue about the formation of zero-valent sulfur in deep sea. *The ISME Journal*, *14*(9), 2261-2274.

APPENDIX

9. APPENDIX

- **Media composition**

1) Lieske's media composition:

Name of composition	**Amount (per litre)**
Filtered Crude salt	31 grams
Na2S2O3	5 grams
KNO3	5 grams
K2HPO4	0.2 gram
MgCl2.6H2O	0.1 gram
NH4Cl	2 grams
CaCl2.6H2O	10 milligrams
FeCl3.6H2O	10 milligrams
Distilled water	1000 ml
Agar	2 %

- **Last minute addition for 200 ml**

Name of composition	Amount (per 200ml)	Concentration
NaHCO3	2 ml	1gm in 10 ml
Vitamin solution	0.05 ml or 2 drops	
Na_2S (freshly prepared)	0.5	5%

- **Vitamin solution composition:**

Name of composition	Amount (in mg) per liter
Calcium pantothenate	100
Pyridoxine HCl	40
Thiamine HCl	20
Riboflavin	20
Nicotinamide	20
Biotin	0.001

Note: Na_2S and Vitamin solution are filter sterilized separately. No need to add Na_2S if adding Na_2S_2O3 in the media.

2) Nutrient broth composition

Name of composition	Amount Gms / Litre
Peptone 10.000	5.000
Sodium chloride	5.000
Beef extract	1.500
Yeast extract	1.500

3) **MR-VP broth composition**

- Preparation of MR/VP broth

Name of Composition	Amount per litre
Peptone	7.0 grams
Glucose	5.0 grams
Dipotassium phosphate	5.0 grams
Distilled water	1000 ml
Final pH	6.9

Dissolve the ingredients, distribute in 5 ml quantities in screw cap bottles, and autoclave at 121°C for 20 minutes.

4) Simmons citrate agar composition

Name of the composition	Amount in gms/liter
Ammonium dihydrogen phosphate	1.000
Magnesium sulfate	0.200
Dipotassium phosphate	1.000
Sodium citrate	2.000
Sodium chloride	5.000
Bromo thymol blue	0.080
Agar	15.000

Final pH (at 25°C) 6.8±0.1

- **Reagents used**

1) 3% KOH- 3 grams of KOH was weighed and mixed in100 ml of distilled water.

2) Preparation of MR reagent

Name of composition	Amount
Methyl red	0.1 gram
Ethyl alcohol	300 ml
Distilled water	200 ml

Dissolve methyl red in Alcohol first and then add distilled water. Store in a brown bottle at 4°C

3) VP reagents (alpha-naphthol and 40% potassium hydroxide)

- Preparation of VP reagent

VP reagent A

Name of composition	Amount
Alpha naphthol	5 grams
Ethyl alhocol	100 ml

Dissolve alpha naphthol in a small amount of alcohol first and then add the remaining alcohol to 100ml. Store in a brown bottle at 4°C.

VP reagent B

Name of composition	Amount
Potassium hydroxide	40 grams
Distilled water	100 ml

Cool the volumetric flask in cold water with 80 ml of water, add KOH, dissolve and make up to 100 ml, and store in polyethylene bottles at 4°C.

4) **Conditioning reagent:**

150gm of NaCl salt, 100 ml of glycerol, 60 ml of concentrated HCl, and 200 ml of 95% ethanol and make up the volume to 1L with deionized water.

5) **30% $BaCl_2$ solution:**

Add 30 grams of $BaCl_2$ in 100 ml of distilled water.

6) **Solution A**

Add 2 grams of DMPD in 200 ml of distilled water to this add 200 ml of concentrated H_2SO_4 and make up the final volume 1 litres.

7) **Solution B**

Add 10 grams of Ammonium iron (III) sulfate into 50 ml of distilled water and add 2 ml of sulfuric acid and make up the final volume to 1000 ml.