

**USE OF SULFATE-REDUCING BACTERIA FOR
THE PRECIPITATION OF HEAVY METAL
CADMIUM FROM SOLUTION**

BY

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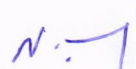
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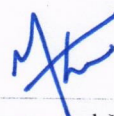
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Dedication

To my parents, Late Usman Ahmad Maidoki, Haj Balaraba Binta Musa, and Alh Yusuf

Kabir.

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LIST OF ABBREVIATION

Cd	:	Cadmium
COD	:	Chemical Oxygen Demand
EDS	:	Energy Dispersive X-ray Spectroscopy
EPA	:	Environmental protection agency
GO	:	Graphene oxide
ICP-OES:		Induced-coupled plasma optical emission spectroscopy
Ni-Cd	:	Nickel-cadmium
rGO	:	Reduced Graphene oxide
SEM	:	Scanning electron microscope
SRB	:	Sulfur-reducing bacteria
XRD	:	X-ray Diffraction analysis

ABSTRACT

Full Name : Ahmad Rufa'i Usman
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Contamination of aquatic environments with heavy metals poses significant global concerns, threatening human health and environmental stability. As such, developing new, sustainable, and environmentally friendly remediation methods for heavy metal pollution is crucial. Using sulfur-reducing bacteria (SRB) for the biological removal of heavy metals presents a holistic approach that is environmentally compatible, cost-effective, selective, and stable over the long term. Cadmium, a toxic heavy metal, significantly threatens ecosystems and human health. This study assesses the ability of SRB to facilitate the formation of cadmium sulfide (CdS) precipitates, which are easily recoverable. Our experiments involved the systematic optimization of conditions for SRB through the integration of graphene oxide (GO). Initially, the SRB demonstrated the capability to precipitate cadmium with an efficiency of up to 59%. Further investigations explored the concentration of cadmium that inhibits bacterial growth. In the final experimental phase, the use of GO was studied, revealing that GO enhanced the cadmium precipitation rate, increasing removal efficiency to 89% within just 5 days—significantly faster than the 25 days required under standard conditions without GO. These findings provide crucial insights into the potential of SRB in mitigating cadmium pollution in water systems, highlighting the significant enhancements brought by GO integration, and underscore the potential scalability of this bioremediation technology for industrial applications.

ملخص الرسالة

الاسم الكامل: أحمد رفاعى عثمان

عنوان الرسالة: استخدام البكتيريا المختزلة للكبريتات لترسيب المعادن الثقيلة من الكادميوم من المحلول

التخصص: علوم الحياة

تاريخ الدرجة ديسمبر 2024

تُعد تلوث المعادن الثقيلة في البيئات المائية مشكلة عالمية كبيرة، تشكل تهديدات خطيرة لصحة الإنسان والبيئة. وعليه، فإن تطوير طرق جديدة ومستدامة وصديقة للبيئة لتنقية تلوث المعادن الثقيلة أمر حيوي. يقدم العملية البيولوجية لإزالة المعادن الثقيلة، خاصة باستخدام البكتيريا المختزلة للكبريتات (SRB)، نهجاً شاملاً يتميز بالتوافق البيئي، وفعالية التكلفة، والانتقائية، والاستقرار طويل الأجل. يُشكل الكادميوم، وهو معدن ثقيل سام، تهديداً كبيراً للنظم البيئية وصحة الإنسان. تقيم هذه الدراسة قدرة الـ SRB على تسهيل تكوين رواسب كبريتيد الكادميوم (CdS)، والتي يسهل استردادها. شملت تجاربنا تحسين الظروف المنهجية لـ SRB من خلال دمج أكسيد الجرافين (GO) في البداية، أظهرت الـ SRB قدرتها على ترسيب الكادميوم بكفاءة تصل إلى 59%. وقد تناولت الأبحاث الإضافية التركيزات التي تعيق نمو البكتيريا. في المرحلة التجريبية النهائية، تم دراسة استخدام أكسيد الجرافين المخفف (rGO)، والذي كشف عن أن rGO قد عزز من معدل ترسيب الكادميوم، مما زاد من كفاءة الإزالة إلى 89% خلال خمسة أيام فقط — وهو أسرع بكثير من الـ 25 يوماً اللازمة تحت الظروف القياسية بدون GO. توفر هذه النتائج رؤى حاسمة حول إمكانيات الـ SRB في التخفيف من تلوث الكادميوم في أنظمة المياه، مما يبرز التحسينات الكبيرة التي أحدثتها دمج rGO ويؤكد على إمكانية تطبيق هذه التكنولوجيا البيولوجية على نطاق صناعي.

CHAPTER 1

INTRODUCTION

Urbanization and industrialization have led to the discharge of different heavy metals into the environment, causing significant environmental pollution [1]. Heavy metals, including cadmium, lead, arsenic, and mercury are non-biodegradable and tend to accumulate significantly in aquatic and terrestrial ecosystems, which can result in diseases and mortality among plants, animals, and humans through the food chain [2], [3], [4]. Heavy metal contamination constitutes a significant environmental concern that affects a substantial portion of global water resources [5]. According to scientific studies, approximately 40% of water bodies across the globe have been contaminated by heavy metals [6]. These toxic pollutants can originate from diverse sources, such as industrial waste, agricultural runoff, and natural weathering of rocks and soils. The average level of heavy metals in the surface water of specific regions significantly exceeds the thresholds set up for potable water safety [7]. Studies conducted in the coastal regions of the Saudi Arabian Gulf have revealed that this region is exposed to various anthropogenic pollutants, such as desalination reject water, steel forging, oil waste, mining, electroplating industries, and solid waste from new coastal construction and landfills [8].

1.1 Cadmium

Cadmium (Cd) is characterized by its toxicity and harmful effects, lacking any known physiological functions in living organisms [9], [10]. It is abundant in the earth's crust in combination with ores of zinc, copper, and lead [11]. Its widespread presence in large

quantities is mostly due to man-made activities, including electroplating, mining operations, plastic stabilization, alloy production, cement manufacturing, pigment production, battery manufacturing, fossil fuel combustion, excessive use of high-phosphate fertilizers, and the incineration of municipal waste and sewage sludge. [12], [13], [14]. Due to its chemical and physical properties, Cd is a highly sought-after material for industrial and consumer purposes. These properties include resistance to corrosion and chemicals, the ability to withstand elevated temperatures, low melting points, and remarkable electrical conductivity. It is highly suitable for incorporation into various products, including alloys, pigments, coatings, and stabilizers. Furthermore, it plays a crucial role as an essential element in rechargeable nickel-cadmium (Ni-Cd) batteries, which constitute its main uses[10].

Various natural processes are significant contributors to environmental contamination by Cd. These processes include rock weathering, volcanic eruptions, wind-borne dust, marine aerosols, forest fires, and erosion [15]. Plants readily absorb Cd from the soil, which leads to its accumulation in the food chain and increases the risk of human exposure to Cd [13]. One of the primary ways of exposure to Cd is through rice consumption [16]. Other harvests, including wheat, potatoes, and vegetables, also accumulate elevated levels of Cd when irrigated with contaminated water [12]. Tobacco plants accumulate considerable amounts of Cd in their leaves, therefore, smokers are at a higher risk [17]. The maximum limit of cadmium intake in drinking water and food as stipulated by the United States Environmental Protection Agency (EPA) is 0.5 µg/kg/day and 1µg/kg/day respectively [18]. Low levels of Cd exposure are associated with various toxic effects, including skeletal

and cardiovascular abnormalities, nephrotoxicity, hepatotoxicity, reproductive disorders in both males and females, teratogenicity, and carcinogenicity[10].

1.2 Removal of Heavy Metals by Sulphur-Reducing Bacteria (SRB)

Numerous physicochemical techniques including reverse osmosis, ion exchange, electrolysis, soil washing, thermal extraction, and coagulation-flocculation have been employed to extract heavy metals from solutions [19], [20]. These techniques effectively remove heavy metals but produce substantial amounts of sludge and are energy-intensive and expensive. Biological methods have several advantages over traditional processes, including high selectivity, cost-effectiveness, enhanced removal performance when treating effluent with low metal concentration, and a lack of secondary pollution [1], [21], [22]. Utilizing biogenic sulfides facilitated by SRB is an effective and appealing method for treating solutions containing heavy metals and sulfates. This enables the precipitation of heavy metals [23], [24], [25]. SRB constitutes a heterogeneous group of microorganisms that use sulfate as the terminal electron acceptor and simple compounds as electron donors [26]. They reduce sulfate to various sulfide forms in the absence of oxygen. This process leads to a reduction in soluble sulfate and the formation of insoluble metal sulfides, facilitating the removal of sulfate and heavy metals from wastewater [27], [28]. Metal sulfides precipitate as dense black sludge and can be recovered from water by sedimentation [28]. The resultant precipitates are more stable than the metal hydroxides formed using chemical techniques. The main drawback of using SRB-based treatment systems is the harmful effects of heavy metals on bacterial growth and activity [29]. Anaerobic environments have a prolonged initiation period and are vulnerable to disturbances caused by organic overload [30].

1.3 Enhancing Heavy Metal Precipitation by SRB

Various methods such as gel entrapment and biofilm formation have been employed to improve microbial activity and protect microorganisms from toxic molecules and adverse conditions. Sodium alginate and polyvinyl alcohol are frequently used as entrapment carriers for SRB [31], [32]. This immobilization enhances the tolerance of the SRB to elevated concentrations of heavy metals [33].

Graphene oxide (GO) has been shown to function as an electron shuffle, accelerating the reduction of some compounds with sulfide as a terminal electron acceptor [34]. Go and rGO have attracted significant attention in various research fields because of their exceptional mechanical strength, electron transport capability, large surface area, and ability to transform and degrade pollutants [4], [34], [35]. Microbial reduction is a cost-effective and green process that converts GO into rGO and produces graphene. This method is more economical and environmentally friendly than the chemical and physical methods [4]. Subsequently, rGO can be used as a framework to arrange the cells into complex structures. Graphene-based materials can significantly improve bioelectricity production, biological substance conversion, and contaminant breakdown by mediating redox reactions and facilitating electron transport [4], [36], [37].

1.4 Objectives of the Study

1. Determine whether SRB can precipitate Cd under normal conditions.
2. The cadmium salt was characterized using SEM, EDS, XRD, and ICP-OES.
3. Kinetic analysis of sulfate, and cadmium salt by ICP-OES
4. Assess the effect of graphene oxide on Cd precipitation.

CHAPTER 2

LITERATURE REVIEW

2.1 Biology and Ecology of SRB:

SRB constitute a diverse group of anaerobic prokaryotes that proliferate in both natural and controlled environments [38], [39]. They are found in marine [40] and freshwater sediments, wastewater treatment systems [41], coastal wetlands [42], underground pipelines, petroleum deposits [39] and mangrove sediments [43]. The sulfur and carbon cycles in marine sediments are significantly influenced by SRB, which are responsible for mineralizing more than half of the organic carbon in these environments [38], [39], [40], [44]. They are also involved in bioremediation of heavy metals from the ecosystem. They reduce sulfate to hydrogen sulfide, which causes pipeline corrosion [26]. The sulfide reacts with dissolved heavy metal ions to form stable metal sulfide precipitates [39], [45], which can be easily recovered.

2.1.1 Metabolic pathways

SRB utilizes oxidized sulfur compounds, including sulfate (SO_4^{2-}), thiosulfate ($\text{S}_2\text{O}_3^{2-}$), sulfite (SO_3^{2-}) and elemental sulfur (S^0) as final electron acceptors in their energy metabolism process [38], [39], [46], [47]. In addition to sulfur compounds, some SRB employ iron, nitrite, nitrate, and other compounds as electron acceptors [48]. Li and his colleagues provided a comprehensive review of electron acceptors and donors for SRB [39].

Sulfate reduction occurs through two pathways: dissimilatory sulfate reduction (DSR) and assimilatory sulfate reduction (ASR) [49]. ASR occurs in four steps: activation of sulfate

to form adenosine phosphosulfate (APS) in the presence of adenosine triphosphate (ATP). This is followed by the formation of 3'-phosphoadenosine-5'-phosphosulfate (PAPS). PAPS is converted to sulfite by PAPS reductase, and sulfite is converted to sulfide. DSR follows the same step without the formation of PAPS, but from APS to sulfite directly [49], [50], [51].

During ASR, sulfate is used primarily for the synthesis of sulfur-containing amino acids (from PAPS intermediate), while it serves as an energy source for ATP generation from the oxidation of organic compounds or hydrogen [50] and electron acceptors in DSR[51].

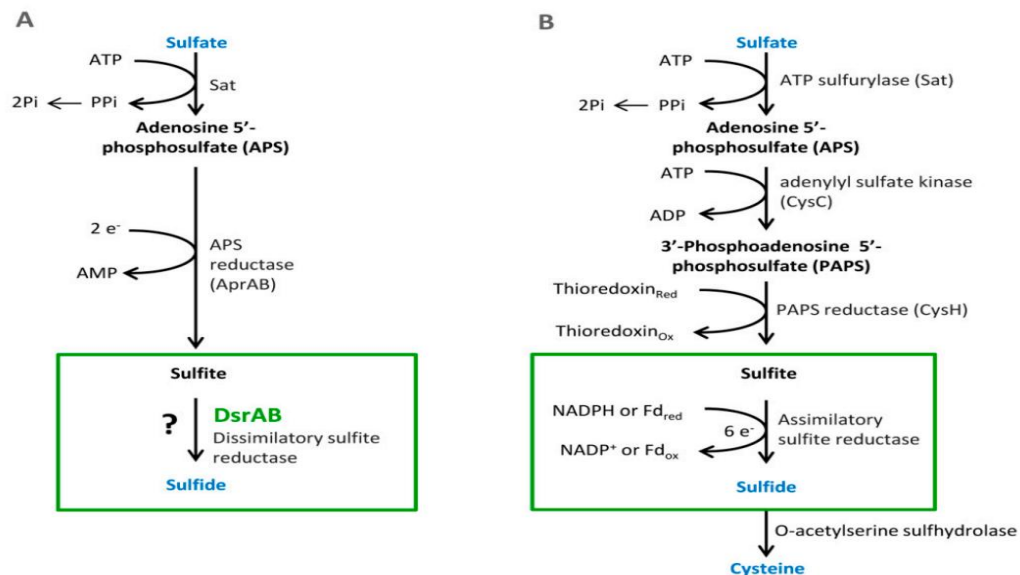


Figure 1. (A) DSR pathway (B) ASR pathway, from Kushkevych et al, 2020 [50].

2.2 Mechanisms of Cadmium Precipitation:

Immobilization of Cd by SRB in anoxic environment involves bio-absorption and chemical precipitation as the main mechanism. Under strict anaerobic conditions, SRB have been shown to have a good fixing effect on Cd, with an immobilization rate >90% under specific

conditions[52]. Wu et al. (2014) discovered that under specific conditions, a biological filter equipped with SRB could effectively remove > 99% of Cd^{2+} from wastewater. This further proves the potential of SRB-based filtration systems for treating industrial effluents containing heavy metals[53].

As previously mentioned, SRB reduces sulfate to sulfide under anaerobic conditions using organic compounds as electron donors [38], [39], [46], [47]. The sulfide ions produced react with Cd^{2+} in the environment to form cadmium sulfide (CdS) which precipitates due to its poor solubility[54], [55], [56].

This mechanism is employed in bioremediation technologies to treat heavy metal contamination in environments such as acid mine drainage and industrial effluents, transforming toxic cadmium into a stable, less bioavailable form[1], [47], [55], [57].



2.3 Factors Influencing SRB Activity:

The efficacy of biological treatment and its successful incorporation into wastewater treatment processes depends on the metabolic activity of SRB. Various environmental factors and conditions, such as temperature, pH, the electron donor, and the chemical oxygen demand to sulfate ratio ($\text{COD}/\text{SO}_4^{2-}$), play an important role in enabling SRB to thrive within anaerobic microbial communities [49].

2.3.1 pH

SRB are divided into neutrophilic and acidophilic groups based on their pH requirement for growth. Neutrophils grow in the range of 7.0 - 7.8 with optimal growth at 7.5[58]. Acidophils maintain metabolic activity between pH 2.0 - 4.0. The metabolic activity of SRB is inhibited above pH 9 and below pH 2[59]. The impact of pH on SRB can be studied from different viewpoints. To begin with, pH precisely affects SRB metabolism by disrupting cellular homeostasis. It also interferes with the internal-external pH gradient and leads to significant energy depletion. Furthermore, it indirectly influences SRB by altering the chemical states of substances like sulfides, organic acids, and heavy metals which affects SRB activity and interactions. Finally, pH plays a role in shaping the metabolic equilibrium among microorganisms in the environment, including acidogenic bacteria, which either coexist with or compete against SRB[49].

2.3.2 Temperature

SRB can thrive in a diverse range of temperature conditions; however, the majority are mesophilic, exhibiting optimal growth at 28-30 °C. [49]. Psychrophilic SRB grow within the temperature range of 7 – 18 °C while the thermophiles grow optimally between 65 - 70 °C, and extreme thermophilic SRB found in marine hydrothermal vents grows only above 80 °C [60].

Low temperature results in the solidification of cell membrane lipids and decreases membrane protein activity, inhibiting transport across the membrane[61]. Temperature influences the solubility of H₂S in wastewater, with elevated temperatures decreasing the solubility. This reduction in solubility subsequently diminishes the inhibitory effect of H₂S on SRB[49].

2.3.3 COD/SO₄²⁻ ratio

By controlling the ratio of accessible electron donors (COD) to sulfate acceptors, the COD/SO₄²⁻ ratio significantly affects SRB activity. Since electrons mostly flow toward sulfate, low ratios—typically below 1.0—favor SRB activity and lead to effective sulfate reduction. For example, maximal sulfate removal rates (up to 87%) under controlled settings were noted at a COD/SO₄²⁻ ratio of 0.8. Alternatively, excess COD can cause byproducts like H₂S to accumulate at greater ratios (>1.5), which can impair system performance[62], [63]. This relationship is made simpler in SRB-only systems due to the lack of competition, highlighting the importance of the COD/SO₄²⁻ ratio in preserving efficient SRB metabolism and maximizing sulfate reduction kinetics. Low ratios maximize SRB development and activity while reducing sulfide toxicity[49].

2.4 Properties of GO:

GO exhibits exceptional chemical and physical properties, making it a promising material for diverse applications. It has a large theoretical surface area, providing numerous binding sites for heavy metal ions [64]. This high surface area, combined with abundant oxygen-containing functional groups such as -COOH, -OH, and -CHO, enables efficient adsorption of heavy metal ions through interactions between these hydrophilic groups and metal cations [64]. The electrical conductivity of GO is a key property that can be influenced by various factors. The reduction of GO to rGO significantly increases its electrical conductivity by removing oxygen-containing functional groups [65]. rGO enhances the overall electrical conductivity of composite materials. For instance, in copper matrix composites, the addition of rGO improved thermal conductivity and mechanical properties while maintaining high electrical conductivity [66]. In summary, GO's high surface area,

tunable electrical conductivity, and functional groups make it an excellent candidate for applications in heavy metal detection and removal. The balance between reduction degree, surface area, and functional groups is crucial for optimizing GO's performance in various applications, including electrochemical sensors and energy storage devices [64], [65], [67]

2.5 Mechanisms of Enhancement:

GO enhances Cd precipitation by SRB through several mechanisms: it acts as a carrier for SRB, allowing the bacteria to self-assemble and form particles with enhanced sulfate reduction activity [4]. Sulfate reduction and growth of SRB are enhanced by the protective properties of GO and its role as an electron transfer mediator [4]. This increased bacterial activity leads to more efficient Cd precipitation. The presence of GO significantly reduces metal toxicity to SRB and accelerates heavy metal removal by up to 50% [4]. This suggests that GO provides a protective environment for the bacteria, allowing them to function more effectively in the presence of toxic metals like Cd. The enhanced electron transfer capabilities of GO contribute to more efficient metal reduction and precipitation processes. Interestingly, while GO enhances SRB activity, it can also be produced by SRB themselves. Some SRB strains, such as *Desulfovibrio desulfuricans*, can reduce GO to rGO in an environmentally friendly manner [68]. This suggests a potential synergistic relationship between rGO and SRB in metal precipitation processes. In conclusion, GO enhances Cd precipitation by SRB through improved bacterial growth and activity, reduced metal toxicity, and enhanced electron transfer. The ability of some SRB to produce rGO from GO further supports the potential for developing efficient, eco-friendly bioremediation systems for heavy metal contamination.

CHAPTER 3

MATERIALS AND METHODS

3.1 Materials

Sodium citrate, sodium lactate, and monopotassium phosphate (KH_2PO_4) used for the culture media were procured from Alfa aesar (Massachusetts, USA). Calcium chloride (CaCl_2), hydrochloric acid (HCl), and magnesium chloride hexahydrate ($\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$) were from Sigma-Aldrich (St Louis, MO, USA). Ammonium chloride (NH_4Cl) is from Mallinckrodt, sodium sulfate (Na_2SO_4) from Alpha Chemika, glycerol anhydrous and yeast extract were from Fluka, cadmium chloride (CdCl_2) and ascorbic acid were acquired from Thermo Fisher Scientific and barium chloride (BaCl_2) was gotten from Loba Chemie (Mumbai, India). All chemicals and reagents used were of analytical grade and had a percentage purity of $\geq 96\%$.

3.2 Microbial Culture and Medium

The bacterial strain *Pseudomonas aeruginosa* SRB1A used in this study was isolated and preserved on agar slants from a previous study conducted in the Life Science Department Laboratory at King Fahd University of Petroleum and Mineral from the work of Aftab et al. 2023 [69].

This strain was activated by inoculation on a standard Postgate medium under anaerobic conditions. Nitrogen gas (99%) was used to sparge the medium and displace oxygen, which is toxic to SRB and prevents the oxidation of sulfide produced by SRB via sulfate reduction[70]. The media contained per liter 0.5g KH_2PO_4 , 1.0g NH_4Cl , 1.0g Na_2SO_4 , 1.0g CaCl_2 , 1.83g $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, 1.0g Yeast extract, 0.1g ascorbic acid, 0.013g sodium

thioglycolate, 6.38g sodium citrate, 1.75ml/g sodium lactate, 3.5% w/v NaCl, 2.0ml resazurin (0.025% P/V). After cooling, previously sterilized 250 μ L FeSO₄.7H₂O stock solution (0.5 g/L or 1.8mM) was added for every 50ml of the previously autoclaved medium, resulting in a final FeSO₄.7H₂O concentration of 0.009mM (equivalent to 0.009mM Fe). The mixture was homogenized by vortexing and autoclaved at 121⁰C, 15 psi for 30 min[71]. The darkening of the medium due to iron sulfide formation indicated growth.

3.3 Growth of SRB as a Function of Bacterial Load

Aliquots of 2.0ml SRB culture were transferred to a 50ml fresh Postgate medium. The culture was incubated at 38⁰C under anaerobic conditions for 12 days. Subsequently, aliquots of 45ml were taken, and drops of HCL (0.01M) were added incrementally to each sample until the black precipitate (FeS) dissolved and the color disappeared. It was centrifuged at 10⁰C for 10 mins at 6000rpm (the relative centrifugal force was 4024 $\times$ g). The supernatant was discarded, and the pellets were collected and washed three times with 0.85% NaCl solution to avoid osmotic lysis. The pellets were resuspended in 3ml Postgate medium bottles to produce the stock culture.

From the stock culture, a series of 10-, 100, and 1000-fold dilutions was conducted, new cultures were initiated with 1ml of each dilution, and the culture that grew the fastest (12 days) was selected as the standard for SRB growth.

To calculate the growth rate, we use the formula:

$$K = \frac{\ln(OD2) - \ln(OD1)}{t2 - t1}$$

where t₁, and t₂, are time points at OD1, and OD2.

Doubling time was then calculated from the growth rate using the equation:

$$Dt = \frac{\ln 2}{K}$$

3.3.1 Optical Density

The absorbance of the stock culture and the diluted concentrations was quantified with a spectrophotometer at 600 nm. While it is commonly reported that an optical density (OD) of 1 unit correlates approximately to 1.0 g/L of dry cell weight, this relationship varies significantly depending on the organism, wavelength, and growth conditions. For instance, in *Escherichia coli*, an OD₆₀₀ of 1 corresponds to a dry weight ranging from 0.36 g/L to 0.56 g/L [72] and for *Saccharomyces ellipsoideus*, an OD₆₀₀ of 1 correlates to approximately 0.62 g/L of dry weight [72]. For this study, the commonly used approximation of 1.0 g/L per OD unit was assumed.

3.4 Precipitation of Cadmium by SRB

Cadmium experiments were conducted using a modified Postgate medium. However, instead of Fe²⁺(FeSO₄·7H₂O), the medium contained Cd²⁺ (CdCl₂) to facilitate the monitoring of Cd precipitation. Different concentrations of 1mM, 2mM, and 5mM Cd²⁺ were added to 50ml culture media. A sterile syringe was used to inoculate 2 ml of SRB culture with an initial OD₆₀₀ of 0.5 and the medium was incubated anaerobically at 37°C for 240 hours. The formed precipitates were collected, dried, and stored for characterization and further analysis.

3.5 Synthesis of GO

GO was prepared with slight modifications to the well-known Hummers' process. Initially, 3g of graphite powder was distributed evenly in a mixture of 6 mL of nitric acid (HNO_3) and 140 mL of sulfuric acid (H_2SO_4) within a beaker set in an ice bath to keep the temperature under 5°C . This mixture was stirred continuously in the ice bath for one hour. Then, 10g of KMnO_4 was slowly added, after which the mixture's temperature was raised to 35°C and stirred continuously for four hours. Subsequently, 240 mL of double-distilled water was added slowly, ensuring the temperature did not exceed 50°C due to the exothermic nature of the dilution. The temperature was later increased to 95°C and maintained for four hours, turning the mixture brown as GO formed. To reduce the remaining oxidant, 15 mL of 30% H_2O_2 was added gradually. The final step involved washing the GO thoroughly with dilute hydrochloric acid and double-distilled water to achieve a pH of 6.5, followed by centrifugation at 10000 rpm for 15 minutes over four days. After extensive cleaning, the GO was dried and prepared for further characterization and experiments [73].

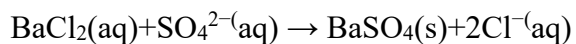
3.6 Characterization of CdS Precipitates and GO using SEM and XRD

After 12 days of culture, the bacterial suspension was centrifuged at 6000 rpm for 10 minutes to separate the precipitate from the supernatant. The precipitate was then washed repeatedly with deionized water and dried in an oven for 24 hours. The resulting dried precipitate was analyzed using Rigaku Ultima IV X-ray Diffractometer (XRD) at a scanning rate of $5^\circ/\text{min}$ in the 2θ range $20-70^\circ$ with $\text{Cu K}\alpha$ radiation ($\lambda = 0.1542\text{nm}$). The morphology and particle size of the CdS were observed by using JEOL JSM-6610 SEM [52] with a 20 kV operating voltage. Samples were prepared by drying and mounting on

aluminum stubs, followed by coating with a thin layer of gold to enhance conductivity and reduce charging effects during imaging, as outlined by Goldstein et al. (2003) [74] and Zhou & Wang (2007) [75]. The SEM provided high-resolution images, revealing surface features and structural details critical for characterizing the CdS precipitates and synthesized GO, consistent with previously reported methodologies for nanomaterials Nain (2024) [76] and graphene oxide Dreyer et al. (2010) [77].

3.7 Sulphate Assay Protocol

In a test tube, 1 mL of culture medium was mixed with the conditioning reagent. The conditioning reagent was prepared by dissolving 15 g of NaCl, 10 mL of glycerol, 6 mL of concentrated HCl, and 20 mL of 95% ethanol in 100 mL of distilled water. Approximately 60 mg of crushed barium chloride crystals were added, and the mixture was vortexed for 30 seconds. The reaction between barium chloride and sulfate ions in the medium resulted in the formation of insoluble barium sulfate (BaSO₄) precipitate, following the reaction:



The conditioning reagent facilitated this process by stabilizing the precipitation. Sodium chloride provided ionic strength to reduce interference from other ions, glycerol controlled the growth of BaSO₄ crystals, hydrochloric acid acidified the solution to prevent interference from carbonate ions, and ethanol minimized BaSO₄ solubility while enhancing precipitation. The absorbance at 420 nm was measured to quantify the light scattering caused by the BaSO₄ particles, which correlated with the sulfate concentration in the sample. A blank solution, consisting of the complete reaction mixture excluding sulfate, was used to account for any background signal. The measured absorbance values were

compared against a standard curve prepared using sulfate concentrations ranging from 0 to 7 mM [78].

3.8 Measurement of Heavy Metal (Cd)

The level of Cd in the PG media was quantified using ICP-OES Thermo Scientific™ iCAP™ 7000 series at 228.802 nm. Before the analysis, the samples were centrifuged, and the supernatant was collected and filtered to remove any solid precipitate or particle to ensure that only dissolved cadmium was quantified. The ICP-OES operates by nebulizing the sample into an argon plasma, where the cadmium atoms are excited to emit light at characteristic wavelengths. The intensity of this light at 228.02 nm directly correlates with the Cd concentration in the sample.

3.9 Effect of Go on cadmium precipitation

As mentioned above, Cd experiments were conducted using a modified Postgate medium. However, instead of Fe^{2+} ($\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$), the medium contained Cd^{2+} (CdCl_2) to facilitate the monitoring of Cd precipitation. A 0.1mg GO was added to the medium to assess its effect on SRB growth. A sterile syringe was used to inoculate 2 ml of SRB with an initial OD_{600} of 0.5, and the medium was incubated anaerobically at 37°C for 240 h.

CHAPTER 4

RESULTS

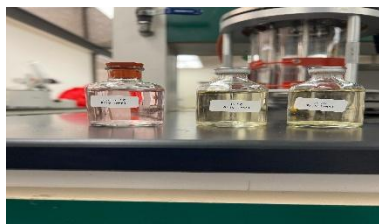
4.1 Evaluation of SRB activity by monitoring Iron Sulfide and Cadmium sulfide precipitation under normal conditions

In this study, a modified Postgate medium was prepared to support SRB in precipitating metal sulfides. This medium contained sulfate (Na_2SO_4) as an electron acceptor, and SRB could be reduced to sulfide during metabolic processes. Organic carbon sources citrate and lactate served as electron donors, enabling the SRB to perform anaerobic respiration and produce sulfide ions essential for metal precipitation.

Initially, iron sulfate ($\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$) was added as a source of Fe^{2+} . During SRB growth, Fe^{2+} reacted with the biogenic HS^- produced through sulfate reduction, resulting in the formation of iron sulfide (FeS). The FeS precipitated as a dark, black solid, visibly darkening the medium. The appearance of this black precipitate is a strong visual indicator of SRB metabolic activity, as it suggests both successful sulfate reduction and FeS formation (Figure 2A). The precipitation reaction is as follows:



A



B

Figure 2 SRB growth in modified Postgate medium showing (A) FeS and (B) CdS formation.

After confirming that the SRB isolate could reduce sulfate and precipitate FeS, the medium was further modified by replacing iron sulfate with cadmium chloride (CdCl₂) to introduce Cd²⁺ ions. This approach facilitated the assessment of the ability of SRB to precipitate CdS under similar conditions.

After 12 d of culture, a yellow precipitate was observed, indicating the formation of CdS. The yellow color of the precipitate is characteristic of CdS, suggesting that SRB activity facilitated the reaction between Cd²⁺ and biogenic HS⁻ to yield CdS (Figure **2B**). The reaction is represented by



4.2 Bacterial Growth Curve

Figure 3 illustrates the relationship between cell concentration, expressed as optical density (OD) at 600 nm, and time for SRB cells cultured in Postgate standard medium with pH adjusted below 2 using dilute HCl. Darkening of the medium, a typical indicator of SRB metabolic activity, was observed after 72 h and during the logarithmic growth phase. The transition from the lag phase to the exponential phase marks the period of active cell division and rapid accumulation of biomass.

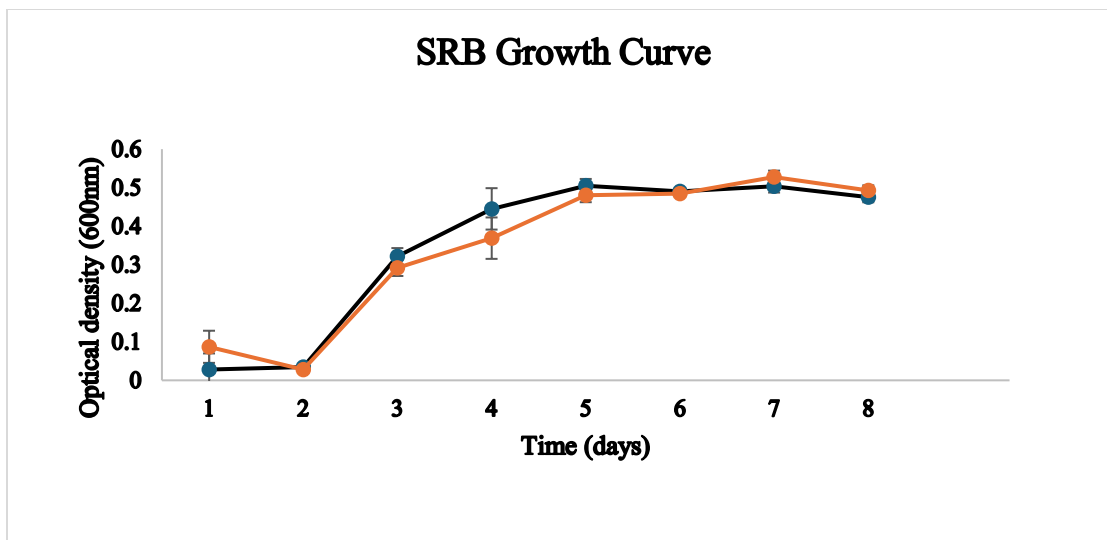


Figure 3 Cell concentration as a function of optical density (absorbance at 600nm)

The growth rate and doubling time were determined at two points during the logarithmic growth phase using the following formula:

$$K = \frac{\ln(OD_3) - \ln(OD_2)}{t_3 - t_2} \quad \quad Dt = \frac{\ln 2}{K}$$

where t_3 and t_2 are time points at OD_3 , and OD_2 .

The growth rate was calculated to be $0.09h^{-1}$ with a doubling time of approximately 7.4 hours.

4.3 Confirmation of CdS precipitate using XRD

Figure 4 shows the XRD spectrum of the CdS precipitate. Diffraction peaks of CdS (25.3° , 26.9° , 28.9° , 36.7° , 43.7° , 47.6° , and 52.8°) were observed in the spectrum, demonstrating that SRB precipitated CdS. These peaks are characteristic of the hexagonal structure of CdS, also called the wurtzite structure (ICDD77-2306). The sharp and intense peaks indicated a high degree of crystallinity. Additional peaks at 34° , 41.9° , 42.7° , and 46.8°

indicate the presence of impurities or secondary phases within the sample, suggesting slight deviations from the pure CdS composition.

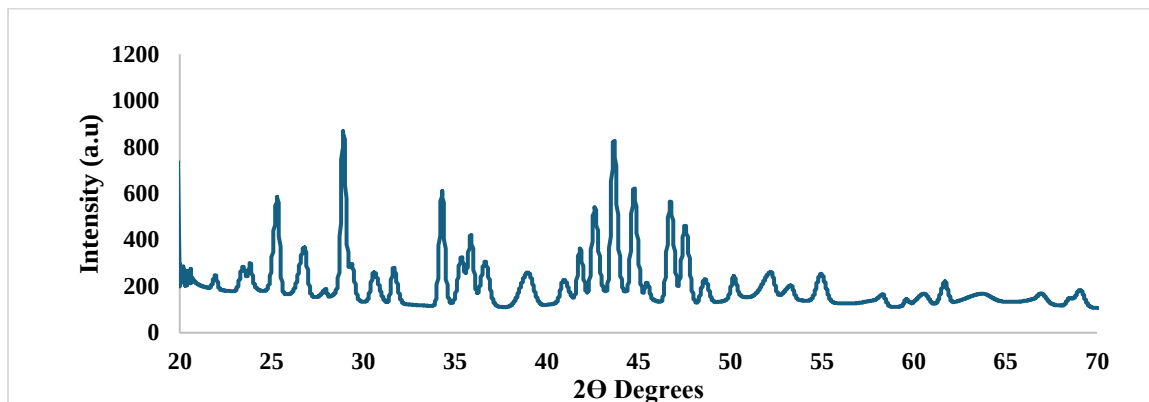


Figure 4 Powdered X-ray diffraction patterns of CdS precipitated by SRB.

Table 1 Comparison of XRD peaks for Hexagonal CdS precipitates.

ICDD 77-2306	24.8	26.5	28.2	36.6	43.7	47.8	51.8	Standard hexagonal CdS ref.
[79]	24.9	26.6	28.2	36.8	43.9	48.0	52.0	Matches experiment closely
Sample	25.3	26.9	28.9	36.7	43.7	47.6	52.3	This study

4.4 SEM Analysis of CdS Morphology

The SEM micrographs in Figure 5 showed irregularly shaped spherical nanoparticles with an average diameter of $0.63\mu\text{m}$. The particles tend to aggregate, due to Van der Waals forces acting between the nanosized particles. The surface morphology appeared smooth with minimal porosity and a dense structural arrangement.

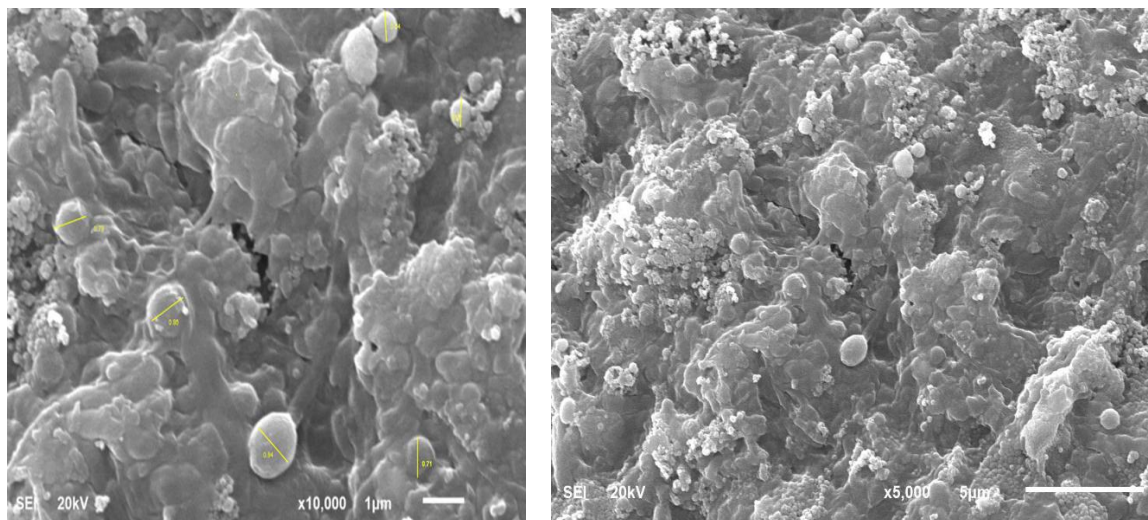


Figure 5 SEM micrographs of CdS nanoparticles

The EDS spectrum (Figure 6) supports the composition of CdS as shown by the significant peaks for cadmium (Cd) and sulfur (S) in the spectrum. Quantitative analysis showed that the sample consisted of 35.2% Cd and 12.0% S, which is in close agreement with the stoichiometry expected for CdS, although an excess of carbon (38.3%) and oxygen (14.5%) was detected. Carbon and oxygen could result from residual organic material from sample preparation or environmental exposure. Overall, the elemental composition aligns with the CdS structure shown by XRD and supports the crystalline morphology observed by SEM.

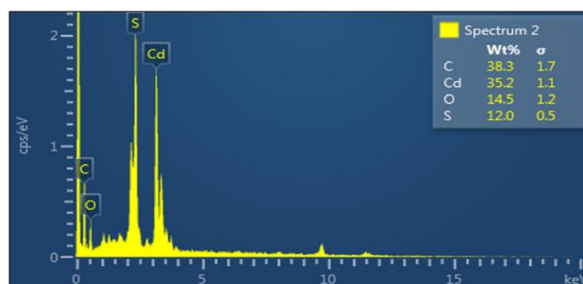


Figure 6 Elemental analysis of CdS

4.5 Sulphate calibration curve

A standard sulfate solution was used to create a calibration curve, and the plot showed a significant correlation with an R^2 value of 0.9951, indicating a dependable linear connection within this concentration range ($y = 0.2688x - 0.2529$)

In the experimental setup, 7.05 mM of sulfate was added to the culture media to serve as an electron acceptor for sulfate reduction. The initial sulfate concentration was determined empirically using the established calibration curve. The optical density (OD) at 420 nm was recorded at 1.825, which, according to our calibration curve, corresponds to a sulfate concentration of approximately 7.73 mM. The close agreement between the added and calculated concentrations highlights the accuracy and reliability of the calibration method used.

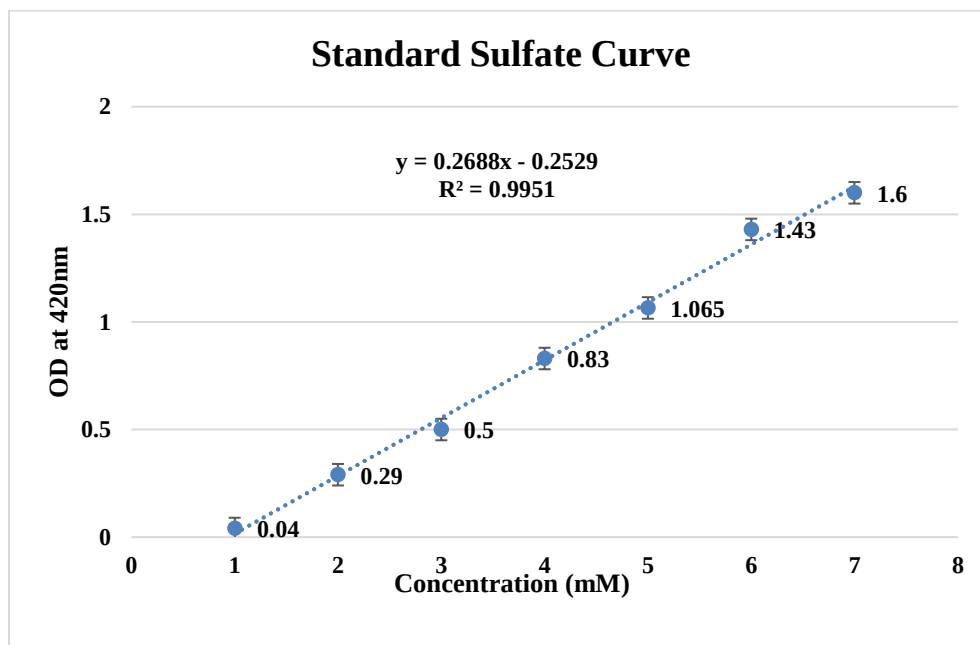


Figure 7 Standard sulfate curve obtained using Na_2SO_4 .

4.6 Kinetic analysis of sulfate and cadmium

Sulfate concentration dropped from 7.73 mM to 6.13 mM after 25 days of culture. The OD values obtained from the measurement were converted to concentrations in mM, and a graph of concentration against time was plotted. A drop of 1.6 mM was recorded in sulfate concentration over 25 days, which is a 20% reduction (Figure 8). The percent reduction of sulphate was calculated using the formula:

$$\% R = \frac{C_o - C_f}{C_o} \times 100$$

Where Co is the initial sulphate concentration and Cf is the final concentration

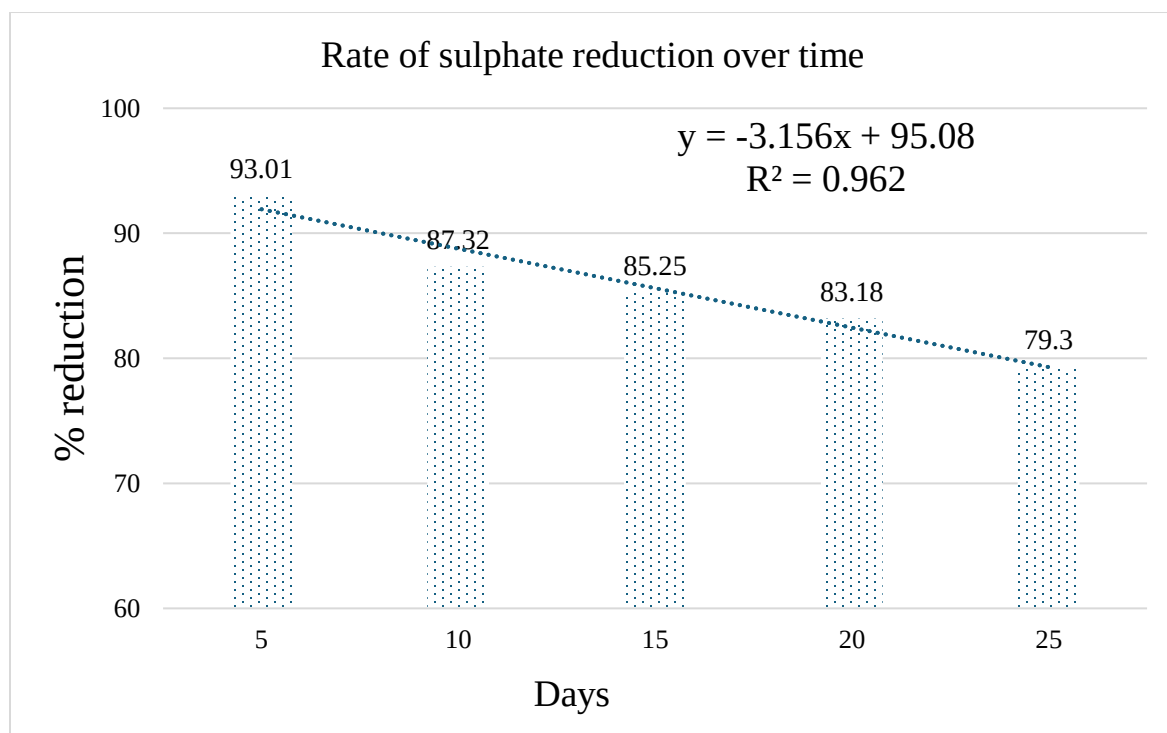


Figure 8 Percent reduction of sulphate concentration over 25 days.

For Cd, 87% was precipitated by SRB as the concentration dropped from 40mg/L to 5.2mg/L after 25 days of culture (Figure 9). Even though it is high, it is lower than the

theoretical complete removal expected under ideal conditions. The following equation was used to calculate the percentage of Cd precipitated.

$$\% R = \frac{C_o - C_f}{C_o} \times 100$$

Where C_o is the initial Cd concentration and C_f is the final concentration

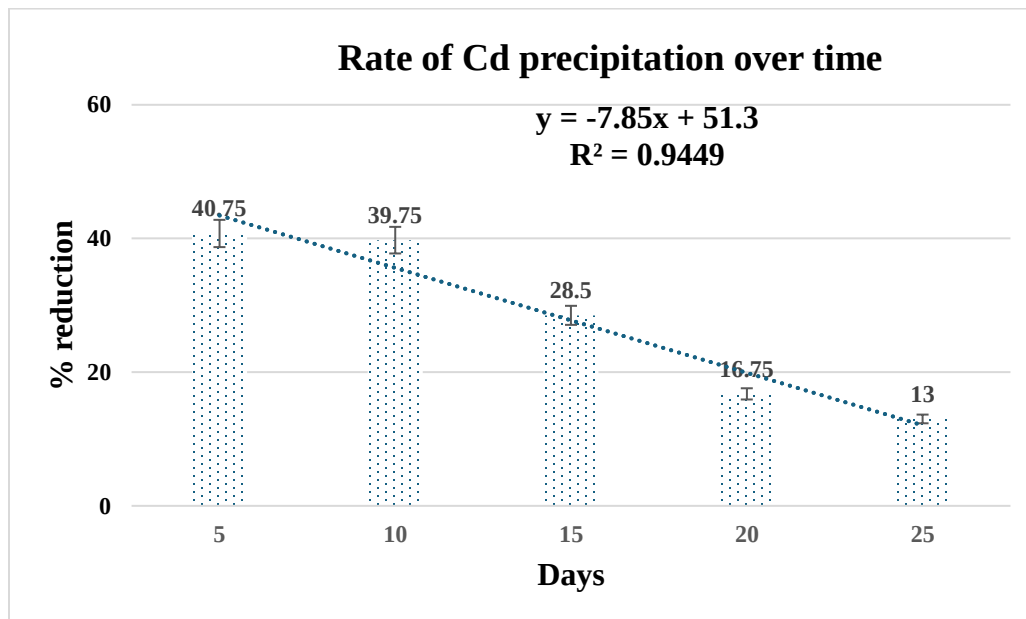


Figure 9 Rate of Cadmium precipitation over time

4.7 Characterization of Graphene Oxide (GO) synthesized.

The XRD pattern of GO synthesized (Figure 10) showed a strong peak at 11.2° , characteristic of the (001) plane, confirming the oxidation of graphite and the increased interlayer spacing due to oxygen functional groups. A second, less intense peak at 42.8° corresponds to the (100) plane, indicating retained graphitic domains.

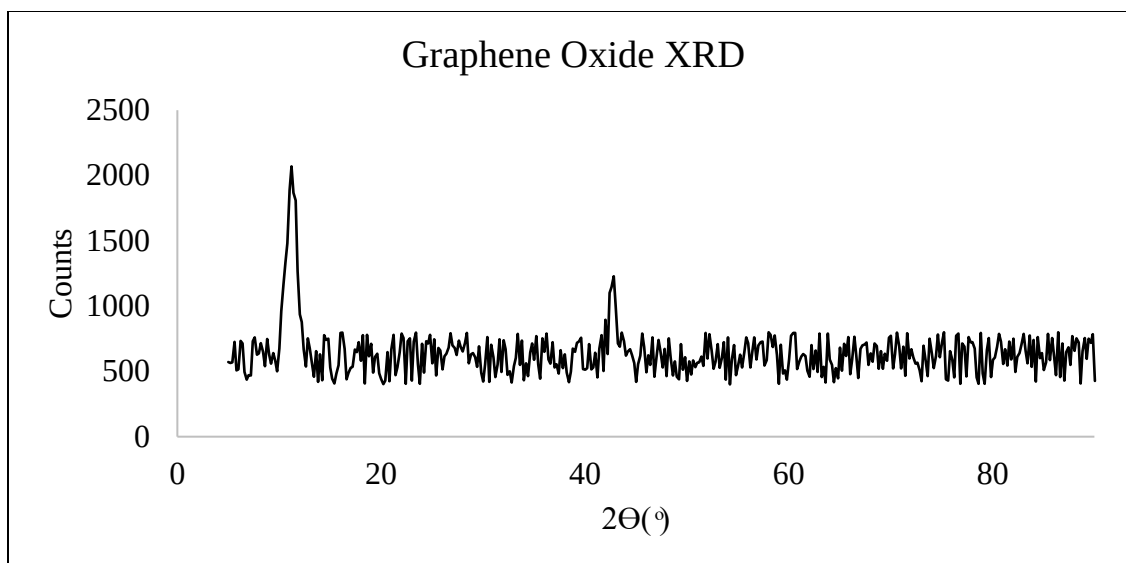


Figure 10 XRD pattern recorded for GO

The SEM micrographs (Figure 11) showed textured, layered sheets, with a rippled surface morphology that is typically found in GO. This makes it desirable for bioremediation as the high surface area increases the binding sites for the metal ions to adhere, facilitating their precipitation from solutions. The EDS analysis (Figure 12) gave 56.8% carbon and 43.2% oxygen. The result aligns with the previous XRD data 11.2°, as oxidation of graphite to graphene oxide takes place shown by expanded interlayer spacing.

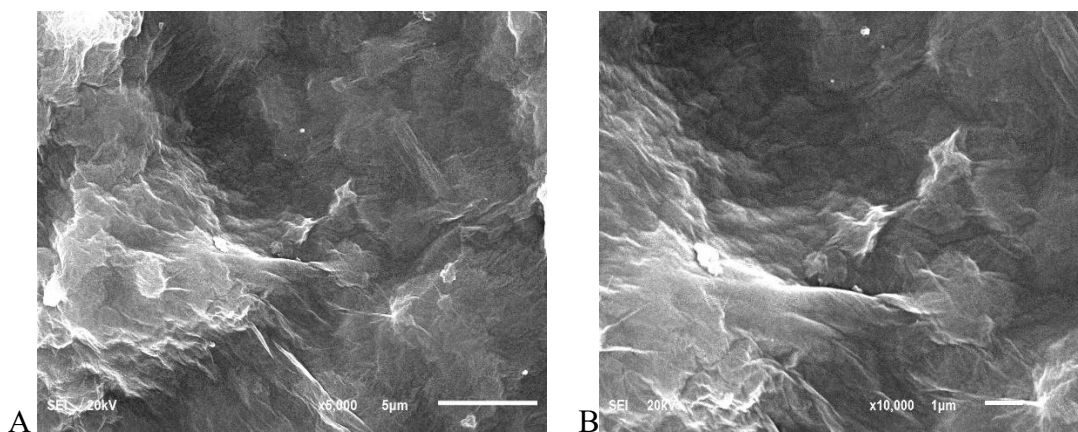


Figure 11. SEM micrographs of GO synthesized

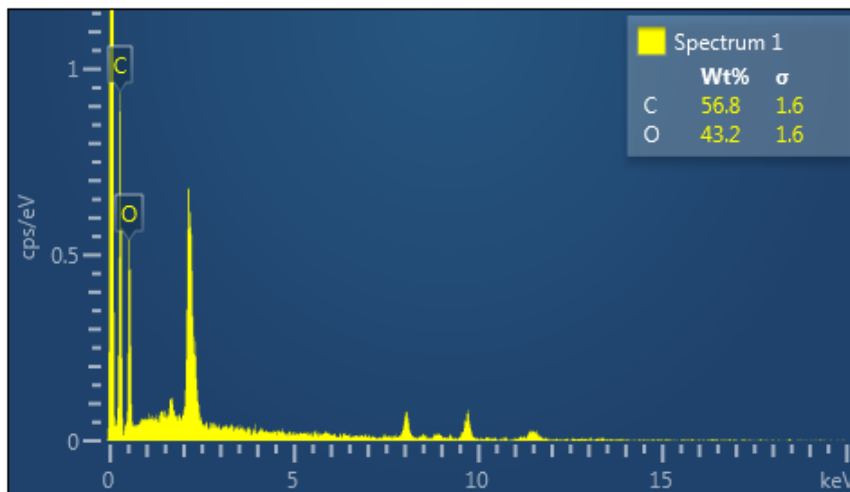


Figure 12 Elemental analysis of GO



Figure 13. GO synthesized by modified Hummer's method

4.8 GO incorporation into the Postgate medium for enhanced CdS precipitation

The addition of 0.1mg GO into the Postgate medium resulted in 89% removal of cadmium after just 5 days of culture. The 40mg/L Cd^{2+} added was reduced to 4.3mg/L in just 5 days and the percent reduction formula was used to calculate the percentage precipitated.

Table 2 Removal of Cd²⁺ by SRB

Bacteria	Initial Cd²⁺	Cd²⁺ Removal	Reference
<i>Pseudomonas aeruginosa</i> SRB1A	40mg/L	59%	This work
<i>Pseudomonas aeruginosa</i> SRB1A + GO	40mg/L	89%	This work

CHAPTER 5

DISCUSSION

The observed growth rate and doubling time suggest that SRB are highly active under the pH conditions used in this study, with a rapid proliferation rate during the logarithmic phase. The growth rate of 0.09 h^{-1} and a doubling time of 7.4 hours show that the cells are capable of efficient metabolic processes, due to optimal nutrient availability and favorable environmental conditions. These results are consistent with those of previous studies on SRB growth in similar media [80], confirming that SRB can thrive under a range of environmental conditions. The fast growth observed here has implications for the potential use of SRB in bioremediation, where their rapid growth is advantageous for sulfate reduction and metal precipitation processes.

The successful precipitation of FeS and CdS highlights SRB's effectiveness in removing heavy metals through biogenic sulfate reduction, forming insoluble metal sulfides. This SRB-mediated process shows strong potential for bioremediation applications [52], [57], [81]. Differences in precipitation efficiency between Fe and Cd may result from variations in solubility products and reaction kinetics [82]. Factors such as medium composition, incubation time, and SRB growth phase significantly impact precipitation efficiency, as nutrient availability influences SRB metabolism, while extended incubation can enhance metal removal [49], [83], [84]. Visual color changes—black for FeS and yellow for CdS—served as direct indicators of successful precipitation, offering a non-invasive method for monitoring SRB activity and metal removal in both lab and field applications [85].

The XRD pattern confirmed the successful precipitation of CdS with peaks that closely match the hexagonal standard reference (ICDD 77-2306). The SEM micrographs support the crystalline structure, showing densely packed spherical nanoparticles with a smooth surface and minimal porosity. The observed aggregation is consistent with Van der Waals forces typically found in nanoparticles. EDS data confirmed the presence of cadmium and sulfur in almost stoichiometric proportions. However, some carbon and oxygen were also detected from organic residuals during sample processing and environmental exposure explaining the slight deviations observed in the XRD spectrum in the form of additional peaks indicating impurities or secondary phases. Dong and his colleagues (2023) [1], and Zhang et al (2022) [55] reported similar findings where SRB-mediated bioremediation yielded hexagonal wurtzite CdS with a high degree of crystallinity and EDS data that supports CdS stoichiometry.

The 87% removal rate recorded in the experiment showed the efficiency of Cd precipitation by this SRB strain. Although higher removal rates have been reported in existing literature, they fall short of the concentration we used. Oporto and his colleagues (2021)[86] reported the highest removal rate of Cd^{2+} up to 99%. However, the concentration was considerably lower at 4.3mg/L. Usually, researchers carry out this experiment around 5-10mg/L as shown in Table 3 Below, however, our experiment went up to as high as 40mg/L.

Table 3 Comparison of Cd²⁺ ion precipitation over different concentrations

Bacteria	Initial Cd²⁺	Cd²⁺ Removal	Reference
<i>Pseudomonas aeruginosa</i> SRB1A	40mg/L	59%	This work
<i>Pseudomonas aeruginosa</i> SRB1A + GO	40mg/L	89%	This work
<i>Clostridium sulfidigenes</i> HY-1	5mg/L	59%	Wu et al. (2022)
<i>Desulfovibrio africanus</i>	5mg/L	80%	Yan et al. (2022)
<i>Desulfovibrio africanus</i>	1-5mg/L	84%	Nguyen et al. (2022)
<i>Desulfovibrio</i> SP	10mg/L	86%	Yan et al. (2022)
<i>Desulfotomaculum</i> Sp	4.3mg/L	99%	Oporto et al. (2021)
<i>Desulfovibrio</i> SP	5mg/L	75%	Kumar et al. (2020)
<i>Desulfovibrio</i> SP	0.3mg/L	84%	Kiran et al. (2017)
<i>Desulfotomaculum</i> Sp	1-5mg/L	75%	Kiran et al. (2017)
<i>Desulfovibrio africanus</i>	5mg/L	47%	Sun et al. (2017)

After adding GO, the results were further enhanced as the precipitation rate increased from 59% to 89% within just 5 days. The high surface area and enhanced electron transfer capabilities of rGO contributed to more efficient precipitation in a shorter number of days. As indicated by the results, 87% precipitation was recorded after 25 days, meanwhile 89% precipitation after adding GO in 5 days.

CHAPTER 6

CONCLUSION

The study effectively demonstrated the synergistic potential of utilizing SRB and GO for the enhanced precipitation of cadmium from high-concentration solutions. The robustness of SRB in reducing cadmium levels, combined with the catalytic advantages provided by GO, points to a promising strategy for the treatment of heavy metal-contaminated industrial effluents. A high level of cadmium (40mg/L) was precipitated as CdS for up to 59% and the rate went as high as 87% in 25 days. The addition of GO increased the rate from 59% to 89% within 5 days.

Future research should focus on optimizing SRB growth conditions, expanding its use to various metal sulfides, and assessing the scalability and long-term stability of precipitated compounds. Exploring mixed-metal systems and selective metal recovery could further enhance its potential for bioremediation.

APPENDIX

Table S1. SRB growth rate data

Time (days)	Optical Density (600nm)
1	0.028
2	0.034
3	0.322
4	0.445
5	0.505
6	0.490
7	0.489
8	0.475

Table S2. Standard Sulfate Curve

Concentration (mM)	Optical Density (420nm)
1	0.04
2	0.29
3	0.50
4	0.83
5	1.065
6	1.43
7	1.60

Table S3. Sulfate Reduction Data

Time (days)	Optical Density (420nm)	Concentration (mM)	Reduction (%)
5	1.68	7.19	6.99
10	1.56	6.75	12.68
15	1.52	6.59	14.75
20	1.48	6.43	16.82
25	1.40	6.13	20.70

Table S4. Cadmium Precipitation Data

Time (days)	ICP-Concentration (mg/L)	Adjusted Concentration (mg/L)	Reduction (%)
5	88.3	16.3	59.25
10	87.9	15.9	60.25
15	83.4	11.4	71.50
20	78.7	6.7	83.25
25	77.2	5.2	87.00

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