MJFAS
Malaysian Journal of Fundamental and Applied Sciences

Hamdan *et al.* | Malaysian Journal of Fundamental and Applied Sciences, Vol. 20 (2024) 1017-1031

RESEARCH ARTICLE

Cd^{2+} Sequestration by Ureolytic Bacteria Isolated from Goat Faeces and Nursery Wastewater

Muhammad Daniel Hamdan[a], Hazlami Fikri Basri[a*], Armstrong Ighodalo Omoregie[b], Mohd Akmali Mokhter[c], Koji Iwamoto[d], Tariq Ouahbi[e]

[a]Department of Water and Environmental Engineering, Faculty of Civil Engineering, Universiti Teknologi Malaysia, 81310 UTM Johor Bahru, Johor, Malaysia; [b]Centre for Borneo Regionalism and Conservation, University of Technology Sarawak, No. 1 Jalan University, 96000 Sibu, Sarawak, Malaysia; [c]Advanced Membrane Technology Research Centre (AMTEC) [HICoE], Faculty of Chemical and Energy Engineering, 81310 UTM Johor Bahru, Johor, Malaysia; [d]Department of Environmental Engineering and Green Technology, Malaysia-Japan International Institute of Technology (MJIIT), Universiti Teknologi Malaysia, 54100, UTM Kuala Lumpur, Malaysia; [e]Université Le Havre Normandie, Normandie Université, LOMC, UMR 6294 CNRS, 53 rue de Prony, 76058 Le Havre Cedex, France

Abstract As modern industrial production makes extensive use of heavy metals, a significant amount of sewage and solid wastes containing heavy metals are released into the environment. There is significant potential for bioremediation of multiple heavy metal contamination using biomineralization to immobilise the toxic metal. In this study, microbially induced carbonate precipitation (MICP) technology was used to treat Cd^{2+} contamination on plants, humans, and the environment. Ureolytic bacteria that collected from goat faeces and nursery wastewater were stimulated using enrichment technique. Next, physicochemical properties of the bacteria including urea agar base test, biomineralization test, conductivity & pH measurement test and optical density test were examined through enrichment subculturing of the bacteria. The morphological characterization of precipitate formed from both samples were analayzed by using scanning electron microscopy- energy dispersive x-ray diffraction method. Ureolytic bacteria of the goat faeces sample demonstrated greater tolerance to Cd^{2+} concentration compared to the nursery wastewater sample by obtaining a higher optical density values and larger amounts of precipitate produced by the goat faeces sample across all tested Cd^{2+} concentrations. As a result, from the atomic absorption spectrophotometry (AAS) analysis, the goat faeces sample showed higher efficiency in removing Cd^{2+} compared to the nursery wastewater sample, with a removal efficiency of 89.06% for the goat faeces sample and 77.75% for the nursery wastewater sample. Hence, this study confirmed that the interaction between ureolytic bacteria isolated from waste sources and heavy metal ions is vital for the overall effectiveness of heavy metal removal.

Keywords: Heavy metal, bioremediation, ureolytic bacteria, MICP, wastewater.

***For correspondence:**
hazlamibasri@gmail.com

Received: 31 Jan. 2024
Accepted: 21 Aug. 2024

Introduction

The surge in industrialization has fueled a concerning escalation in heavy metal contamination, presenting imminent ecological hazards [1]. According to the World Health Organization (WHO), allowable concentrations of heavy metals in drinking water are tightly regulated, with maximum limits set at 50 g/L for chromium, 3 g/L for cadmium, 3 g/L for mercury, 10 g/L for lead, 10 g/L for arsenic, and 700 g/L for nickel [2]. Industries such as paint, fertilizer, textile, and electrochemical production contribute to the problem by discharging wastewater containing alarming concentrations of unbound heavy metals. When left untreated or partially treated, this toxic cocktail infiltrates aquatic and terrestrial ecosystems, posing threats to both wildlife and human populations [3].

The repercussions of heavy metal contamination extend globally, impacting biodiversity, human health, and the overall stability of ecosystems [4]. Modern industrial practices release significant amounts of sewage and solid wastes laden with heavy metals into the environment, compounding the pollution crisis [4]. Mining, smelting operations, chemical pesticide use, and the application of sewage sludge as fertilizer also contribute substantially to heavy metal contamination [5]. The persistence of non-biodegradable heavy metals in ecosystems poses health risks as these contaminants accumulate in organisms and traverse the food chain [6].

Traditional wastewater treatment methods, while prevalent, have limitations. Techniques such as chemical precipitation, electrochemical precipitation, membrane technology, adsorption, coagulation, and evaporation recovery can lead to secondary pollution and incur high costs [7]. Biological treatment methods, like ecological wetland restoration or biosorption, are effective only at low metal concentrations and require extended processing cycles [8]. Recognizing the need for more sustainable and cost-effective alternatives, researchers are turning to bioremediation approaches.

Microbially Induced Carbonate Precipitation (MICP) emerges as a promising bioremediation technology due to its effectiveness, environmental friendliness, and economic benefits [6]. This innovative method involves the precipitation of calcium carbonate minerals within the soil matrix by ureolytic bacteria, converting free metal ions into stable forms and reducing the mobility and toxicity of harmful metals [9].

While existing studies on MICP primarily focus on individual heavy metals, using ureolytic bacteria with inherent heavy metal resistance, this study takes a unique approach. Native ureolytic bacteria from waste sources will be screened to assess their potential in immobilizing heavy metals in soil particles through biocementation. Previous studies, such as the isolation of Bacillus sp. WA from metal-contaminated soil in Guiyu, China, or the utilization of Sporosarcina ureilytica ML-2 during the land application of sewage sludge, have shown promise [1]. However, the current research seeks to address the gap in existing studies by specifically targeting ureolytic bacteria from waste sources for heavy metal bioremediation.

The study's objectives encompass screening native ureolytic bacteria, evaluating their performance in immobilizing heavy metals, and examining the influence of various conditions, including bacterial species, concentration, pH, temperature, and heavy metal concentrations on MICP efficiency. By employing such a comprehensive approach, this research aims to contribute to a deeper understanding of MICP and its potential to mitigate heavy metal contamination while enhancing soil properties. The results are expected to provide valuable insights into sustainable and effective strategies for addressing the global challenge of heavy metal pollution.

Materials and Methods

The experimental work conducted for this study consists of three major parts. The first part of the experiment was to stimulate urease producing bacteria from waste sources. Samples collection was taken from local goat farm and plant nursery. Afterwards, the waste samples that had been collected were used to prepare and inoculate the nutrient broth that contains urea. Each culture solution was maintained in an aerobic environment for the enrichment culturing and subculture of the bacteria. In the second part, the subculture underwent a few tests such as urea agar base test, biomineralization test, conductivity & pH measurement test and optical density test. This allowed the investigation of the physicochemical properties of ureolytic bacteria through selective enrichment subculturing. Next, the study focused on the tolerance of Cd^{2+} concentration towards the isolated bacteria from wastewater. The last part of the experimental work concentrated on the effect of using the local isolate ureolytic bacteria from goat faeces and nursery wastewater source towards soil strength during the treatment of heavy metal via MICP. Hence, the overall framework for this study as shown in Figure 1 was illustrated to give an overview of experimental works done to achieve the objectives of this study.

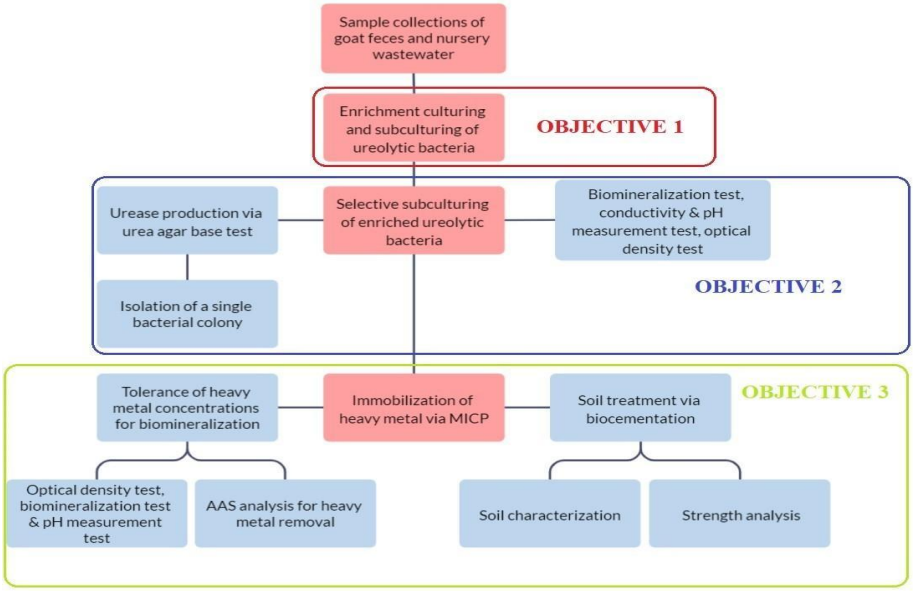


Figure 1. Flowchart of the experimental plan for this study

Collection of Samples

Waste samples were meticulously collected from a local goat farm in Senai, Johor (1.6453747110092993° N, 103.72483872242563° E), and a nursery in Skudai, Johor (1.5433399681829336° N, 103.6270644995925° E) for this study focusing on the identification of potential ureolytic bacteria for heavy metal immobilization. The collected goat faeces were carefully placed into zip-lock bags, while nursery wastewater samples were stored in 1-liter sampling bottles to accommodate their diverse characteristics. To preserve sample integrity during transportation to the laboratory, both the zip-lock bags and sampling bottles were securely sealed to prevent leakage or contamination. Attention was given to maintaining appropriate temperatures during transit to avoid alterations in microbial composition or other sample properties. Upon arrival at the laboratory, the samples underwent additional analysis to identify and characterize ureolytic bacteria, ensuring the reliability and accuracy of the study's subsequent findings.

Medium Preparation

To ensure transparency regarding the source and quality of urea used in the preparation of the nutrient broth medium, 13 g/L of Hmedia nutrient broth purchased from Atama tech Sdn. Bhd. and 10 g/L of ammonium chloride were initially added to 1 L of distilled water, and this mixture was subsequently sterilized using an autoclave machine. The urea, a critical component of the medium, was introduced after the medium had cooled to prevent any potential degradation caused by heat. It is essential to highlight that this procedure, including the incorporation of urea, was meticulously conducted within a biosafety cabinet. This precautionary measure was implemented to maintain a controlled and sterile environment, minimizing the risk of contamination and ensuring the safety of the researchers and the integrity of the experimental setup.

Enrichment Culture and Subculture of Samples

Enrichment culture was crucial to select and maintain the population of ureolytic bacteria among the microbial community in the samples. This can be achieved by giving nutrients for only ureolytic bacteria to inhibit the growth of other microbes or bacteria. To start, 10 mL of each sample was inoculated into 125 mL of Hmedia nutrient broth medium purchased from Atama Tech Sdn Bhd. mixed with and 10 g/L of ammonium chloride. The contents were mixed by gently shaking the flasks that had been sterilized. Then, the enriched culture was incubated under aerobic batch conditions at 30 °C for 3 days which shaken at 130 rpm by using KS4000 i incubator shaker. The rationale behind selecting a 3-day incubation period for the initial enrichment culture is important in optimizing conditions for the selection and maintenance of ureolytic bacteria within the microbial community present in the samples. The enrichment culture serves the crucial purpose of providing specific nutrients favoring the growth of ureolytic bacteria while inhibiting the proliferation of other microbial species or bacteria.

Subculturing involved providing further growth of the ureolytic bacteria where more nutrients were given to ensure only ureolytic bacteria were dominant. Firstly, 12.5 mL of enriched culture was added into 112.5 mL of same medium as in enrichment culture process. The enriched culture was incubated under aerobic batch conditions at 30 °C for 24 hours and was shaken at 130 rpm. The decision to employ a 24-hour incubation period for the subculture was driven by the intention to further nurture and enhance the dominance of ureolytic bacteria. This relatively shorter duration, compared to the initial 3-day incubation, was strategically chosen to capture a specific growth phase, ensuring robust proliferation of ureolytic bacteria without prolonged competition from other microbial species.

Urea Agar Base Test

The Urea agar base test is a microbiological test designed to culture bacteria which can produce the enzyme urease. Urease brings about the conversion of urea to ammonia and CO2 resulting into a change of the pH of the medium. The test is performed by streaking a bacterial sample from goat faeces and nursery wastewater on a urea agar base that incorporates a pH sensitive dye – phenol red. Then Incubation of the sample at 32°C for 1 hour, presence of microorganisms that produce urease results in a change of colour of the medium from yellow/orange to pink/red accompanied by an increase in pH. If the medium remains yellow/orange then the test is negative thus the ability to infer that the bacterium does not produce the enzyme urease.

Biomineralization Test

The aim of this test was to investigate the biomineralization or precipitation potential of the ureolytic bacteria after mixing urea and water with calcium. 45 mL of cementation solution was prepared in centrifuge tube that consist of 40g/L of urea and 40g/L of calcium chloride. Later, 5 mL of subculture was added into the cementation solution by using micropipette. The precipitation process was observed while the time for each sample will be recorded. Hence, the mass of precipitate formed can be calculated using Equation 1.

$$\text{Mass of precipitate} = X_1 - X_2 \quad (1)$$

where X_1 is mass of empty centrifuge tube and X_2 is the mass of centrifuge tube with precipitate.

Optical Density Test

Optical density test was used to determine the bacterial biomass or concentration of bacteria present in the solution. The optical density biomass concentration was measured using a spectrophotometer at a wavelength of 600 nm (OD600). Firstly, three millilitres of the culture were sampled and placed into clean 10 mm cuvettes before reading the values. The spectrophotometer was calibrated using un-inoculated growth media as blanks before the optical density of bacterial cultures grown in the medium was measured.

Urease Activity via Conductivity Test & Ph Measurement Test

The conductivity analytical method was employed to assess urease activity [11]. This test was carried out to investigate the urease activity during hydrolysis which related with the conductivity. Other than that, measuring the pH of the bacterial culture was necessary to determine the acidity or alkalinity of the environment. In this conductivity test, a conductivity meter was used to determine the electrical changes after bacterial cultures were inoculated into the cementation solution. The investigation started with preparation of urea solution containing 69.07 g of urea and 1 L of distilled water. Afterwards, 10 mL of each bacterial culture was inoculated into 250 mL sterile beakers containing 90 mL of 1.5 M urea solution. The probe of the conductivity meter was immersed into the beakers containing the solution to achieve the conductivity readings and measured the changes in conductivity (mS/cm). The conductivity and pH were recorded for a duration of 6 min at 25±2 °C. The conductivity (mS/cm) of bacterial-urea solution was measured and converted into urease activity (mM urea hydrolysed.min-1) and specific urease activity (mM urea hydrolysed.min-1.OD-1) of the ureolytic bacteria using the following Equation 2 and Equation 3, respectively.

$$Urease\ Activity = \frac{C6}{Co}\ x\ df\ x\ 11.11 \quad (2)$$

where C6 and Co represented the EC measured at 6 minute and 0 minute, respectively.

$$Specific\ urease\ activity = \frac{Urease\ activity}{Biomass} \quad (3)$$

From Equation 2, urease activity was calculated by multiplying the conductivity variation rate (mS.cm-1.min-1) by dilution factor (df) and 11.11 (correlation rate). 1 mS.cm-1.min-1 corresponds to a hydrolysis activity of 11 mM urea.min^{-1} in the measured range of activities considering the dilution of the culture during the activity measurement by a factor of 10.

Tolerance of The Bacterial Strains to Heavy Metals

In the heavy metal tolerance test, the investigation of toxicity involved preparing 45 mL of yeast extract solution in three different test tubes, with Cd^{2+} concentrations set at 0 g/L, 2 g/L, and 4 g/L. For the 2 g/L concentration, 0.1 g of cadmium sulphate was added, while 0.2 g was added for the 4 g/L concentration. Subsequently, 5 mL of bacterial culture was inoculated into each test tube using a micropipette. However, the provided information lacks details on the duration of the test and the specific criteria for assessing toxicity. It is recommended to explicitly state the test duration (e.g., 72 hours) and the criteria used for toxicity evaluation. To assess the impact of heavy metals on bacterial growth, optical density, biomineralization, and pH tests were conducted. The optical density was measured using a spectrophotometer at a specific wavelength, while biomineralization was assessed by quantifying calcite production through an appropriate method such as titration or gravimetric analysis. pH tests were performed using a designated pH meter or indicator to gauge the influence of heavy metals on solution acidity.

Removal of Heavy Metals Through MICP Process

The decision to assess heavy metal removal using the supernatant of the biomineralization test in the heavy metal tolerance study is based on the rationale of understanding the impact of ureolytic bacteria on heavy metal immobilization. The supernatant, representing the liquid phase after biomineralization, serves as a crucial indicator of the bacteria's efficacy in influencing heavy metal concentrations in the solution. In the specific case of Cd^{2+} content determination, Atomic Absorption Spectrophotometry (AAS) was employed. This analytical method involves exposing the sample to a specific wavelength of light, enabling the quantification of Cd^{2+} content in the treated samples. The AAS procedure includes calibrating the instrument with known standards and measuring the absorbance of the treated samples. Utilizing 1.5 mL of the supernatant in clean microcentrifuge tubes, the Cd^{2+} content was determined, and the removal efficiency of heavy metals was quantified using Equation 4, below.

$$\text{Removal efficiency (\%)} = \frac{Ci - Cf}{Ci} \; x \; 100 \quad (4)$$

where Ci and Cf represent the initial and final concentrations of heavy metals (mg/L), respectively.

Statistical Analysis

All experiments were conducted in triplicate, and the data were arithmetically arithmetic mean. This study's data analysis and figure plotting were performed using GraphPad Prism® software (version 9). The significance of the difference was determined using two-way analysis of variance (ANOVA) and post hoc. The significance threshold was set at 0.05.

Scanning Electron Microscope- Energy- Dispersive X-Ray (SEM-EDX) Analysis

SEM is used to investigate the microstructure and chemistry of range materials. This study employed a Hitachi SU8230 Regulus ultra high-resolution field emission scanning electron microscope (SEM) operating in variable pressure mode (50–70 Pa) to capture high magnification images of the treated wastewater by MICP. It should be emphasized that the untreated specimens were imaged in backscattered electron (BSE) mode without any coating, utilizing a 15 kV accelerating voltage. Conversely, for the imaging of treated samples, a 3 kV accelerating voltage was employed, and secondary electron images were obtained. In addition, the treated samples were coated with a 4 nm layer of iridium (conductive material) to prevent charging and improve the sample's conductivity under the electron beam.

Energy-dispersive X-ray analysis (EDX), often paired with scanning electron microscopy (SEM), serves to scrutinize elemental composition and quantity at or near the surface of nanomaterials, facilitating specimen mapping. However, its limitation arises from X-rays being generated within a shallow 2-micrometer depth, making it unsuitable for precise surface characterization. The technique involves traversing the electron beam across the sample, a process typically spanning several hours. Despite this, EDX proves effective in quantifying heavy metal ions within nanoparticles situated close to or on the sample surface, while detecting elements with atomic numbers below 11 poses challenges. In recent times, diverse X-ray characterization methodologies, including small-angle X-ray scattering, X-ray

absorption fine structure (XAFS), X-ray diffraction, and X-ray photoelectron spectroscopy (XPS), have gained prominence for characterizing nanomaterials, enabling in-depth chemical analysis, such as X-ray absorption near-edge structure, XPS, X-ray fluorescence spectroscopy, EDX, and X-ray absorption fine structure.

Results and Discussion

Screening Of Ureolytic Bacteria from Goat Faeces and Nursery Samples

A total of 2 samples were collected from goat faeces and nursery wastewater for the screening of highly active ureolytic bacteria. Ureolytic bacteria are defined as microorganisms that are capable of secreting enzymes for urea hydrolysis, resulting in biocalcification in the presence of calcium ions [12]. According to [13], consideration or selection of waste sources with suitable conditions (urea as a substrate and an alkaline pH) was crucial so that the desired bacteria could grow and generate urease. In this study, both sampling source were chosen based on the conditions and suitability for ureolytic bacteria growth. Goat faeces and nursery wastewater are considered as a waste from natural environments. They have high presence of urea as substrate, optimum alkalinity for microbial growth as well as diverse microbial community [14]. According to [15], ureolytic bacteria are likely to be found in soils that have a consistent supply of urea. Urea is the end result of the nitrogen metabolism in mammals. Hence, enrichment culture designed to select ureolytic bacteria suitable for microbial-induced reaction should be supplemented with enough urea substrate.

Enrichment culture method was conducted to screen bacteria that have high production of urease. This method helps to create competition among different types of bacteria for the nutrients they need to grow, and helps to remove bacteria that cannot survive in high urea concentrations [16]. We used a 6% urea solution to specifically target bacteria that can break down urea for energy and nitrogen. Figure 2 illustrate the images of enriched culture of goat faeces and nursery wastewater samples. They observed that during the 120-hour incubation period, there was a strong smell of ammonia gas, which is a sign that bacteria were breaking down urea and producing ammonia as a byproduct. Afterward, the enrichment culture was subcultured and tested for urea agar base test to further investigate the ability of the bacteria culture in producing urease. The urea agar base medium used in the test contains urea and a pH indicator, phenol red. The accumulation of ammonia in the media because of urea hydrolysis raises the pH of the surrounding medium. A favourable result for urea hydrolysis is indicated when the pH indicator moves from pale orange to pink as the pH rises. Several studies have indicated that urea agar base media is the preferred qualitative urease assay for isolating and distinguishing ureolytic microorganisms [24]. Within 48 hours of incubation, a bacterial culture grown from goat faeces and nursery had transformed the urea agar base medium from pale orange to pink as shown in Figure 3, indicating the production of urease enzyme. In contrast to nursery wastewater, bacterial colonies isolated from goat faeces were found to alter colour more rapidly.

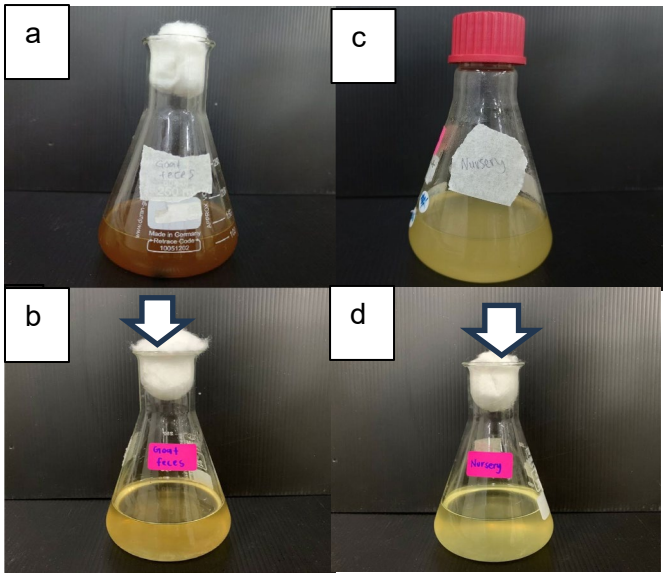


Figure 2. Enriched culture of goat faeces (a) before (b) after and nursery wastewater (c) before and (d) after

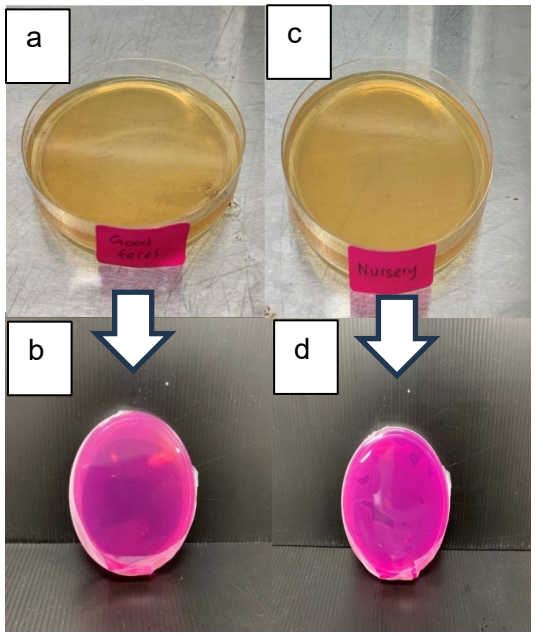


Figure 3. Presence of urease inside the agar plate (orange to pink colour indicating of urease present) of goat faeces (a, b) and nursery wastewater (c, d)

Biomineralization

The biomineralization test is commonly used to evaluate the potential of microorganisms to be used in bioremediation, soil enhancement as well to produce sustainable building materials. The process of biomineralization occurs when living cells biologically create minerals as a by-product of microbial activity in environments that promote interaction with specific cations [17]. In this study, the bacteria cultures from goat faeces and nursery wastewater sample were mixed with a cementation solution containing urea and $CaCl_2$. The mix process called MICP leads to a formation of neutral minerals settling at the bottom of the falcon tube. After five minutes of reaction, minerals appeared as white murky solids at the bottom of the falcon tubes in Figure 4, indicating the presence of MICP nucleation sites as a result of the addition of bacterial solution to encourage urease enzyme production. The visual observation made during the ureolysis-driven reaction time was in line with the findings that had been reported in the literature [18], [19].

The formation of these minerals is dependent on the activity of specific microbial enzymes, such as urease, which break down the urea to produce ammonia and carbon dioxide. The carbon dioxide then reacts with the calcium to produce calcium carbonate, which is deposited in the presence of microbial cells [20]. According to Omoregie ***et al***. [19] ureolytic bacteria play two crucial roles in the biomineralization test: first, they produce urease for urea hydrolysis, which then creates an alkaline environment suitable for carbonate precipitation; and second, they provide nucleation sites necessary for the formation of carbonate crystallisation, morphology, and yield of the precipitation. The capacity of the bacteria to travel freely throughout the water's pore spaces and an adequate amount of particle-particle interaction per unit volume are necessary for microbial cementation to have an impact on granular behavior. As a result, the faster development of calcium carbonate precipitates occurred at the injection points of the bacterial and cementation solution flowing freely down the columns, leading to a non-uniform distribution of calcium carbonate precipitates that hardened at the bottom surface of the falcon tubes[4].

The goat faeces sample produced different masses of precipitate at varying Cd^{2+} concentrations: 0.61 grams at 0 g/L, 0.49 grams at 2 g/L, and 0.58 grams at 4 g/L. Similarly, the nursery wastewater sample produced 0.49 grams at 0 g/L, 0.43 grams at 2 g/L, and 0.50 grams at 4 g/L Cd^{2+} concentrations. By analyzing the results of the biomineralization test, the tolerance of ureolytic bacteria to Cd^{2+} concentration can be assessed. The goat faeces sample consistently exhibited higher quantities of precipitate across all tested Cd^{2+} concentrations compared to the nursery wastewater sample. This suggests that the ureolytic bacteria in the goat faeces sample were more effective in inducing biomineralization and precipitate formation in the presence of Cd^{2+} [1].

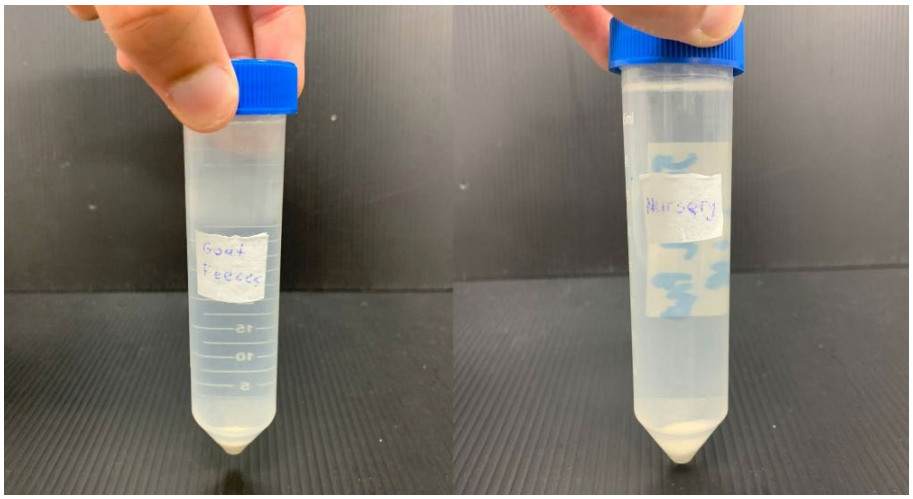


Figure 4. Precipitates formed after biomineralization process in goat faeces and nursery wastewater

Determination Of Urease Activities

Urease is an enzyme that catalyzes the hydrolysis of urea into ammonia and carbon dioxide. Urease activity is a measure of the amount of urease enzyme present in a sample and is commonly expressed in units of activity. The higher the urease activity, the greater the potential for urea hydrolysis to occur. Specific urease activity is a measure of the activity of urease per unit of protein or biomass present in the sample [21]. This measure takes into account the amount of biomass or protein in the sample, which can affect the overall urease activity. Specific urease activity is typically expressed in units of activity per milligram of protein or biomass. Both urease activity and specific urease activity are important parameters in the study of MICP, which is a biomineralization process that uses bacteria to precipitate calcium carbonate from soluble sources of calcium and bicarbonate.

Figure 5 shows the result of specific urease activity where both samples showed higher values in the pre-selection stage compared to the post-selection stage of a single bacteria colony. Specifically, for the goat faeces sample which obtained the higher specific urease activity, decreased from 25.34 to 3.64 mM urea hydrolyzed.min- 1.OD-1 after selecting a single bacterial isolate. Likewise, for the nursery wastewater sample, the specific urease activity decreased from 2.46 to 1.28 mM urea hydrolyzed.min-1.OD-1. These findings indicate that the selected bacterial isolate had lower urease activity compared to the initial diverse bacterial population. These results suggest that the process of selecting a single bacterial isolate resulted in the identification of a strain with reduced urease activity and less efficiency in urea hydrolysis. While the specific reasons behind this difference require further investigation, it is crucial to consider that the selection process may have led to the isolation of a bacterial strain lacking the same level of urease activity as the original sample.

The goat faeces sample exhibited higher urease activity and specific urease activity compared to the nursery wastewater sample before and after selecting a single bacterial isolate. A higher level of urease activity leads to increased precipitation of CaCO3. Various factors such as temperature, pH of the medium, nutrient concentration, incubation period, and urea concentration can impact the enzymatic activity of ureolytic bacteria. In a different study, it was observed that decreased urease activity results in carbonate minerals with larger crystal shapes[22]. This could be attributed to slower metabolic activity and nucleation during soil biocementation, indicating a longer duration for the formation of CaCO3 content in the soil matrix. Conversely, higher urease activity is preferable for achieving solidified soil biocementation[23]. These results suggest that the process of selecting a single bacterial isolate led to the identification of a strain with reduced urease activity and less efficiency in urea hydrolysis.

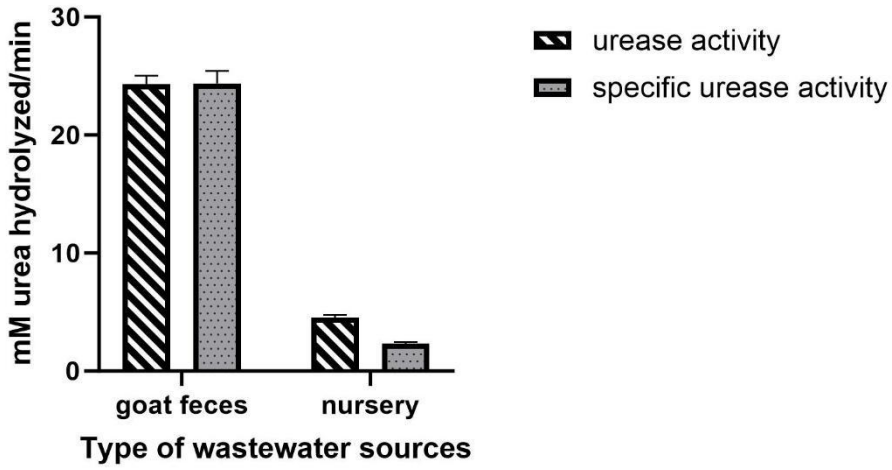


Figure 5. Urease activity and specific urease activity of ureolytic bacteria from goat faeces and nursery wastewater sample

Tolerance of Ureolytic Bacteria Towards Heavy Metal

The provided data allows for a comprehensive comparison and justification of the tolerance of ureolytic bacteria in goat faeces and nursery wastewater towards heavy metals. The parameters of conductivity, pH, and optical density (OD) were examined, with the concentration of all heavy metals fixed at 1000 mg/L. Table 1 indicates the comparison of ureolytic bacteria goat faeces and nursery wastewater sample on the tolerance towards cadmium.

Table 1. Tolerance performance of ureolytic bacteria from goat faeces and nursery wastewater towards cadmium

Sample	OD 600			Mass of precipitate (g)			Supernatant pH		
Cd^{2+} concentration(g/L)	0	2	4	0	2	4	0	2	4
Control	0.32	0.68	0.95	0.25	0.3	0.16	7.4	7.2	7.1
Goat faeces	0.54	1.06	1.70	0.61	0.49	0.58	7.0	7.20	7.13
Nursery wastewater	0.41	1.03	0.24	0.49	0.43	0.50	6.96	6.97	6.99

The selection of different concentrations of cadmium ions (Cd^{2+}) for heavy metal tolerance testing is motivated by the need to identify levels at which bacterial growth is hindered, as determined by optical density measurements. Heavy metals, including cadmium, can exert toxicity on bacteria involved in bioremediation processes. By examining bacterial responses across varying Cd^{2+} concentrations, the study aims to assess the impact of heavy metal toxicity on microbial growth. The optical density values obtained for different Cd^{2+} concentrations in both the goat faeces and nursery wastewater samples provide insights into the bacterial tolerance to cadmium and its potential implications.

The observed bacterial growth patterns in the presence of different Cd^{2+} concentrations reveal distinct responses in the two samples. In the goat faeces sample, there is a consistent increasing trend in optical

density with higher Cd^{2+} concentrations, indicating a positive influence on bacterial growth. Conversely, the nursery wastewater sample exhibits a different pattern, with bacterial growth increasing up to a certain Cd^{2+} concentration (2 g/L) and then declining at the higher concentration (4 g/L). These results suggest that the nursery wastewater bacterial culture tolerates Cd^{2+} up to a concentration of 2 g/L, beyond which the higher concentration has a detrimental effect on bacterial growth.

The biological and environmental significance of these differences in heavy metal tolerance between the goat faeces and nursery wastewater samples lies in their respective abilities to adapt and thrive in the presence of cadmium ions. The goat faeces sample demonstrates better tolerance to Cd^{2+} concentrations, as evidenced by consistently increasing bacterial growth even at higher Cd^{2+} concentrations. This indicates a higher level of Cd^{2+} tolerance and adaptability in the bacterial population from goat faeces. The ability of the goat faeces sample to maintain increasing bacterial growth and produce larger amounts of precipitate at all examined Cd^{2+} concentrations implies a higher proficiency in triggering biomineralization when Cd^{2+} ions are present.

While the pH responses to Cd^{2+} are similar in both samples, with both maintaining a neutral pH environment, the optical density and biomineralization test results provide a clearer distinction in Cd^{2+} tolerance. The ability to maintain bacterial growth and induce biomineralization at higher Cd^{2+} concentrations in the goat faeces sample underscores its superior tolerance compared to the nursery wastewater sample. Understanding these tolerance mechanisms is crucial for optimizing bioremediation strategies, and further investigations are needed to elucidate the underlying mechanisms and implications of bacterial responses to Cd^{2+} concentrations.

Analysis of Heavy Metal Removal

Figure 6 shows the removal percentages of cadmium by ureolytic bacteria through the MICP process in two different types of wastewaters: goat faeces and nursery wastewater. Overall, the ureolytic bacteria from goat faeces recorded a better removal performance compared to nursery for the cadmium. Statistical analysis using t test indicates there is significant difference between the removal performance of ureolytic bacteria from goat faeces and nursery wastewater for cadmium removal, P= 0.0009, p (0.0009 > 0.0001), R2 = 0.9981. The R-squared value of 0.9981 indicates that the model explains 99.81% of the variance in the data. This suggests that the variability in the removal percentages can be partially explained by the differences between the wastewater sources.

The profile reveals that control concentration of Cd^{2+} in the samples was 909.8 mg/L. The nursery wastewater sample had a Cd^{2+} concentration of 202.4 mg/L in the supernatant, resulting in a removal efficiency of 77.75% as shown in Figure 6. On the other hand, the goat faeces sample had a Cd^{2+} concentration of 99.49 mg/L in the supernatant, yielding a higher removal efficiency of 89.06%. These findings indicate that both samples were capable of effectively removing $Cd^{2+,}$ with the goat faeces sample exhibiting a higher removal efficiency compared to the nursery wastewater sample. The reason behind the superior Cd2+ removal performance of the goat faeces sample compared to nursery wastewater sample can be attributed to several factors. One possible explanation is that the goat faeces sample may contain a higher concentration of beneficial microorganisms with specific capabilities for removing heavy metals [4]. Furthermore, experimental variability and slight variations in the test conditions could have influenced the outcome as goat faeces was expected to obtained higher values of Cd^{2+} removal. Small differences in handling, measurement techniques, or sample preparation may have contributed to the observed differences in Cd^{2+} removal efficiency. Therefore, these as specific bacterial strains could enhance the bioremediation process and improve the efficiency of Cd^{2+} removal.

Additionally, the goat faeces sample might contain additional compounds or substances that facilitate the precipitation or adsorption of Cd^{2+} ions [25] These compounds, which could include organic matter, humic acids, or other chemical constituents, can bind with the heavy metals and aid in their removal from the solution. Moreover, the nutrient content of the goat faeces sample may support the growth and activity of microorganisms involved in Cd^{2+} removal, further enhancing the removal efficiency [26]. The physical and chemical characteristics of goat faeces sample, such as pH, conductivity, and buffering

capacity, may also create a more favorable environment for Cd^{2+} removal by influencing the precipitation or sorption of Cd^{2+} ions. In summary, the combination of a favorable microbial composition, additional Cd^{2+}-binding compounds, nutrient availability, and suitable environmental conditions likely contributed to the superior Cd^{2+} removal performance of the goat faeces sample compared to the nursery wastewater sample.

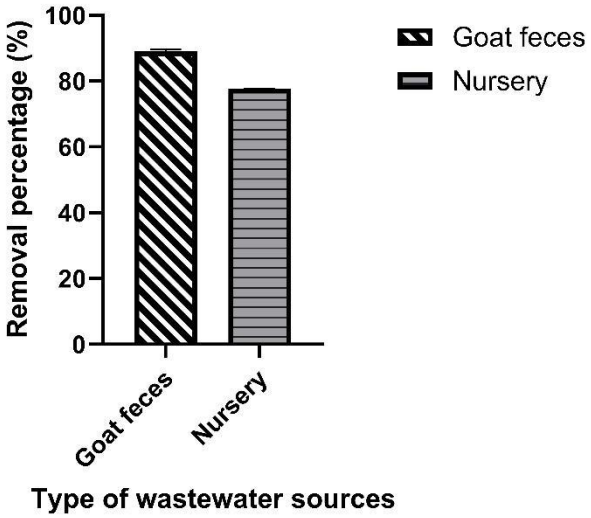


Figure 6. Removal percentage of ureolytic bacteria from goat faeces and nursery wastewater for cadmium heavy metals.

SEM Analysis

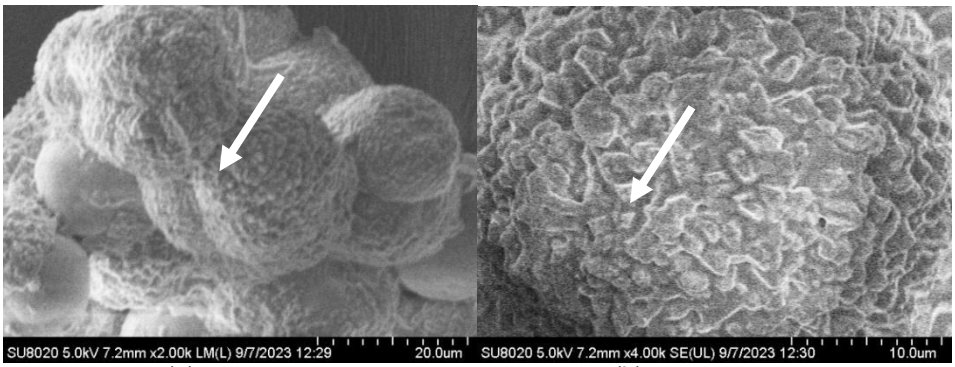
The Scanning Electron Microscopy (SEM) results presented in Figure 7 reveal the intricate morphological characteristics of the metallic calcite nanocrystals formed through MICP process. The nanocrystals exhibit a remarkable degree of uniformity, smoothness, and spherical shape [27]. This consistency in morphology is indicative of a well-controlled and organized biomineralization process driven by the ureolytic bacteria present in the Greywater samples obtained from both goat faeces and nursery wastewater.

The smooth, homogenous, and spherical features of the metallic calcite nanocrystals point towards a systematic and controlled nucleation and growth during the MICP process. The bacterial strains in the samples appear to induce the formation of calcite crystals with distinct characteristics, suggesting a specific microbial influence on the mineralization process. The homogeneity in the size and shape of the nanocrystals indicates a uniform and controlled biomineralization environment, emphasizing the precision with which the ureolytic bacteria are able to orchestrate the MICP process.

The observed morphology of the metallic calcite nanocrystals holds significant implications for the efficiency and mechanism of MICP in heavy metal bioremediation. The smooth and spherical characteristics of the nanocrystals suggest that the ureolytic bacteria play a crucial role not only in inducing calcite precipitation but also in promoting the formation of well-defined and stable mineral structures [27]. This structural stability is essential for the immobilization of heavy metals, as the formed crystals can create a robust framework that effectively captures and retains metal ions within the soil matrix.

Furthermore, the SEM results provide insights into the potential application of these metallic calcite nanocrystals in enhancing soil properties. The development of a skeletal mesh of micro bones between soil grains, as evidenced by the SEM images, implies that the MICP process can contribute to soil stabilization and improvement. This may have broader implications for environmental remediation and sustainable land management practices.

In conclusion, the SEM results showcasing smooth, homogenous, and spherical metallic calcite nanocrystals highlight the precision and efficacy of the MICP process mediated by ureolytic bacteria from Greywater samples. The observed morphology not only underscores the controlled nature of biomineralization but also suggests the potential for these nanocrystals to play a pivotal role in heavy metal immobilization and soil enhancement. Understanding the detailed characteristics of the formed nanocrystals provides valuable insights into the mechanisms underlying MICP-driven bioremediation and opens avenues for further exploration of its environmental applications [27].



(a) (b)

Figure 7. SEM results showing the precipitate obtained from treated wastewater sample for (a) goat faeces and (b) nursery wastewater sample

EDX Analysis

The selection of Energy-Dispersive X-ray analysis (EDX) for elemental analysis in this study serves as a powerful tool for discerning the constituent elements within the precipitate formed [28]. The EDX technique provides a direct and non-destructive method to investigate the elemental composition of materials, making it particularly suitable for analysing the intricate mineral structures produced during the MICP process.

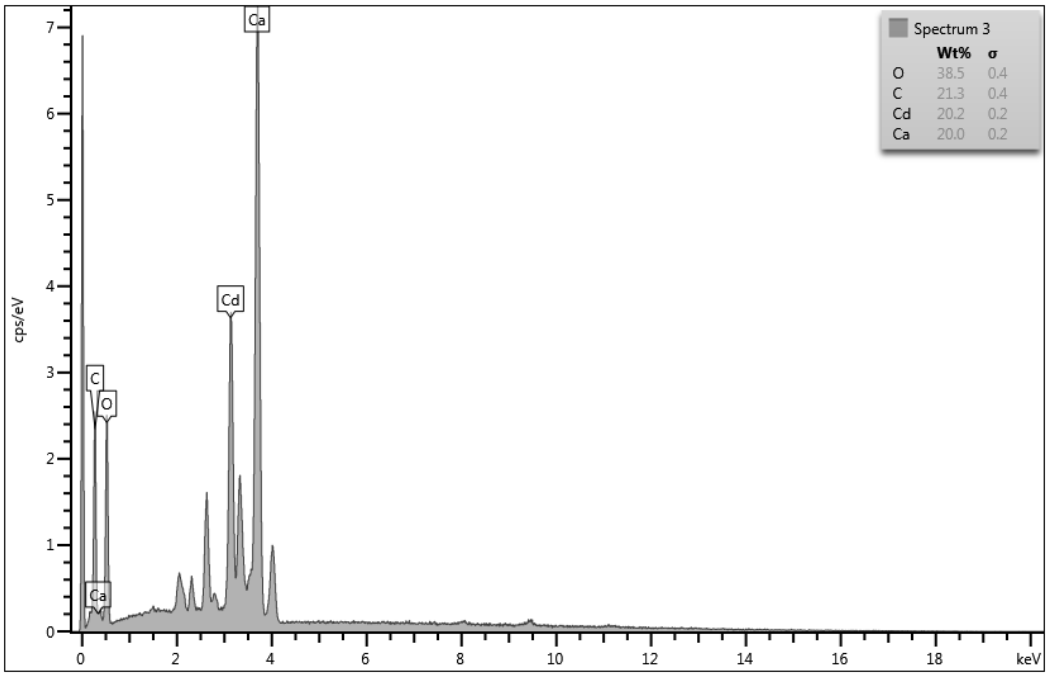
The EDX analysis of the precipitate, as depicted in Figure 8 (a) and (b), reveals a diverse array of elements present, with a notable abundance of calcium. This observation aligns with expectations, considering that calcium is a key component of calcium carbonate, the primary mineral formed during MICP. The naturally rich calcium content within the precipitate is crucial for its stability and effectiveness in immobilizing heavy metals.

However, when focusing on the EDX spectra of cadmium-adsorbed, showcased in Figure 8, a distinct peak emerges, specifically indicating the presence of cadmium. This new peak corroborates the process of cadmium sorption onto the surface of the precipitate, signifying an alteration in the elemental composition induced by the interaction with cadmium [29]. The appearance of this additional peak provides direct evidence of the immobilization of cadmium within the precipitate, supporting the efficacy of MICP in sequestering heavy metals.

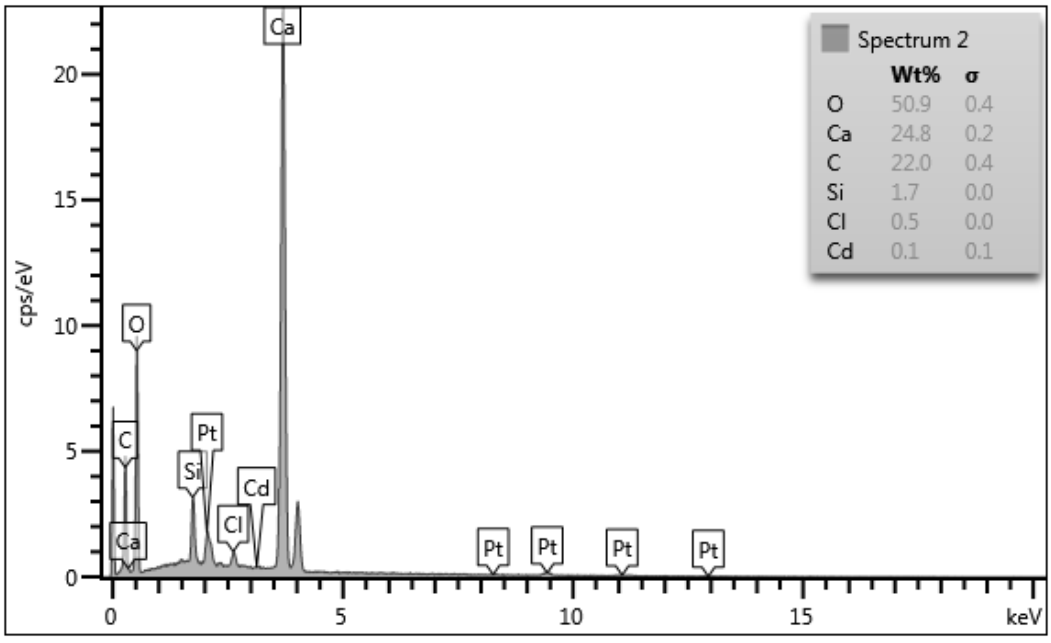
The data further reveals that cadmium is more highly concentrated in the precipitate from goat faeces compared to the nursery wastewater sample [28]. This disparity in cadmium immobilization suggests variations in the efficiency of the MICP process between the two types of wastewaters. The higher concentration of cadmium in the goat faeces sample indicates a more effective immobilization of this heavy metal by ureolytic bacteria from goat faeces compared to nursery wastewater. The reasons behind this difference could be attributed to variations in the microbial composition, nutrient content, or other environmental factors that influence the MICP process in different wastewater sources.

In summary, the EDX analysis provides valuable insights into the elemental composition of the precipitate, confirming the successful immobilization of cadmium within the formed mineral structures. The observed differences in cadmium concentration between goat faeces and nursery

wastewater samples emphasize the need to consider the specific characteristics of different wastewater sources when designing MICP-based bioremediation strategies. Understanding these variations contributes to refining and optimizing MICP processes for effective heavy metal immobilization in diverse environmental contexts.



(a)



(b)

Figure 8. EDX results showing the precipitate obtained from treated wastewater sample for (a) goat faeces and (b) nursery wastewater sample

Conclusions

This study successfully harnessed ureolytic bacteria derived from waste materials for effective heavy metal immobilization and soil enhancement. The research objectives were intricately aligned with key outcomes, emphasizing the potential applications of waste-derived ureolytic bacteria in environmental remediation and sustainable soil management. Notably, ureolytic bacteria sourced from goat faeces exhibited heightened tolerance to Cd^{2+} concentration and superior cadmium removal compared to those from nursery wastewater, which displayed lower Cd^{2+} removal efficiency and soil compressive strength. The findings underscore the intricate interplay between waste-derived ureolytic bacteria and heavy metal

ions, highlighting their crucial role in metal removal and soil improvement. The enriched urease-producing bacteria were thoroughly characterized, confirming their ability to immobilize Cd^{2+} and solidify soil particles. Morphological analysis using SEM and elemental analysis with EDX provided insights into the biomineralization process, showcasing the transformation of cadmium heavy metals into carbonate minerals. Acknowledging study limitations, future research directions should address these constraints, exploring microbial communities, optimizing treatment conditions, and assessing long-term effects on soil health. This study lays the groundwork for future endeavors, urging researchers to delve into diverse waste sources and collaborate for a sustainable future in environmental management and soil remediation.

Conflicts of Interest

The author(s) declare(s) that there is no conflict of interest regarding the publication of this paper.

Acknowledgement

The authors wished to thank the Ministry of Education Malaysia – Higher Education and Universiti Teknologi Malaysia (UTM) for the financial support of this 2024 Fundamental Research Grant Scheme (FRGS) (Grant No: R.J130000.7822.5F727).

References

[1] Zeng, Y., *et al.* (2023). Microbiologically induced calcite precipitation for in situ stabilization of heavy metals contributes to land application of sewage sludge. *Journal of Hazardous Materials, 441*, 129866. https://doi.org/10.1016/j.jhazmat.2022.129866

[2] Baby, R., Saifullah, B., & Hussein, M. Z. (2019). Carbon nanomaterials for the treatment of heavy metal-contaminated water and environmental remediation. *Nanoscale Research Letters, 14*(1). https://doi.org/10.1186/s11671-019-3167-8

[3] Kumar, V., *et al.* (2019). Pollution assessment of heavy metals in soils of India and ecological risk assessment: A state-of-the-art. *Chemosphere, 216*, 449–462. https://doi.org/10.1016/j.chemosphere.2018.10.066

[4] Qiao, S., *et al.* (2021). Multiple heavy metals immobilization based on microbially induced carbonate precipitation by ureolytic bacteria and the precipitation patterns exploration. *Chemosphere, 274*, 129661. https://doi.org/10.1016/j.chemosphere.2021.129661

[5] Duarte-Nass, C., *et al.* (2020). Application of microbe-induced carbonate precipitation for copper removal from copper-enriched waters: Challenges to future industrial application. *Journal of Environmental Management, 256*, 109938. https://doi.org/10.1016/j.jenvman.2019.109938

[6] Chen, M., *et al.* (2021). Study on soil physical structure after the bioremediation of Pb pollution using microbial-induced carbonate precipitation methodology. *Journal of Hazardous Materials, 411*, 125103. https://doi.org/10.1016/j.jhazmat.2021.125103

[7] Guo, H., *et al.* (2010). Bioremediation of heavy metals by growing hyperaccumulator endophytic bacterium *Bacillus* sp. L14. *Bioresource Technology, 101*(22), 8599–8605. https://doi.org/10.1016/j.biortech.2010.06.085

[8] Dan, A., *et al.* (2017). Removal of heavy metals from synthetic landfill leachate in lab-scale vertical flow constructed wetlands. *Science of the Total Environment, 584–585*, 742–750. https://doi.org/10.1016/j.scitotenv.2017.01.112

[9] Bhattacharya, A., Naik, S. N., & Khare, S. K. (2018). Harnessing the bio-mineralization ability of urease producing *Serratia marcescens* and *Enterobacter cloacae* EMB19 for remediation of heavy metal cadmium (II). *Journal of Environmental Management, 215*, 143–152. https://doi.org/10.1016/j.jenvman.2018.03.055

[10] Fang, L., *et al.* (2021). Ca-mediated alleviation of Cd2+ induced toxicity and improved Cd2+ biomineralization by *Sporosarcina pasteurii*. *Science of the Total Environment, 787*. https://doi.org/10.1016/j.scitotenv.2021.147627

[11] Whiffin, V. S. (2004). Microbial CaCO3 precipitation for the production of biocement.

[12] Al Disi, Z., Attia, E., Ahmad, M. I., & Zouari, N. (2022). Immobilization of heavy metals by microbially induced carbonate precipitation using hydrocarbon-degrading ureolytic bacteria. *Biotechnology Reports, 35*, e00747. https://doi.org/10.1016/j.btre.2022.e00747

[13] He, Z., *et al.* (2023). Synergistic mechanism and application of microbially induced carbonate precipitation (MICP) and inorganic additives for passivation of heavy metals in copper-nickel tailings. *Chemosphere, 311*, 136981. https://doi.org/10.1016/j.chemosphere.2022.136981

[14] Basri, H. F., Omoregie, A. I., & Mokhter, M. A. (2023). Influence of enriched urease producing bacteria from leachate and restaurant wastewater on heavy metal removal. *Malaysian Journal of Fundamental and Applied Sciences, 19*(6), 956–969. https://doi.org/10.11113/mjfas.v19n6.3130

[15] Omoregie, A. I., *et al.* (2023). Impact of palm oil mill effluent as an economic medium for soil fixation via microbially induced carbonate precipitation. *Biomass Conversion and Biorefinery*. https://doi.org/10.1007/s13399-023-03889-4

[16] Miles, S. M. (2023). A review of microbial induced carbonate precipitation treatment in the remediation of diverse mine tailings.

[17] Chen, X., *et al.* (2021). Microbially induced carbonate precipitation techniques for the remediation of heavy metal and trace element–polluted soils and water. *Water, Air, & Soil Pollution, 232*(7). https://doi.org/10.1007/s11270-021-05206-z

[18] Omoregie, A. I., Muda, K., Bin Bakri, M. K., Rahman, M. R., Yusof, F. A. M., & Ojuri, O. O. (2022). Calcium carbonate bioprecipitation mediated by ureolytic bacteria grown in pelletized organic manure medium. *Biomass Conversion and Biorefinery*. https://doi.org/10.1007/s13399-022-03239-w

[19] Ighodalo Omoregie, A., Khoshdelnezamiha, G., Ek Leong Ong, D., & Morin Nissom, P. (2017). Microbial-induced carbonate precipitation using a sustainable treatment technique.

[20] Almajed, A., Lateef, M. A., Moghal, A. A. B., & Lemboye, K. (2021). State-of-the-art review of the applicability and challenges of microbial-induced calcite precipitation (MICP) and enzyme-induced calcite precipitation (EICP) techniques for geotechnical and geoenvironmental applications. *Crystals, 11*(4). https://doi.org/10.3390/cryst11040370

[21] Pawar, V. S., Bhande, D., Pawar, S. D., Mudila, H., Kaushik, A., & Kumar, A. (2022). Investigating purification and activity analysis of urease enzyme extracted from jack bean source: A green chemistry approach. *Analytical Biochemistry, 659*, 114925. https://doi.org/10.1016/j.ab.2022.114925

[22] Murugan, R., Suraishkumar, G. K., Mukherjee, A., & Dhami, N. K. (2021). Insights into the influence of cell concentration in design and development of microbially induced calcium carbonate precipitation (MICP) process. *PLoS ONE, 16*(7), e0254536. https://doi.org/10.1371/journal.pone.0254536

[23] Chen, Q.-Y., *et al.* (2020). Response of sulfhydryl compounds in subcells of *Cladophora rupestris* under Pb stress. https://doi.org/10.1007/s11356-020-11577-3

[24] Mugwar, A. J., & Harbottle, M. J. (2016). Toxicity effects on metal sequestration by microbially-induced carbonate precipitation. *Journal of Hazardous Materials, 314*, 237–248. https://doi.org/10.1016/j.jhazmat.2016.04.039

[25] Song, M., Ju, T., Meng, Y., Han, S., Lin, L., & Jiang, J. (2022). A review on the applications of microbially induced calcium carbonate precipitation in solid waste treatment and soil remediation. *Chemosphere, 290*. https://doi.org/10.1016/j.chemosphere.2021.133229

[26] Cuthbert, M. O., *et al.* (2012). Controls on the rate of ureolysis and the morphology of carbonate precipitated by *Sporosarcina pasteurii* biofilms and limits due to bacterial encapsulation. *Ecological Engineering, 41*, 32–40. https://doi.org/10.1016/j.ecoleng.2012.01.008

[27] Ashraf, M. S., Azahar, S. B., & Yusof, N. Z. (2017). Soil improvement using MICP and biopolymers: A review. In *IOP Conference Series: Materials Science and Engineering*. Institute of Physics Publishing. https://doi.org/10.1088/1757-899X/226/1/012058

[28] Jain, M., Garg, V. K., Garg, U. K., Kadirvelu, K., & Sillanpää, M. (2015). Cadmium removal from wastewater using carbonaceous adsorbents prepared from sunflower waste. *International Journal of Environmental Research, 9*(3), 1079–1088.

[29] Memon, J. R., Memon, S. Q., Bhanger, M. I., Memon, G. Z., El-Turki, A., & Allen, G. C. (2008). Characterization of banana peel by scanning electron microscopy and FT-IR spectroscopy and its use for cadmium removal. *Colloids and Surfaces B: Biointerfaces, 66*(2), 260–265. https://doi.org/10.1016/j.colsurfb.2008.07.001