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RESEARCH ARTICLE

Immobilization of Cadmium Via Ureolytic Bacteria Isolated from Greywater Waste and Horse Faeces

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Abstract With the constant growth of technology and pollution caused by urban expansion, heavy metal contamination has become an alarming concern. The performance of Microbially induced carbonate precipitation (MICP) technology in immobilizing heavy metal has been demonstrated by several researchers. While various studies have successfully isolated ureolytic bacteria from a variety of sources, there are very limited studies that have focused on isolating them from local waste sources, specifically Greywater and Horse Faeces for MICP application, which benefits in terms of economical, sustainability, and efficiency for engineering purposes. The study aims to investigate the effect of urease-producing bacteria derived from Greywater and horse faeces on heavy metal immobilization. The methods include collecting waste samples, isolating and subculturing of ureolytic bacteria, testing the physiological characteristics of ureolytic bacteria, examining the tolerance test and evaluating heavy metal removal efficacy through Atomic Absorption Spectrophotometry (AAS) analysis, and last but not least, Scanning Electron Microscopy (SEM) and Energy-Dispersive X-ray (EDX) analyses of morphological and mineralogical properties of biominerals formed after heavy metal immobilization treatment. The study found that bacteria from Greywater were more effective at heavy metal immobilization than those from Horse Faeces. Additionally, AAS analysis indicates the greywater-derived bacteria from the residential area sample's exceptional competence in Cd^{2+} removal, with a significant 80.19% removal rate exceeding the horse faeces bacteria sample's removal rate of 65.26%. The morphological analysis confirmed the presence of heavy metal carbonate in treated heavy metal samples, as well as presenting insight into the effectiveness and application of local ureolytic bacteria's potential for heavy metal toxicity degradation.

Keywords: Heavy Metal, bioremediation, ureolytic bacteria, MICP, Horse Faeces, Greywater.

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Introduction

The pervasive issue of heavy metals in the environment is an urgent matter, given the widespread presence and detrimental consequences associated with their toxic nature. The root cause of heavy metal toxicity in the environment can be attributed to human activities, which continually introduce these hazardous elements into various ecosystems. Industrial processes, the extraction of minerals through

mining, and the extensive application of heavy metals in manufacturing and agriculture are all major contributors to this environmental contamination [1] [2]. Notable heavy metals that raise significant concern include lead, mercury, cadmium, arsenic, and chromium [3]. Over time, these heavy metals accumulate in soil, water, and even the atmosphere, infiltrating the intricate web of the food chain and creating substantial threats to both human well-being and the ecological balance [4]. The consequences of heavy metal toxicity are profound and extend across a wide spectrum. Human exposure to these toxic elements typically takes place through the ingestion of contaminated food and water, the inhalation of polluted air, or direct contact with tainted soil [5]. This exposure, unfortunately, opens the door to a host of health problems, encompassing neurological disorders, severe organ damage, developmental complications, and an elevated susceptibility to various forms of cancer [6]. Particularly vulnerable segments of the population, such as children and pregnant women, face an elevated risk, making the imperative for mitigating heavy metal exposure even more critical to safeguard the well-being of these susceptible groups. In the environment, heavy metals disrupt ecosystems and have detrimental effects on both terrestrial and aquatic life. They can lead to reduced biodiversity, harm fish and wildlife, and damage aquatic habitats [5]. Furthermore, contaminated soil can affect crop growth, reducing agricultural productivity and potentially compromising food safety [7]. The long-term consequences of heavy metal pollution in the environment can be severe, impacting ecosystem resilience and the availability of safe and nutritious food.

Conventional methods for immobilizing heavy metals in water or soil are essential for mitigating the adverse effects of heavy metal contamination. However, they come with certain disadvantages. One common approach to remove heavy metals from water is precipitation, which involves the addition of chemicals that form insoluble compounds with heavy metals, reducing their solubility and mobility [8]. The disadvantage here lies in the need for careful handling and disposal of the chemicals used, which can themselves pose environmental risks if not managed properly. Ion exchange, another method, utilizes resins to capture heavy metal ions, but these resins can be expensive, and their capacity to remove heavy metals may diminish over time, requiring replacement [8]. Adsorption using materials like activated carbon or zeolites is effective but may require frequent regeneration or replacement of the adsorbents, leading to increased operational costs. Stabilization and solidification techniques are reliable for immobilizing heavy metals in solid matrices like cement, but they can be resource-intensive and may not be suitable for large-scale applications [9]. Moreover, some of these methods might not eliminate heavy metal contaminants, leading to long-term monitoring and maintenance requirements. Phytoremediation, while eco-friendly, can be time-consuming, and the growth of hyperaccumulator plants can be slow [10]. Additionally, the method may not be suitable for all types of contaminants or in regions with adverse environmental conditions.

MICP, or Microbial Induced Carbonate Carbonate Precipitation (MICP), is a biotechnological process that involves the use of specific bacteria to induce the precipitation of calcium carbonate ($CaCO_3$) [11]. MICP is a biotechnological process that harnesses the concept of naturally occurring or engineered bacteria to immobilize heavy metals in contaminated environments. This eco-friendly approach involves the bacteria metabolizing calcium-rich compounds and producing carbonate ions, which then react with heavy metal ions, causing them to precipitate as insoluble metal carbonates [12]. MICP offers a range of distinct advantages as an environmentally responsible solution for heavy metal immobilization. It significantly reduces the need for harsh chemicals and energy-intensive processes, thereby minimizing the ecological footprint [13]. The technique is versatile and effective across various contamination scenarios, offering in situ treatment capabilities, long-term stability, and cost-efficiency [14]. By relying on the inherent mechanisms of microorganisms, MICP aligns with sustainable remediation practices, making it a promising and eco-conscious strategy for mitigating heavy metal contamination while preserving the health of the ecosystems.

Numerous studies have effectively utilized MICP technology to immobilize heavy metals [15]. One of the noteworthy investigations such as conducted by [16], explores the removal of heavy metals (Cd^{2+}, Cu^{2+}, and Pb^{2+}) from aqueous solutions through MICP driven by acclimatized *Sporosarcina pasteurii*. The experimental approach involves the sequential inoculation of *S. pasteurii* in nutrient broths supplemented with escalating concentrations of heavy metals to enhance resistance. The outcomes reveal that, when coprecipitated with Ca^{2+}, the removal efficiency for Cd^{2+}, Cu^{2+}, and Pb^{2+} ranged from 98.0% to 99.0%, 78.1% to 82.1%, and 98.0% to 100.0%, respectively. Not only that, a study [17] investigated the performance of *Bacillus pasteurii* enriched from the soil to remediate lead-zinc tailing caused by mining and the results of the experiments have shown a significant success of heavy metal removal in which removal rate of Fe^{3+}, Zn^{2+}, Pb^{2+}, Cu^{2+}, Cd^{2+} reaches 100 %, respectively and the removal rate of Mn^{2+} and Cr^{3+} reaches 98.24 % and 95.56 % respectively when adding 1 cm of lead–zinc tailings. Hence, MICP technology performance in immobilizing heavy metal has been proven to succeed in many records.

Despite all the outstanding studies of MICP in immobilizing heavy metals, the uneconomical cost of commercial MICP bacteria is one of the challenging issues that cannot be neglected. Therefore, one promising solution involves isolating native bacteria from local waste sources. The reuse of waste from greywater and animal manure, such as horse faeces were used in this study due to its suitable avenue for MICP bacteria. Greywater, a byproduct of domestic activities excluding toilet waste, and horse faeces serve as a potential reservoir of ureolytic bacteria. The utilization of these waste streams aligns with eco-conscious practices, offering a dual benefit for both the environment and research endeavors. By repurposing waste materials, studies contribute to reducing environmental burdens while presenting a cost-effective and practical approach to isolating ureolytic bacteria. The use of locally available bacteria from waste aligns with the principles of sustainability and the circular economy. This approach not only minimizes waste but also transforms it into a valuable resource for environmental research. Therefore, this study not only addresses the immediate concern of heavy metal contamination but also explores a sustainable pathway for waste utilization, emphasizing the interconnectedness of environmental preservation and scientific inquiry. Moreover, this study on waste reutilization aligns with the United Nations Sustainable Development Goal (SDG) 12: "Responsible Consumption and Production," by promoting sustainable practices that reduce waste and contribute to the efficient use of resources.

The laboratory procedures will be divided into three objectives to be acquired for the completion of the research study. The first objective is to collect the different types of waste samples and stimulate the ureolytic bacteria by the process of enrichment culturing and subculturing. The next objective is to investigate the physicochemical characteristics of ureolytic bacteria for heavy metal immobilization through four laboratory tests which are the Urea Agar Base Test, Biomineralization Test, Conductivity and pH test, and Optical Density test. Then, the following objective is to be carried out which is to determine the performance of the selected ureolytic bacteria in heavy metal immobilization by heavy metal tolerance test and identifying heavy metal removal via AAS analysis. Finally, to conduct a several analyses of morphological and minerology of treated solution via MICP by using SEM and EDX.

Materials and Methods

The overall framework for this study as shown in **Figure 1.**

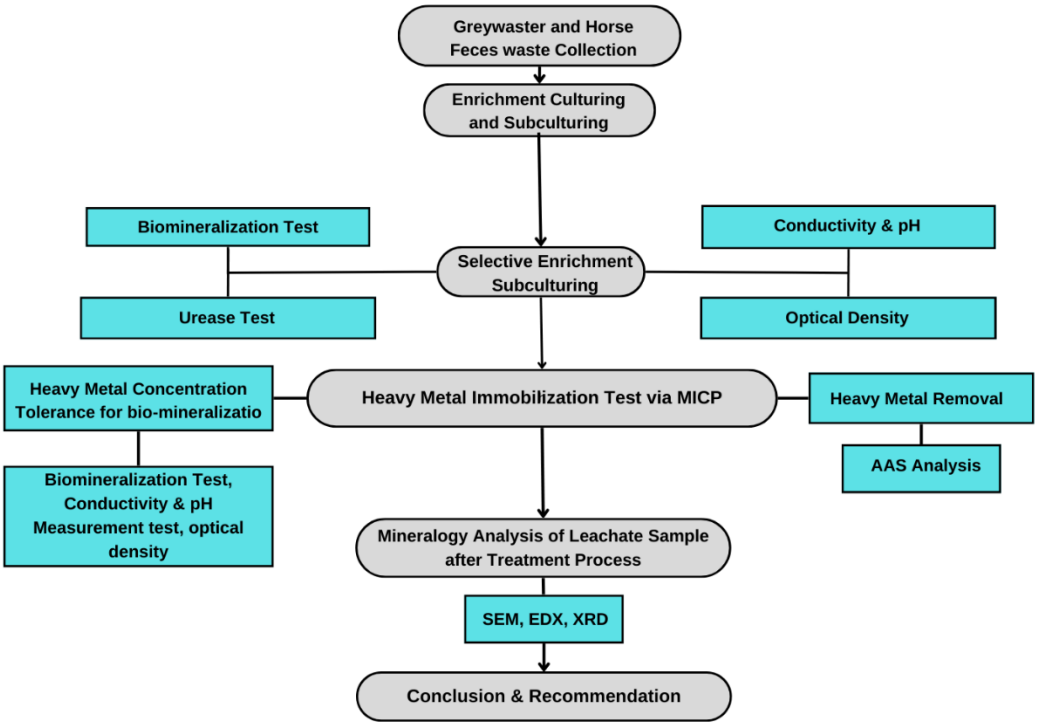


Figure 1. Flowchart of the experimental plan for this study

Collection of Samples

Two distinct types of wastewater samples were gathered for the purpose of conducting the Microbially Induced Calcium Carbonate Precipitation (MICP) experiment. In **Fig 2** and **Fig 3**, the initial specimen was collected at Arked Cengal, University Teknologi Malaysia, situated in Skudai, Johor Bahru, Johor (1.561763° N, 103.632284° E). This particular wastewater primarily comes from residential regions. To ensure the removal of undesired suspended solids or inert particles, each sample underwent meticulous filtration using a 1.0-millimeter mesh sieve. The second sample was derived from horse faeces at UTM Equine Park (1.555695° N, 103.641493° E) as shown in **Fig 4** and **Fig 5**, which is also affiliated with the Universiti Teknologi Malaysia. These two distinct sources of waste were chosen to facilitate the MICP experiment and enable a comprehensive assessment of microbial activities.

Figure 2. Google maps of Arked Cengal, UTM, Skudai, Johor

Figure 3. Location of sampling for Greywater at Arked Cengal, UTM, Skudai, Johor

Figure 4. Google maps of Nadi Equestrian Centre, UTM Equine Park, Skudai, Johor

Figure 5. Location of sampling for Horse Faeces at Nadi Equestrian Centre, UTM Equine Park, Skudai, Johor

Enrichment Culture of Samples

Enrichment culture serves as an isolation technique specifically crafted to create highly conducive growth conditions for a targeted organism, while concurrently establishing an unfavorable environment for potential competitors [18]. This method involves employing specialized growth media that promote the multiplication of a particular microorganism, thereby enhancing the concentration of the desired organism in a sample. The enrichment process involved introducing specific nutrients or manipulating environmental conditions to selectively support the growth of the organism of interest, fostering its dominance over other microbial entities in the culture. During this process, 1 L of cultivation growth medium was prepared containing distilled water, 13 g/L nutrient broth, 10 g/L ammonium chloride, and 60.06 g/L urea (added after autoclaving was done). Next, for each solid waste sample, 125 mL of nutrient broth medium will be inoculated with 10 g of waste samples in a sterilized flask and has been prepared inside the biosafety cabinet to provide personnel, environmental and product protection. The substances were put under aerobic batch conditions at 30 °C for 120 hours by using IKA KS 4000i control incubator shaker at 150 rpm. After 120 hours, ureolytic bacteria were expected to digest the substrate, and any other unrelated microorganism types were killed in the process. Furthermore, the sub-culturing of enriched culture onto new sterilized glassware was conducted by taking 12.5 mL of incubated enriched culture and transferring it into the 112.5 mL of nutrient broth medium for the purpose of population growth of the bacteria. The enriched cultures were incubated under aerobic batch conditions at 30 °C for 120 hours and will be shaken for its condition at 150 rpm. After 120 hours, the enriched cultures were observed by their turbidity and smell. The pungent smell indicated urea hydrolysis activity in which native bacteria from the waste source were able to break down the urea while the turbidity observed from the flasks were representing cell propagation.

Urea Agar Base Test

The Urea agar base was implemented as a qualitative urease analysis. This medium was used as an instant test to detect bacteria that secrete urease by observing the colour changes of the medium due to elevation of pH resulted by ammonia produced from the reaction taken placed [19]. The urea agar base test was conducted by pouring approximately 90 mL of urea agar base onto the petri dish. Then, 0.5 mL of each enriched culture was pipetted and gently inoculated onto the center of the Petri dish containing the urea agar base. The colour changes of the Petri dishes were observed in which the transformation of orange to pink indicated the presence of urease and the time taken for the changes to complete wholly was recorded. On the other hand, if there is no change of colour, urease does not present.

Optical Density and Ph

For decades, spectrophotometers have been used to assess the density of bacterial populations with turbidity called as optical density (OD) typically at a wavelength of 600 nm by Beer-Lambert law [20] . To conduct the experiment, 1 L of nutrient broth medium was prepared and sterilized under 121 °C for at least 30 mins by using saturated steam under 15 to 30 psi of pressure using the Hirayama Manufacturing Co. autoclave machine. Then, 10 mL of bacterial cultures were inoculated into sterile shake flask containing 125 mL of 1.5 M urea solution. Using a mechanical shaker, the solution was incubated under aerobic batch conditions at 30 °C for 24 hours at 150 rpm. 3 mm of the culture was sampled and placed into clean 10 mm cuvettes. After that, the optical density of the sample in the cuvette was measured using a spectrophotometer at OD_{600}. Before the OD of the bacterial cultures' growth in the medium was measured, the un- inoculated growth media was calibrated by the spectrophotometer as blanks.

Biomineralization test

The biomineralization test is an initial examination aimed at observing whether the isolated bacteria have the capability to generate calcite precipitates through chemical reactions with cementation solutions and urea. This specific test is crucial for assessing the quantity of precipitates produced by the ureolytic bacteria, providing insights into their potential for urease production. 45 mL of cementation solution was prepared in a falcon tube that consists of 40 g/L urea and 55 g/L $CaCl_2$. Then, 5 mL of bacterial cultures was poured into the cementation solution. The precipitation process was observed, and the time for the occurrence of precipitation to complete was recorded for each sample. Then, the pH of the supernatant (the liquid above the precipitates was taken by pouring it into a small beaker, and the falcon tube was oven dried briefly to obtain a solid precipitate within. The dried precipitate was weighed and recorded to obtain the amount of calcium carbonate attained from bacterial activity.

Specific Urease Activity via Conductivity Test and Optical Density

Urease activity is determined by measuring the relative conductivity changes in a solution containing urea (1.0–1.5 M) and bacterial cultures at room temperature [21]. One unit of urease activity represents the amount of enzyme required to catalyze the dissolution of 1 mM urea per minute. [21]. Besides, determining the acidity or alkalinity of the environment is required for measuring the pH of the bacterial culture when the chemical reaction between the bacteria and the urea solution occurs . This is because the relative change of conductivity is due to the reaction of ammonium and hydroxide ions. A conductivity metre was used in this conductivity test to evaluate the electrical changes that occurred after bacterial cultures were introduced into the cementation solution.To prepare the test, 10 mL of bacterial cultures were inoculated into 250 mL sterile beakers containing 90 mL of 1.5 M urea solution. The conductivity meter probe was immersed in the solution to obtain the initial conductivity reading (mS/cm). After a duration of 6 minutes and at 25±2 °C, the second conductivity reading, and pH of the solution was recorded. 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 1.0** and **Equation 2.0**, respectively.

$$\text{Urease activity} = \frac{C_6 - C_0}{5} \times d_f \times 11.11 \quad (1)$$

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

$$\text{Specific urease activity} = \frac{urease\ activity}{Biomass} \quad (2)$$

Urease activity was determined using Equation 1.0 by multiplying the conductivity changes rate (mS.cm^{-1}.min-1) with the dilution factor (df) and correlation rate, 11.11. 1 mS.cm^{-1}.min^{-1} is equivalent to a hydrolysis activity of 11 mM urea.min-1 in the recorded range of activities when the culture is diluted by

a factor of 10 during the activity measurement. While specific urease activity represents the amount of urease activity per biomasss (OD_{600}) as defined in equation 2.0.

Tolerance of the Bacterial Strains to Heavy Metals

Some of microorganisms living in effluent-polluted environments are known for acquiring resistance to heavy metals [22]. For example, the bacteria *Stenotrophomonas maltophilia* was discovered to be multi-metal resistant, which could potentially be beneficial in bioremediation treatments [23]. Therefore, this experiment was aimed to determine the tolerance of isolated bacteria towards different concentration of selected heavy metal. For each bacteria sample, 3 falcon tubes were prepared and sterilized. 45 mL of cementation solution was prepared and poured in a sterilized falcon tube that consists of 40 g/L urea and 55 g/L $CaCl_2$. Then, in each falcon tube, 0 g/L, 2g/L, and 4g/L of $CdCl_2$ concentration were weighed, poured, and stirred evenly. After that, 5 mL of bacterial cultures was poured into each of the falcon tubes. The precipitation process was observed, and the time for the occurrence of precipitation to complete was recorded for each sample. Then, the pH of the supernatant (the liquid above the precipitates) was taken by pouring it into a small beaker, and the falcon tube was oven dried briefly to obtain a solid precipitate within. The dried precipitate was weighed and recorded to obtain the amount of calcium carbonate attained from bacterial activity. Then, 3 mL of the supernatants were pipetted and placed into clean 10 mm cuvettes. 3 mL of control solution without bacteria cultures were prepared and pipetted into different clean cuvettes and was calibrated by the spectrophotometer as blanks. After that, the optical density of the sample in the cuvette was measured using a spectrophotometer at a wavelength of 600 nm (OD_{600}).

Removal of Heavy Metals Through MICP Process

Following the assessment of bacterial tolerance to varying concentrations of heavy metals, a test on heavy metal immobilization was performed by selecting the most suitable concentration of heavy metal for the bacterial culture. To assess the heavy metal removal test, the supernatants from the biomineralization test that included 2 g/L of cadmium were used to determine the percentage of heavy metal that is still present in the supernatants after the precipitation of biomineralization test was completely done. A control sample was created and pipetted into a 1.5 mL polypropylene microcentrifuge tube. It contained a cementation solution and 2 g/L of cadmium as a heavy metal and no bacterial cultures. Each of the bacterium samples used in the biomineralization test had its supernatants pipetted into a similar tube before being teste d. Then, Atomic Absorption Spectroscopy (AAS) analysis using the wavelength of light was conducted to determine the heavy metal concentration in the sample as the Beer-Lambert Law applies. Atomic absorption spectroscopy (AAS) is a versatile method that uses absorption, emission, or fluorescence techniques to qualitatively and quantitatively analyse 70-80 elements, with detection limits ranging from ppm to ppt, and is recognised for its accuracy and precision down to trace (g/ml) and ultra-trace (sub-g/ml) levels [24]. The removal efficiency for heavy metals were calculated as shown in **Equation 3.0**:

$$\text{Removal efficiency (\%)} = \frac{Ci-Cf}{Ci}\ x\ 100 \quad (3)$$

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

Morphological and Mineralogical Properties of Biominerals

The morphological and mineralogical analysis of biominerals through techniques like scanning electron microscopy (SEM) and energy-dispersive X-ray spectroscopy (EDX) holds a great importance in detailed understanding of the mechanism in MICP treatment technology. In this study, SEM and EDX analysis were performed to obtain a graphical and microstructure displays of treated heavy metal solutions. Together, these analytical methods contribute to a comprehensive understanding of biomineralization processes taken placed throughout the experiments.

SEM-EDX Analysis

According to [25], to detect the presence of $CaCO_3$ crystals in soil samples subjected to MICP treatment with enriched ureolytic bacterial cultures, a sequence of imaging and analytical methods was employed. Initially, the soil samples were dried at 60 °C in an oven and subsequently coated with a conductive material like gold or carbon using sputter coating to prevent charging and improve image quality by enhancing surface conductivity. These prepared samples were then placed on the stage of a scanning electron microscope (SEM) and imaged using an electron beam at an operational voltage of 15.0 kV and a working distance of 10.6 mm.

For the identification and characterization of $CaCO_3$ crystals, high-resolution images of the sample surface were generated using the backscattered electrons detector, and analysis was conducted using

an energy-dispersive X-ray spectrometer (EDX). EDS analysis involves detecting X-rays emitted from the sample upon electron bombardment, with these X-rays being characteristic of the elements present in the sample. The energies of these X-rays were utilized to determine the chemical composition of the $CaCO_3$ crystals. The SEM–EDX analysis was conducted at various surface areas of the samples to ensure analysis accuracy and confirm the presence of $CaCO_3$ crystals, facilitating the identification of their elemental composition. The SEM instrument utilized was a TM4000 from Hitachi, Ltd., Tokyo, Japan, operating at a deceleration voltage of 0 V, a vacuum of 30 Pa, and an emission current of 90,000 nA. EDX spectra were acquired by focusing the electron beam on the area of interest and collecting X-ray signals across a range of energies. Data processing was carried out using ESPRIT 2 software from Bruker, MA, USA, to identify the elemental components of the crystals, and all analyses were replicated to ensure result accuracy and consistency [25] .

Statistical Analysis

This study's data analysis and figure plotting were performed using GraphPad Prism® software (version 9), California, United States of America. All experiments consist of two variables; therefore, the significance of the difference was determined using t-test analysis. The statistical significance was set at 0.05.

Results and Discussion

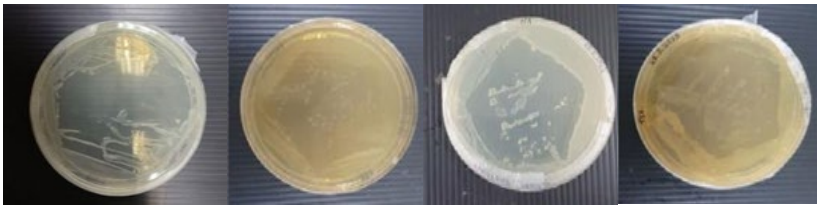
Screening Of Ureolytic Bacteria from Greywater and Horse Faeces Samples

Bio stimulation or enrichment culturing is performed for screening MICP bacteria by promoting the growth of indigenous bacteria with favourable metabolic traits using a specialized enrichment and nutrient medium [26]. The benefits of bio stimulation for obtaining targeted bacteria traits have been highlighted by several research and one of the advantages is to improve the adaptability and survivability of ureolytic bacteria [27] within the provided growth medium (by eliminating undesirable microorganism) and therefore lesser competitive environment will consequently improve the bacteria density through their rapid growth. The concentrated bacteria provided with growth medium will be prone to improve in metabolic activity and ultimately will optimize the effectiveness of MICP process [28] [29]. Additionally, urea is an important substrate for screening ureolytic bacteria during enrichment culture since it promotes microbial metabolism and stimulates the MICP process [28]. Urea, a nitrogen-rich molecule, is the principal source of nitrogen for ureolytic bacteria throughout their development and metabolic activity.

The enrichment culture technique was employed to identify bacteria with elevated urease production. This approach induces competition among various bacterial types for essential nutrients, facilitating the elimination of bacteria incapable of surviving in high urea concentrations [30] [31]. The urease enzyme produced by ureolytic bacteria hydrolyzes urea in nutrient broth medium to produce ammonia and carbonic acid. Ammonia is equilibrated in water to form ammonium and hydroxide ions. The hydroxide ions resulted in an increase in pH to create an alkaline environment. The pungent smell from the incubated bacteria subcultures suggested the presence of ammonia and therefore indicates the presence of potential ureolytic bacteria. Not only that but the difference of turbidity of the bacteria culture after incubated for 24 hours indicates the growth of targeted bacteria in the medium. **Figure 6** indicate the turbidity difference between before and after the enrichment culturing of the bacteria samples. The cloudiness of the turbidity after the enrichment culturing were associated with the proliferation of bacteria in a liquid culture medium [32]. This is because as bacteria multiply and grow in a liquid medium, the number of bacterial cells in the medium increases. Bacterial cells, being microscopic, are too small to be individually visible. However, as their numbers grow, they collectively contribute to the formation of suspended particles in the liquid medium, resulting with the changes of haziness in the medium. The pure, serial diluted colony for both bacteria sources was shown in **Figure 7** below.

(a) (b) (c) (d)

Figure 6. (a) to (b) Horse Faeces sample changes before and after 24 hours, (c) to (d) Greywater changes before and after 24 hours



(a) (b) (c) (d)

Figure 7. (a) to (b) ureolytic bacteria of Horse Faeces in Nutrient Agar (NA) and Tryptic Soy Agar (TSA) petri dish respectively, (c) to (d) ureolytic bacteria of Greywater in NA and TSA petri dish respectively

The urease test determines the presence of ammonia due to the hydrolysis of urea by the urease enzyme in the agar base that contributed to the changes in agar base colour due to the alkaline environment. Based on **Fig 8,** the urease test for both Greywater and Horse Faeces has shown the presence of urease and it is confirmed that the bacteria culture is indeed a ureolytic bacteria that produce urease enzyme to hydrolize urea to form ammonia and carbon dioxide that alkalinize the medium and influence the changes of colour of UAB from light orange to magenta. Urea hydrolysis in Urea Agar Base by ureolytic bacteria using urease as an enzyme can be described based on the equation below,

$(NH_2)2CO + 2\ H_2O \rightarrow CO_2 + H_2O + 2\ NH_3$

Urea → Carbon dioxide + Water +Ammonia

Ammonia and CO_2 are produced when urea is hydrolyzed. The solution becomes more alkaline due to the creation of ammonia, and the pH change is indicated by phenol red, which turns from pale orange at pH 6.8 to magenta (pink) at pH 8.1 [33]. The result of urease test for this study can be supported by following paper [34] with similar outcomes in which, out of the 31 isolates subjected for urease test, only five bacteria strains displayed ureolytic activity.

The outcomes of screening performance for both bacteria strains are closely related with the wise choice of bacteria sources. The circumstances and suitability of the two sampling sources used in this experiment to foster the growth of ureolytic bacteria were taken into consideration. Greywater and horse faeces emerge as distinctive types of waste with highly potential due to their high urea content as well as the ammonium production, offering a suitable avenue for the cultivation of microorganisms capable of MICP. Greywater, often loaded with various organic and inorganic compounds from residency, and horse faeces, recognized for their richness in urea, collectively provide an ideal environment for the growth of ureolytic bacteria that catalyze the MICP process. Urea, a nitrogen-rich compound, becomes a pivotal substrate for microbial metabolism, fostering the release of byproducts that stimulate the precipitation of calcite, a crystalline form of calcium carbonate. Therefore, the presence of urea as substrate in screening the ureolytic bacteria during the enrichment culturing is vital for the isolation of bacteria itself.

(a) (b) (c) (d)

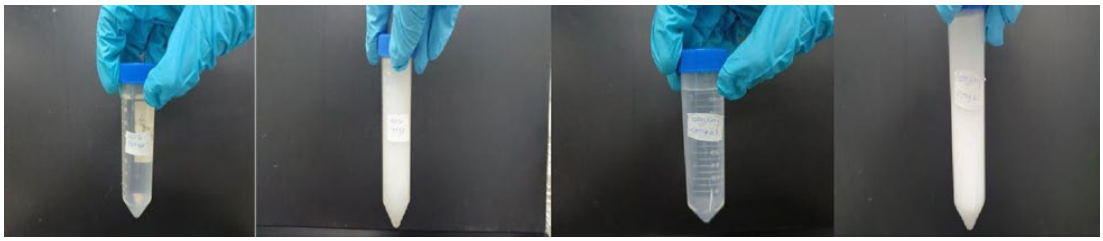
Figure 8. (a) to (b) Horse Faeces petri dish changes from orange to pink, (c) to (d) Greywater petri dish changes from orange to pink

Biomineralization

To test the bacteria potential as ureolytic bacteria that can induce calcite precipitates, several laboratory works must be done to ensure that the isolated bacteria can perform the heavy metal immobilization treatment by the end of this study. One of the tests is the biomineralization experiment. The biomineralization test was conducted to assess the bacteria capability for producing carbonate precipitates, $CaCO_3$ in the presence urea and calcium chloride with the optimal molar ratio of 1:1 [35] known as cementation solution. The dynamics of $CaCO_3$ precipitation are determined by the bacteria's urease production which influences the general efficacy of biocementation performance [36]. Hence, this test aimed to determine the capability of the ureolytic bacteria present in the waste samples to cultivate calcium carbonate precipitates and subsequently predetermine its urease producing performance.

The preliminary test yielded reliable results, as shown in **Figure 9** indicating a chemical reaction between the subculture and the cementation solution. The moment when bacterial cultures were injected during the biomineralization test in the Falcon tubes, visible cloudy precipitation was observed and the integration with bacterial culture resulted in immediate precipitation. Crystalline deposits or flocculation were formed at the bottom of the Falcon tubes after the incubation, showing similar results with previous study by Omoregie *et al*. (2022) [21]. Based on the provided data, the Greywater sample appears to give the best result in the biomineralization test. This conclusion is based on two factors: the weight of the precipitate and the pH value of the supernatants. The Greywater sample produced a higher weight of precipitate (0.44 g) compared to the Horse Faeces sample (0.36 g), indicating a potentially more efficient biomineralization process. Additionally, the pH reading for the Greywater sample (7.98) was slightly higher than that of the Horse Faeces sample (7.95), suggesting a more favorable alkaline environment for the biomineralization process to occur. With higher weight of precipitate and a slightly higher pH value, the outcomes have supported the conclusion that the Greywater sample exhibited a better outcome in the biomineralization test, indicating the presence of more effective ureolytic bacteria and a more favorable environment for mineral precipitation.

There are several factors influencing the dynamic of urease producing performance that partially contributed to the significant result of biomineralization test by ureolytic bacteria isolated from greywater and a huge content of inorganic matter urea is one of its major reasons. Greywater sample contains considerable amount of urea-derived waste that typically found in living organism residue such as urine and effluent from bathroom [37]. Various ureolytic bacteria hydrolyze urea into ammonia, particularly utilized as a nitrogen source, in which required for the bacteria growth and metabolism [38]. Therefore, a great amount of urea supplied to the microbial community will encourage the thrive density of ureolytic bacteria in greywater source and ultimately increase the urease production activity.



(a) (b) (c) (d)

Figure 9. (a) to (b) Horse Faeces changes during biomineralization test, (c) to (d) Greywater changes during biomineralization test

Determination of Specific Urease Activity

Urease activity is determined by measuring the relative conductivity changes in a solution containing urea (1.0–1.5 M) and bacterial cultures at room temperature and Specific urease activity is a measure of how rapidly urea molecules are converted into ammonia and carbon dioxide by urease enzyme per biomass (OD_{600}) [39] . The greater the specific urease activity, the more frequently urea molecules are transformed into ammonia and carbon dioxide in a short period of time. As the urea is hydrolyzed, the pH of the solution increases due to the concentration of hydroxide ions and ammonium ions. To determine the efficiency of the urease enzyme of the bacteria samples to hydrolyze the urea per unit of biomass, specific urease activity has been calculated by measuring the conductivity and pH using a conductivity meter and the density of bacteria growth using OD_{600}. Since conductivity is a measure of ion exchange membranes [40], the presence of more ions, such as ammonium and hydroxide ions, increases the conductivity. Therefore, when urease activity is high, there will be a faster increase in conductivity due to the rapid hydrolysis of urea and the subsequent release of ammonia, leading to an increased concentration of pH in solution.

Figure 10 shows the results of urease activity and specific urease activity for two types of waste samples: Greywater and Horse Faeces. In this analysis, Greywater exhibited higher values for both urease activity and specific urease activity which is 4.22 mM urea hydrolyzed/min and 9.10 mM urea hydrolyzed/min respectively, indicating a greater ability to break down urea per unit of protein compared to Horse faeces, which had a urease activity and specific urease activity values of 2.22 mM urea hydrolyzed/min and 2.25 mM urea hydrolyzed/min respectively. The measurements of conductivity change over time (Ms/cm/min), diffusion factor (df), urea hydrolyzed per minute (mm), and optical density (OD_{600}) further support the assessment of specific urease activity. These findings highlight the notable result of urease activity by Greywater's urealytic bacteria, suggesting its potential as a more efficient catalyst for urea hydrolysis compared to Horse faeces-derived bacteria.

The complex character of urban greywater can help explain the high urease activity in bacteria cultures from greywater near a restaurant and residential area, as opposed to horse manure. Greywater from restaurant and residential outlets in urban areas collects a wide range of organic and inorganic substances from cleaning supplies and leftover kitchen garbage. This diverse mixture creates a nutrient-rich environment that is favourable for the growth of bacteria while boosting the specific urease activity. Furthermore, the high values of inorganic matter such as nitrogen from urine contamination, and phosphorus from dishwashers and laundry are not only increase the alkaline pH levels in greywater, but also increase the conductivity of the greywater waste. The assertions made can be supported by the findings presented in the study by Potivichayanon *et al*. (2021) [41] where greywater was said to have high in electrical conductivity (EC) due to high inorganic matter.

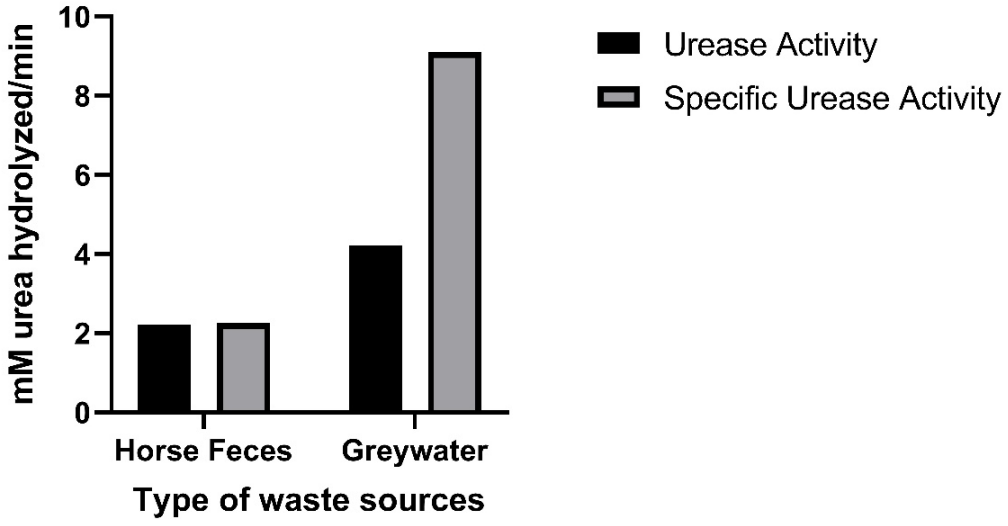


Figure 10. Urease activity and specific urease activity of ureolytic bacteria from Greywater and Horse Faeces

Tolerance of Ureolytic Bacteria Towards Heavy Metal

The purpose of the heavy metal tolerance test is to produce pre assessment of bacteria resilience toward specific heavy metal by evaluating its biomineralization, pH and bacteria density in the presence of metallic ions [42]. Cadmium, being the heavy metal used in this study, is one of the top five most hazardous heavy metal [43], in which suitable to be demonstrated due to its infamous contribution for many environmental risks. The data provided below enables a thorough analysis and explanation of the ureolytic bacteria's resistance to heavy metals in horse faeces and Greywater. For this comparison, several tests were conducted with varying amounts of cadmium (0 g/L, 2 g/L, and 4 g/L), and characteristics such as biomineralization, pH, and optical density (OD) were examined. The comparison of tolerance between the ureolytic bacteria in Greywater and Horce Faeces towards heavy metals is depicted in **Table 1**.

Table 1. Tolerance performance of ureolytic bacteria from Greywater and Horse Faeces for Cadmium

	Weight of precipitates (g)			pH			Optical Density (OD_{600})		
Concentrations of Cd^{2+} (g/L)	0 g/L	2 g/L	4 g/L	0 g/L	2 g/L	4 g/L	0 g/L	2 g/L	4 g/L
Greywater	0.762	0.599	0.654	9.27	8.14	7.97	0.44	0.67	0.67
Horse Faeces	0.564	0.475	0.473	9.45	8.81	8.00	0.38	0.52	0.41

Based on the provided data on the weight of precipitates, the Greywater sample appears to give the best result in the biomineralization test. This conclusion is based on two factors: the weight of the precipitate and the pH value. The Greywater sample consistently produced higher weights of precipitates across all cadmium concentrations compared to the Horse Faeces sample. The t-test statistical analysis revealed that there were significant differences in biomineralization test. Specifically, the analysis of biomineralization test for both samples showed that the calculated P-value of 0.0174 was lesser than the significance level of 0.05 and degrees of freedom, df of 2. This indicates that the biomineralization between Greywater and Horse Faeces is statistically significant.

The correlation between the decrease in pH levels and the escalating concentration of heavy metals signifies the lower adaptability of bacteria in higher concentrations of cadmium heavy metal, demonstrating their limitation in sustaining performance in MICP at certain concentrations and therefore reducing their tolerance to heavy metal exposure. However, compared to the previous pH reading of bacteria physicochemical characteristic conducted earlier, the pH values in the presence of heavy metals are relatively higher than the pH values in the absence of heavy metal ions. This phenomenon can be attributed to the bacteria's ability to produce increased amounts of ammonia as the pH elevates, consequently creating an alkaline environment. Despite the challenging presence of high concentrations of heavy metals, the bacteria showcase resilience by exhibiting an upsurge in urease enzyme production.

Furthermore, **Table 1** indicates that ureolytic bacteria in horse faeces tend to have lower OD_{600} values than those in Greywater. This implies that in the presence of heavy metals, ureolytic bacteria in Greywater accumulate more biomass and cellular growth. Not only that, but the bacteria of both samples also displayed increase of growth in the increase of Cadmium concentrations. Some bacteria often possess adaptive mechanisms that allow them to thrive in challenging environments, including the presence of heavy metals [22]. Based on the previous study by [23], constant exposure to elevated concentrations of heavy metals over time can lead to the development of defence mechanism of bacteria with genetic traits that confer resistance or tolerance to these metals and this concept applies to the bacteria isolated from greywater and horse faeces that often accumulate and able to strive in the toxic environment.

The tolerance test provides compelling evidence for the hypothesis that ureolytic bacteria, particularly those found in Greywater, have a remarkable tolerance to the heavy metal cadmium. This exceptional phenomenon is further supported by the repeated observations that the Greywater samples had higher biomineralization, pH, and optical density values than the Horse Faeces sample. There are multiple reasons for the increased tolerance. First off, the organic chemicals and nutrients such as lipids, proteins, and carbohydrates that are frequently discovered in the wastewater of nearby restaurants give the bacteria more energy sources, boosting their metabolic activity and encouraging resilient development

even in the presence of cadmium toxicity [44]. The ureolytic bacteria benefited from this abundance of varied nutrients, which also makes them competent to withstand harsh conditions. Identifying the mechanisms that explain the high cadmium tolerance of ureolytic bacteria in Greywater offers important perspectives for creating innovative wastewater treatment approaches and reducing heavy metal contamination by urbanisation.

Analysis of Heavy Metal Removal

The percentage of cadmium removed by ureolytic bacteria using the Microbially Induced Calcium Carbonate Precipitation (MICP) technology in two distinct wastewater types which were Greywater and Horse Faeces is shown in **Figure 11**. In the investigation of heavy metal removal using ureolytic bacteria, the efficacy of Greywater and Horse Faeces samples was evaluated for a solution containing 2 g/L of Cadmium. The results revealed a significant disparity in their performance, with Greywater-derived bacteria demonstrating an exceptional ability to remove Cadmium compared to ureolytic bacteria isolated from Horse Faeces. Specifically, the Cadmium removal efficiency was measured at 80.19% for Greywater's bacteria, while Horse Faeces's bacteria exhibited a slightly lower removal efficiency of 65.26%.

The discrepancy in performance could be attributed to several factors. Ureolytic bacteria present in Greywater may possess a more robust enzymatic activity, particularly urease production, facilitating the precipitation of Cadmium in the form of insoluble compounds as proven in previous biomineralization test. Additionally, the microbial community in Greywater might be more adapted to metal bioremediation processes as proven in the previous tolerance test, due to the exposure with abundance of substances from detergents and cleaning agents that contributes to the microbial community's high adaptation to heavy metals, leading to Cadmium removal capabilities. Not only that, Horse Faeces, although containing ureolytic bacteria, have a lower density of these microorganisms or a less favorable microbial composition for efficient Cadmium removal compared to Greywater as mentioned in the previous Optical Density test for ureolytic bacteria's' tolerance of heavy metal. Therefore, the exceptional cadmium removal performance of ureolytic bacteria from Greywater can be attributed to their high tolerance towards cadmium, excelling in all tolerance parameters, including biomineralization performance, pH, and OD_{600}.

The ability of ureolytic bacteria in Greywater to withstand heavy metals better than bacteria in animal manure can be attributed to several factors. one of the key factor is due to the environmental exposure in which Greywater is often exposed to a variety of substances, including detergents, cleaning agents, and food residues [45]. These conditions may lead to the development of a microbial community that has adapted to a broader range of environmental stressors, including heavy metals. In contrast, bacteria in animal manure may not have encountered heavy metals as frequently, resulting in a lower tolerance. Moreover, ureolytic bacteria in Greywater have a higher capacity for binding and sequestering heavy metals. This is due to the presence of specific biomolecules or functional groups as defence mechanism on the bacterial cell surfaces that facilitate metal binding, reducing their toxic effects on the bacteria [46].

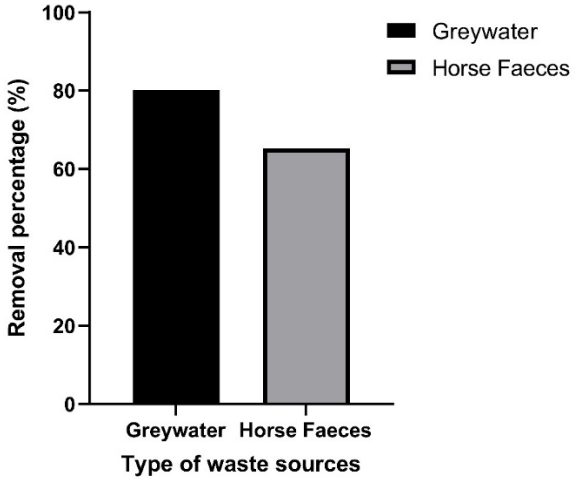


Figure 11. Removal percentage of ureolytic bacteria from Greywater and Horse faeces waste for cadmium heavy metals

Microscopic Analysis of Mineral Composition Analysis of Heavy Metal Immobilization Precipitation by Ureoltic Bacteria

Microstructure tests are carried out using microscopic analysis including scanning electron microscopes (SEM) and Energy-Dispersive X-ray Spectroscopy (EDX), to identify and describe embedded materials after the heavy metal immobilization by the ureolytic bacteria isolated from Greywater and Horse faeces.

Figure 12 shows the image of the SEM of the crystals formed by isolate Horse Faeces. SEM showed that calcite nanocrystals produced by ureolytic bacteria of horse faeces are homogeneous in shape and size. According to SEM analysis, it seems that calcite nanoparticles have been integrated by the bacteria trapped inside them. Moreover, the size of these particles was in the range of 13-30 µm. Meanwhile, the SEM image of the crystals produced by ureolytic bacteria that have been isolated from greywater waste is displayed in **Figure 13.** SEM revealed that, in **Figure 13** (a), (b), and (c), the metallic calcite nanocrystals formed by the ureolytic bacteria of Greywater are smooth, homogenous, and spherical in size; however, in (d), irregular smooth spherical forms are seen. Based on the provided morphological analysis, these particles had a size between 15 and 20 µm.

The existence of metallic calcite component elements in the precipitates formed by ureolytic bacteria from Greywater and horse faeces is demonstrated by the energy dispersive X-ray (EDX) spectrum shown in **Figure 14** and **Figure 15,** demonstrating the presence of its constituent elements of calcite.The EDX spectrum of the selected area of the representative microstructure of both Greywater and Horse Faeces displayed in **Figure 14 and Figure 15** (a) and (c) showed the main composition was Ca, Cd, C and O. The spectrum graph plotted using EDX results shows the elements corresponding to each of their peak values. The higher the peak in a spectrum, the larger the concentration of elements in the specimen. Hence, by analyzing and comparing the peak value of chemical composition in EDX results for both Greywater and Horse Faeces, it is proven that the performance of heavy metal immobilization by Greywater is greater than Horse Faeces due to the amount of Cadmium calcite is higher in Greywater indicating the mass of ionic Cadmium degraded to metallic calcite is more efficient by ureolytic bacteria isolated by Greywater. Nonetheless, EDX analysis has proven the formation of metallic calcites are present in the precipitates and therefore enhances the validity of selected ureolytic bacteria performance in immobilizing cadmium heavy metal. The result of SEM and EDX for this study are equivalent with the previous study and based on the similarity of morphological analyses, it is possible to assume that the microbial community or bacterial identity of ureolytic bacteria isolated from both greywater and horse faeces is *Sporosarcina pasteurii* as compared to several studies [47] [48] [49]**.** .

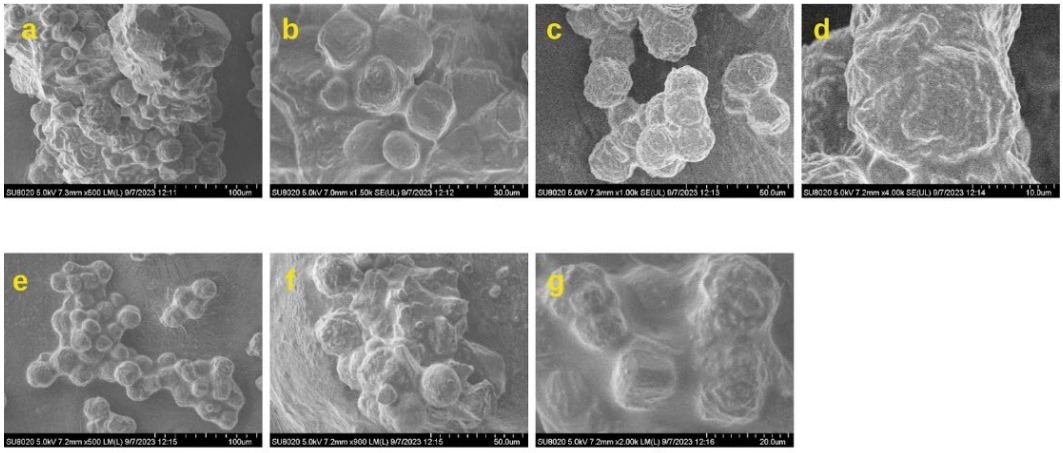


Figure 12. SEM images of aggregation of nanocrystals produced by Horse faeces on bacterial surfaces in precipitating medium with different degrees of magnification

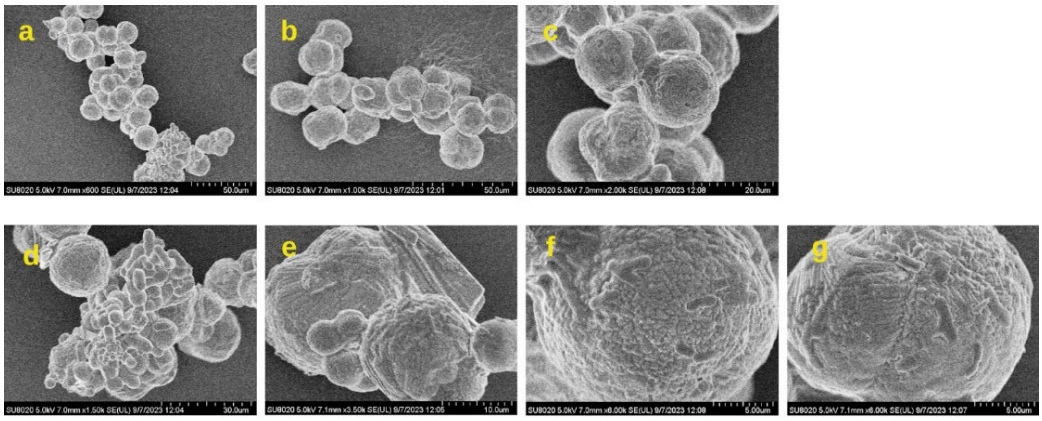


Figure 13. SEM images of aggregation of nanocrystals produced by Greywater on bacterial surfaces in precipitating medium with different degrees of magnification

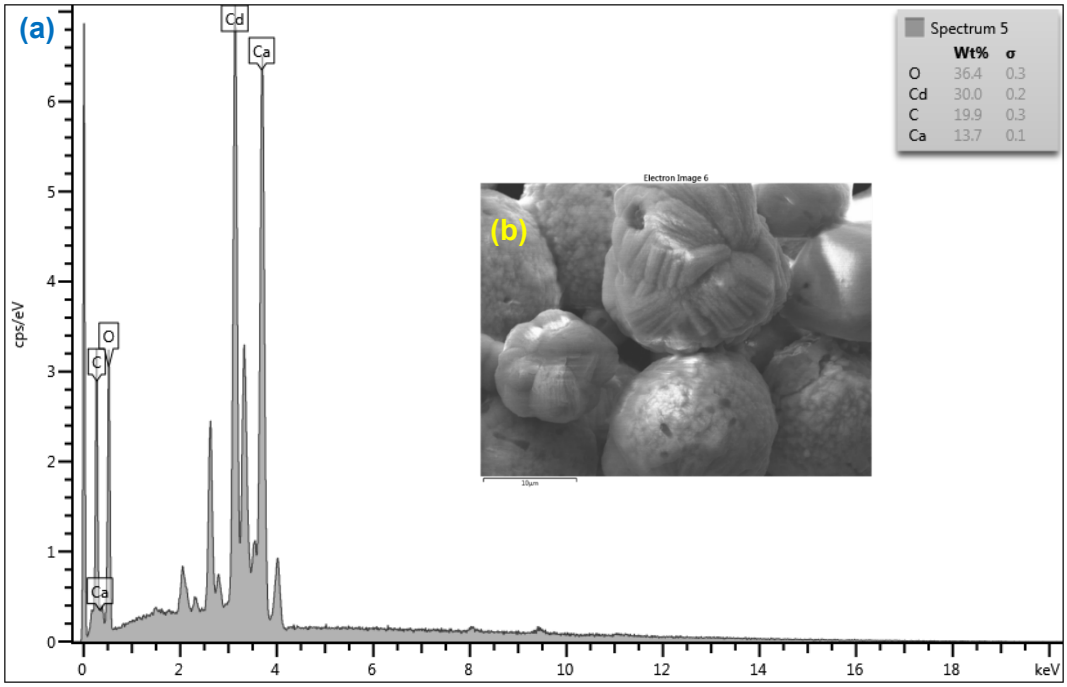


Figure14. SEM microstructures and EDX spectrums of Greywater samples prepared from precipitates of metallic calcite. In (a), EDX spectrums recorded from the sites showing the highest peak of chemical composition marked in corresponding SEM microstructures of (b)

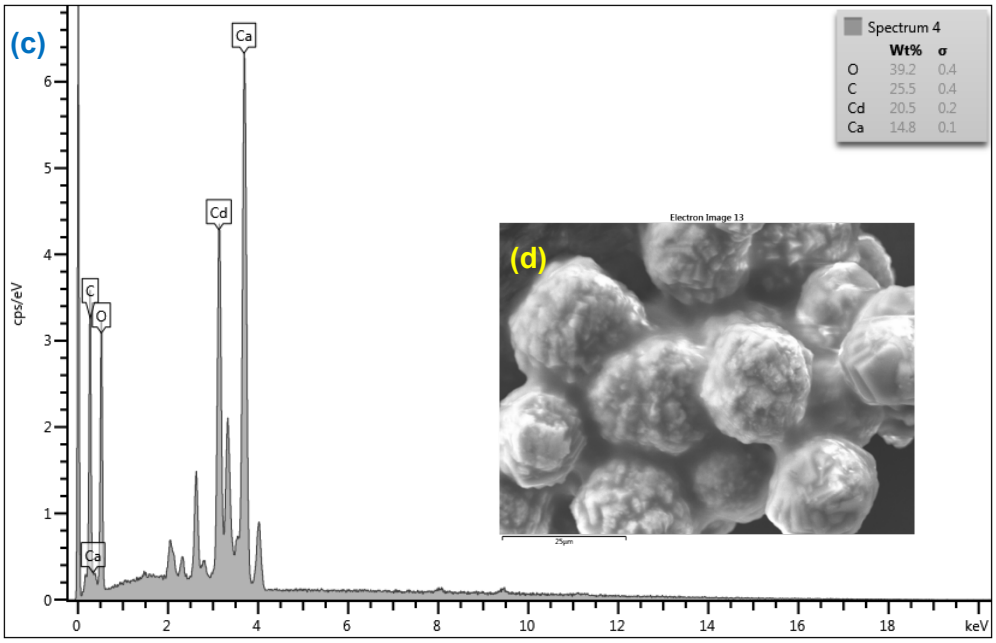


Figure 15. SEM microstructures and EDX spectrums of Horse Faeces samples prepared from precipitates of metallic calcite. In (c), EDX spectrums recorded from the sites showing the highest peak of chemical composition marked in corresponding SEM microstructures of (d)

Conclusions

The ureolytic bacteria utilised in this investigation to immobilise heavy metals were extracted from horse faeces and Greywater. The AAS analysis's results indicate that the ureolytic bacteria derived from Greywater sample is remarkably proficient in removing Cd^{2+}, as demonstrated by its substantial 80.19% removal rate, which greatly outperforms the 65.26% removal rate of the ureolytic bacteria from horse faeces sample. This suggest that ureolytic bacteria isolated from Greywater has the potential to be used for immobilizing heavy metals using MICP technology. The morphological analysis by SEM and EDX of biominerals produced after the treatment of heavy metal immobilization has successfully shown the presence of Cadmium heavy metal being degraded to carbonate minerals as well as the visualisation of the heavy metal carbonate formation and hence becoming additional clarifications that elevate the view and understanding on the mineral binding occurrence of calcite production by MICP technology.

Scaling up the MICP technology for environmental remediation, particularly heavy metal immobilization involves challenges such as achieving consistent microbial activity over large areas, addressing cost and infrastructure needs, and reducing environmental impacts, in which not being discussed in this study. Regulatory approval and gaining public trust are also critical for large-scale application, while ensuring long-term stability and durability, while adapting the technology to different environmental conditions, such as different seasons and humidity are important factors to be considered for strategic implementation. For future consideration, addressing these challenges is crucial for successfully implementing MICP on a wider scale.

Conflicts of Interest

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

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