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RESEARCH ARTICLE

Influence of Enriched Urease Producing Bacteria from Leachate and Restaurant Wastewater on Heavy Metal Removal

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Abstract The escalation of heavy metal pollution in natural ecosystems due to industrialization presents a critical environmental concern, endangering the well-being of living organisms. Microbially Induced Carbonate Precipitation (MICP) technology, an emerging innovation, has gained attention from the scientific community for its potential in biocementation and bioremediation applications. However, a substantial gap in understanding exists regarding the utilization of ureolytic microbial strains from waste sources capable of effectively immobilizing high concentrations of heavy metals. This study endeavors to explore the latent potential of indigenous ureolytic bacteria derived from leachate and restaurant wastewater, possessing bioremediation capabilities for heavy metal immobilization. The investigation includes microbial screening, physiological characterization of ureolytic bacteria, assessment of their tolerance levels, and evaluation of heavy metal removal efficacy through Atomic Absorption Spectrophotometry (AAS) analysis. Notably, the results reveal that ureolytic bacteria from restaurant wastewater are more tolerant to Cd2+ concentrations compared to their leachate counterparts, manifesting optimum conductivity, pH, and optical density (OD). More so, AAS analysis demonstrates the restaurant wastewater-derived sample's remarkable proficiency in Cd2+ removal, achieving a substantial 95% removal rate, significantly outperforming the leachate wastewater sample's removal rate of 53%.

Keywords: Heavy Metal, Bioremediation, Ureolytic Bacteria, MICP, leachate.

Introduction

The rapid acceleration of industrialization and urbanization over the past few decades has created a global environmental challenge. Heavy metals are discharged into the environment due to metalliferous material mining, metal smelting, metallurgical sector operations, waste disposal, and metal corrosion. Heavy metals are continuously discharged into the environment and assimilated into natural resources, posing grave harm to species and leading to bioaccumulation [1]. Arsenic (As), Zinc (Zn), Lead (Pb), Copper (Cu), Cobalt (Co), Chromium (Cr), Selenium (Se), Nickel (Ni), Cadmium (Cd), Mercury (Hg), and Magnesium (Mg) are a few dangerous heavy metals created by industrial operations. The toxicity of each metal influences the amount of metal in organisms, the metal's journey, the concentration level, and the time of exposure [1]. Because they cannot be broken down and transformed into non-toxic forms by the body, heavy metals accumulate in the environment, including human bodies, via the food chain, posing health risks [2]. Excessive levels of heavy metals have devastating effects, including toxic and carcinogenic effects, and contribute to the development of several chronic and acute diseases, including hypertension [3], atherosclerotic disease, cardiovascular disease, endocrine dysfunction, renal failure, and infertility [4]. According to the World Health Organization (WHO), the recommended allowed limit for

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Zn, Cu, Cr, Pb, and Ni heavy metals is 0.6, 10, 1.6, 2, and 10 mg/Kg, respectively. Under the National Water Quality Standards Malaysia (NWQSM), the maximum concentration of certain heavy metals in Malaysia water is 0.05 mg/L for As, 0.01 mg/L for Cd, 0.20 mg/L for Mg, 0.010 mg/L for Pb, 1 mg/L for Zn, and 1 mg/L for Iron (Fe). Unlike organic contaminants, heavy metals cannot be cleared through chemical or biological processes. Therefore, their transformation is the sole option to lessen their toxicity [5].

Several techniques for treating environmental contaminants have been established, including using physical, chemical, and biological processes. The main drawbacks of physical and chemical procedures are their higher energy consumption and requirement for additional chemicals, even though they can eliminate a wide range of contaminants [6]. Conventional remediation methods are often not economically viable for wastewater treatment due to the volume and metal concentration of the wastewater, as well as its salinity and other properties [7]. In the biological process, bioremediation is a promising technique for the removal and immobilization of heavy metals from contaminated environments. The microorganisms' metabolic processes help break down pollutants into non-toxic chemicals during bioremediation. This method can completely dissolve the organic pollutants, possibly leading to mineralization [8]. Bioremediation is one of the most prevalent techniques for eliminating and recovering heavy metals from contaminated areas. The method utilizes natural biological mechanisms to eliminate hazardous pollutants by employing bacteria, plants, or commodities to remediate contaminated environments [4, 9, 10].

To reduce heavy metals' mobility, a novel bioremediation method known as MICP includes encasing them for an extended period within Calcium Carbonate ($CaCO_3$) mineral crystal. This strategy for minimizing heavy metal pollution is both cost-effective and economical. MICP can be produced through several processes, the most well-known pathway is urea hydrolysis (ureolysis), nitrate reduction, methane generation, sulfate reduction, ferric oxide reduction, and cyanobacterial photosynthesis. The ureolysis process has stood out among the others due to the ease with which it can be controlled, the rapid reaction rate that it can achieve, and the great chemical conversion efficiency it can achieve [11]. One of the key advantages of MICP over more conventional techniques is its resilience to redox-insensitive solutions. It ensures that heavy metal carbonates are insoluble, non-toxic, and immobile [12]. A considerable amount of research over the last five years has greatly improved our understanding of heavy metal removal and the elements that influence it when utilizing MICP technology. To remove heavy metals from contaminated locations or to change them from a soluble to an insoluble form, numerous studies have demonstrated that microorganisms may remove heavy metals (up to 98%) by MICP employing ureolysis. Despite its advantages, MICP has not been widely implemented for the remediation of heavy metal pollution in wastewater [8, 13–15]. One of the main drawbacks for the heavy metal application via MICP is closely related with the suitableness and cost of ureolytic bacteria species [16] Therefore, this study aims to investigate the potential source of native ureolytic bacteria with bioremediation capabilities for heavy metal immobilization in wastewater.

Materials and Methods

The experimental work conducted for this study consists of three major parts. The experiment was to isolate ureolytic bacteria from waste sources. Samples collection was taken from leachate and restaurant wastewater. Afterwards, the collected waste samples were used to prepare and inoculate nutrient broth containing 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. Lastly, the study investigated the tolerance of the ureolytic bacteria to different heavy metals (Cu^{2+}, Cd^{2+}, Ni^{2+}) and their heavy metal removal performance. Figure 1 shows the overall framework for this study.

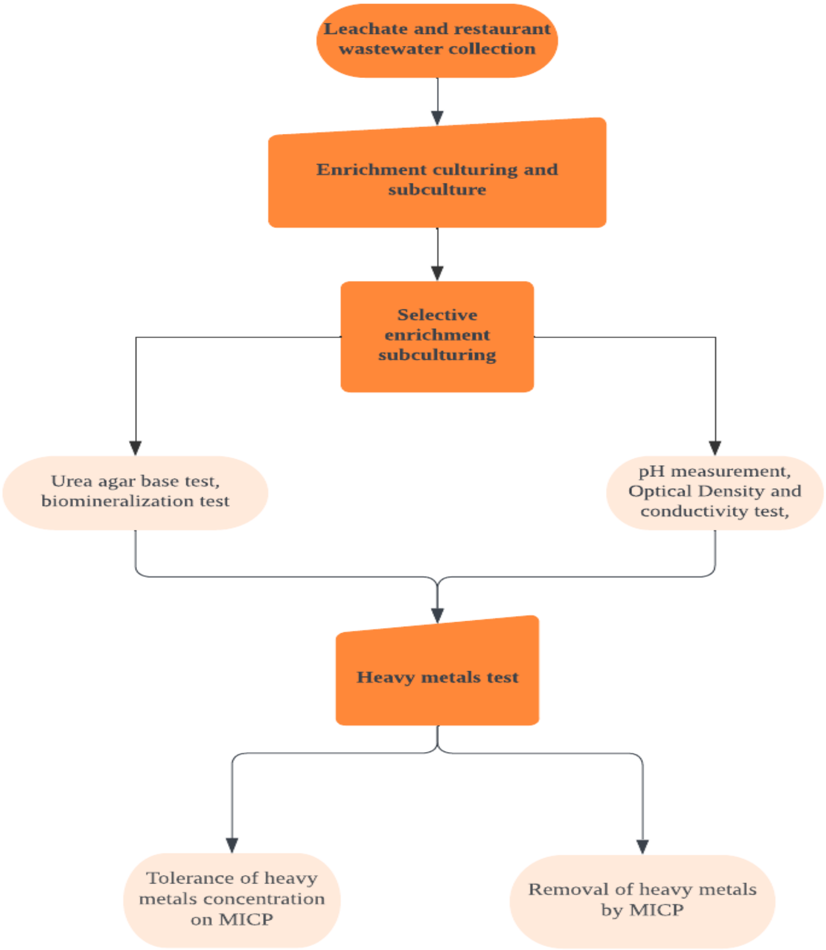

Figure 1. Flowchart of the experimental plan for this study

Collection of Samples

Waste samples were collected from a local restaurant at Universiti Teknologi Malaysia Skudai, Johor (1.5670492281121604° N, 103.63484136698504° E) and leachate treatment plant, Seelong landfill, Senai, Johor (1.659826° N, 103.719749° E). The restaurant wastewater and leachate sample were used in this study to identify potential ureolytic bacteria for heavy metal immobilization. All the collected samples were placed into 10 L sampling bottles transported to the lab for additional analysis.

Enrichment Culture of Samples

Restaurant wastewater and leachate samples were enriched for urease-producing bacteria by inoculating 1 g or 1 mL of each sample into 50 mL of nutrient broth medium (HiMedia, Laboratories Pvt. Ltd) containing 6% urea in 250 mL shaking flasks. Then, the bacterial culture was incubated under aerobic batch conditions at 30 °C for 120 hr while being shaken at 130 rpm. Before sterilisation, the original pH of each medium was adjusted to 8.0 using 0.1 M NaOH. After autoclaving, a sterile urea substrate (through 0.45 m filter sterilisation) was applied to prevent chemical degradation under autoclaving conditions.

Optical Density and pH

During cultivation, the OD and pH profiles of ureolytic bacteria cultivated in restaurant and leachate mediums were examined. Spectrophotometer (Thermo ScientificTM GENESYSTM 20) was used to measure absorbance (optical density) at a wavelength of 600 nm to track changes in cell density (biomass concentration) (OD600). Three millilitres of the culture were sampled and placed in clean 10 mm cuvettes before the values were read. Before measuring the optical density of bacterial cultures grown in these two respective media, the spectrophotometer was calibrated using uninoculated growth media (GM-1 and GM-2) as blanks. Using a pH metre (SevenEasyTM–Mettler Toledo), the pH of the bacterial cultures was measured. The pH electrode was calibrated with buffer solutions of known pH 4, 7, and 10.30 before any measurements were taken (Sigma-Aldrich). The optical density and pH levels were measured at 6-hour intervals till the completion of the incubation period (48 h).

Biomineralization Test

This biomineralization test assessed the potential of ureolytic bacteria grown in restaurant wastewater and leachate medium for MICP applications. In a beaker containing 1 L of deionized water, 1 M of urea, and 1 M of calcium chloride ($CaCl_2$), were mixed together to make a cementation solution (Atama, Shd. Bhd., Malaysia). The solution was properly mixed before being distributed (40.5 mL) into separate centrifuge tubes (Biologix® KS, United States). The tubes were then inoculated with 4.5 mL of ureolytic bacterial cultures cultivated overnight in the restaurant's wastewater and leachate medium. After inoculating the mineralization solution with bacterial culture, the weight of precipitates and pH of the effluents were assessed. To correctly evaluate the effects of various parameters on the bioprecipitation of $CaCO_3$ by bacterial activity during the MICP process, just a single variable was controlled throughout each comparative experiment.

Urease Activity via Conductivity Test

The conductivity method is a straightforward and cost-effective approach for determining the enzymatic rate reaction of the bacterial-urea solution. The assay was conducted by adopting procedures from [17]. The relative variations in conductivity in the urea solution were used to gauge the urease activity of ureolytic bacteria cultivated in restaurant and leachate mediums. 10 mL of bacterial suspension was introduced to a 250-mL sterile beaker containing 90 mL of urea solution (1.11 M). The mixture was then shaken, and the conductivity was measured with a tabletop conductivity metre (MI806, Milwaukee, WI, USA). The relative conductivity changes (mS/min) were obtained by measuring the reaction (bacterial cells-urea solution) at 1 and 7 minutes at 25 2 °C. Before calculating urease activity (mM urea hydrolyzed per minute), the conductivity was multiplied by the dilution factor (10). The dilution factor is the ratio of the original bacterial concentration to the final bacterial concentration following the addition of urea solution. Each 1 mS/min corresponds to 11.11 mM urea/min of hydrolysis activity. Consequently, the observed conductivity fluctuation rate was translated to urease activity (mM urea hydrolyzed per minute). After the cultivation period concludes, specific urease activity was measured by dividing the conductivity by bacterial growth cell values taken from optical density measurements (OD600).

Tolerance of the Bacterial Strains to Heavy Metals

The tolerance of bacterial strains to Cu, Cd, and Ni heavy metals was investigated using Luria-Bertani culture medium (LB). NaCl 5 g/L, tryptone 10 g/L, and yeast extract 5 g/L constituted LB. The media were disinfected by autoclaving for 20 minutes at 121°C. Increasing amounts (0–10 mM) of heavy metal salts (copper sulphate, nickel chloride, and cadmium chloride) were added to the growth media. Each bacterial strain with an initial OD_{600} of 0.15 was injected into the culture media using an inoculum generated by incubating 20 mL of liquid medium overnight with a separate, old colony. After an overnight incubation at 30 C, the inoculum has an OD600 of ~3. The cultures were incubated for 72 hours at 30 C with continuous agitation at 150 rpm. The minimal inhibitory concentration (MIC) was defined as the lowest concentration of heavy metal that caused a >90% reduction in bacterial growth. Each concentration of heavy metal was evaluated in triplicate.

Removal of Heavy Metals through MICP Process

The heavy metal stock solutions were prepared by using CdCl2, NiCl2, CuSO4 (purchased from Atama Sdn. Bhd) and was further diluted in distilled water. All the chemicals and reagents used in this study were analytical grade. 5 mL of bacterial suspension were aseptically transferred to a 15 mL mixture of 0.85% normal saline, 3 M urea solution, and heavy metal at a concentration of 100 mg/L. The final volume of the reaction system was maintained to 20 mL by adding normal saline. The heavy metal contents of the treated samples were determined by flame atomic absorption spectrophotometry (FAAS) (Thermo, ICE3000) after incubation at 30ºC. The removal efficiency of heavy metals by selected MICP bacteria was optimized by investigating the effects of treatment time (0.5h - 24h). The removal efficiency for heavy metals were calculated as shown in equation 1:

$$\text{Removal efficiency (\%)} = \frac{Ci-Cf}{Ci}\ x\ 100 \qquad \text{(Eqn. 1)}$$

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.

Results and Discussion

Screening of Ureolytic Bacteria from Restaurant and Leachate Wastewater Samples

Two samples were collected from restaurant and leachate wastewater to screen for 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 [18]. According to [19], 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 sources were chosen based on the conditions and suitability for ureolytic bacteria growth. Restaurant wastewater and leachate are waste products from natural environments. They have a high presence of urea as substrate, optimum alkalinity for microbial growth as well as a diverse microbial community [20, 21]. According to [22], 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 sufficient amounts of urea substrate.

Enrichment culture method was conducted in order 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 also helps to remove bacteria that cannot survive in high urea concentrations [23]. We used a 6% urea solution to specifically target bacteria that can break down urea for energy and nitrogen. Figure 2 illustrates the images of enriched culture of restaurant and leachate 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. Urea hydrolysis produces ammonia, which 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 restaurant wastewater and leachate had transformed the urea agar base medium from pale orange to pink as shown in Figure 3, indicating the production of urease enzyme. Bacterial colonies isolated from restaurant wastewater altered colour more rapidly than those isolated from leachate wastewater.

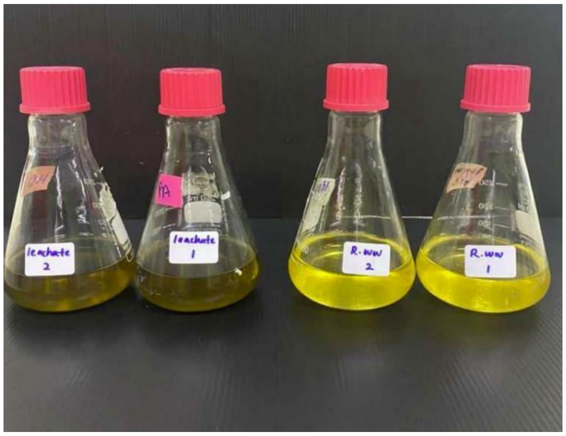

Figure 2. Enriched culture of leachate and restaurant wastewater

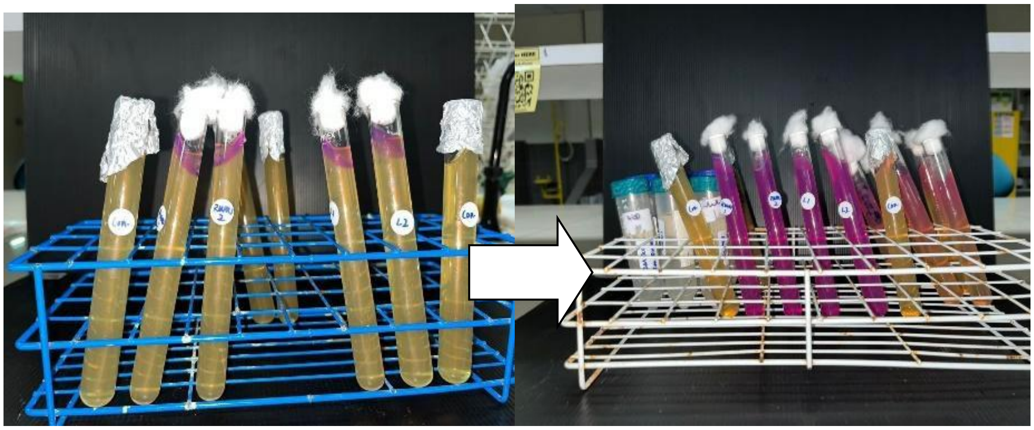

Figure 3. Presence of urease inside the test tubes (orange to pink colour indicating of urease present)

Biomineralization

The biomineralization test is commonly used to evaluate the potential of microorganisms to be used in bioremediation, soil enhancement as well as for the production of sustainable building materials. Biomineralization is the biological formation of minerals by living cells in environments that promote interaction with specific cations [25]. In this study, the bacteria cultures from restaurant and leachate wastewater samples were mixed with a cementation solution containing urea and $CaCl_2$. The mix process called microbial-induced calcium carbonate precipitation (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 (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 [16, 18, 26].

The formation of these minerals depends on the activity of specific microbial enzymes, such as urease, which break down 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 [27]. According to Omoregie *et al*. [18] 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. Microbial cementation requires that bacteria be able to travel freely through the water's pore spaces and that there be an adequate amount of particle-particle interaction. 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 weight of minerals after drying in the oven was used to determine the reaction of the cementitious materials (urea and $CaCl_2$) containing calcium carbonate with the bacteria sample from each source. Biomineralization was more reactive with ureolytic bacteria from restaurant wastewater than with those from leachate wastewater, as evidenced by the higher weight of dried sediments from restaurant wastewater (1.7591 g) compared to leachate wastewater (1.0568 g). This suggests that ureolytic bacteria from restaurant wastewater have a higher potential for cementitious interaction with the cations within their cell walls.

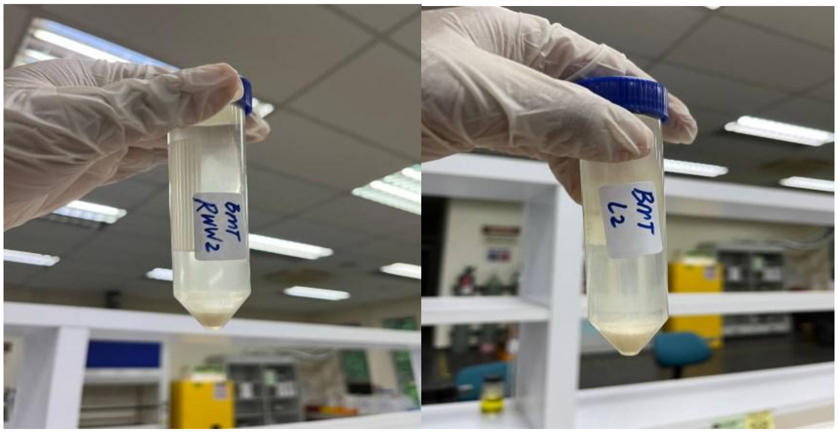

Figure 4. Precipitates formed after biomineralization process in Leachate and Restaurant wastewater

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. Urease activity plays a crucial role in MICP, as it catalyzes the hydrolysis of urea, a key step in the production of the necessary components for calcium carbonate precipitation. 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 [28]. Specific urease activity is a valuable parameter because it considers the influence of biomass or protein content on overall urease activity. This measure is often expressed in units of activity per milligram of protein or biomass 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 microbial-induced calcium carbonate precipitation (MICP), which is a biomineralization process that uses bacteria to precipitate calcium carbonate from soluble sources of calcium and bicarbonate.

Figure 5 displays the results of urease activity and specific urease activity for two distinct types of wastewater samples, shedding light on the variations in microbial ureolytic activity between restaurant wastewater and leachate. The data indicates that restaurant wastewater has a higher urease activity and specific urease activity compared to leachate. Specifically, the mean urease activity for restaurant wastewater is 10.095 mM urea hydrolyzed/min, while the mean urease activity for leachate is 7.765 mM urea hydrolyzed/min. The mean specific urease activity for restaurant wastewater is 9.14 mM urea hydrolyzed/min/OD_{600}, while the mean specific urease activity for leachate is 7.473 mM urea hydrolyzed/min/OD_{600}. The one-way ANOVA statistical analysis revealed that there were significant differences in both urease activity and specific urease activity between the restaurant wastewater and leachate samples. Specifically, the analysis of urease activity for both samples showed that the calculated F-value of 39.239 was higher than the critical F-value of 4.965 (with a significance level of 0.05 and degrees of freedom of 1 and 10). Similarly, for specific urease activity, the calculated F-value of 7.163 was higher than the critical F-value of 4.965 (with a significance level of 0.05 and degrees of freedom of 1 and 10). The significant differences observed in both urease activity and specific urease activity, as indicated by the calculated F-values surpassing the critical F-value, underscore the substantial disparities in microbial ureolytic activity between restaurant wastewater and leachate samples.

The higher urease and specific urease activities observed in restaurant wastewater samples are consistent with previous studies that have shown that organic-rich wastewater can enhance the activity of microorganisms involved in microbial-induced calcium carbonate precipitation (MICP) [29]. Both urease activity and specific urease activity are pivotal parameters in the context of MICP, as they are indicative of the microbial capacity to initiate the precipitation of calcium carbonate from soluble sources of calcium and bicarbonate. The enhanced urease activity and specific urease activity in restaurant wastewater can be attributed to its organic-rich nature, providing a nutrient-rich environment that encourages the growth and activity of urease-producing bacteria. Organic-rich wastewater contains high levels of carbon and nitrogen, which can serve as nutrients for bacteria and stimulate microbial growth and urease activity. As a result, the microbial activity in organic-rich wastewater can lead to higher rates of calcium carbonate precipitation and more effective MICP. The high urease activity and specific urease activity in restaurant wastewater may be attributed to the presence of urease-producing bacteria in the wastewater. Restaurant wastewater typically contains high levels of organic matter and nitrogen, which can provide a favorable environment for the growth and proliferation of microorganisms, including urease-producing bacteria. Previous studies have reported the presence of urease-producing bacteria in various types of wastewater, including domestic wastewater, hospital wastewater, and industrial wastewater [30]. For example, a study by Yahya *et al*. [31] found that domestic wastewater contained high levels of urease activity, which was attributed to the presence of urease-producing bacteria such as Klebsiella pneumoniae and Proteus vulgaris. Similarly, a study by Alam *et al*. [32] found that hospital wastewater contained high levels of urease activity, which was associated with the presence of urease-producing bacteria such as Pseudomonas aeruginosa and Proteus vulgaris. In addition, the use of certain cleaning agents and disinfectants in restaurants may also contribute to the presence of urease-producing bacteria in the wastewater. For example, a study by Zhou *et al*. [33] found that the use of quaternary ammonium compounds as disinfectants in food service establishments was associated with increased levels of urease activity in the wastewater. Studies by Yahya *et al*. [31] and Alam *et al*. [32] highlighted the presence of urease-producing bacteria in domestic and hospital wastewater, demonstrating the broad distribution of such microorganisms across different wastewater types.

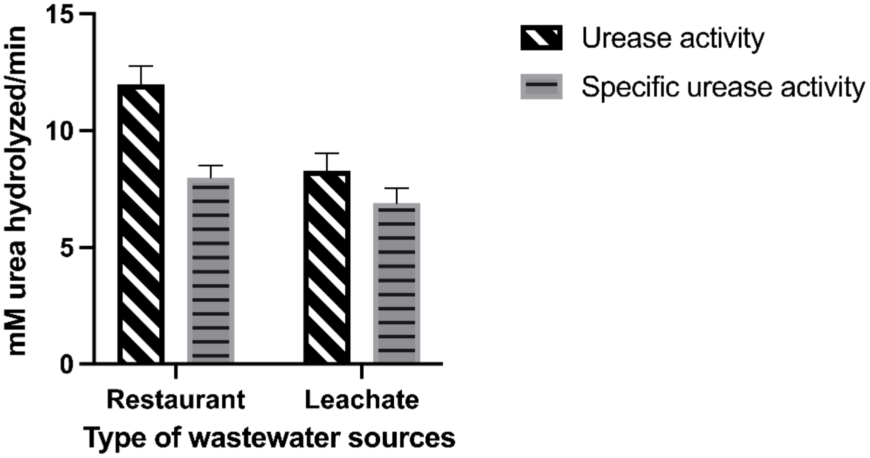

Figure 5. Urease activity and specific urease activity of ureolytic bacteria from restaurant and leachate wastewater

Tolerance of Ureolytic Bacteria towards Heavy Metal

The provided data allows for a comprehensive comparison and justification of the tolerance of ureolytic bacteria in restaurant wastewater and leachate wastewater towards heavy metals. The parameters of urease activity, pH, and optical density (OD) were examined, with the concentration of all heavy metals fixed at 1000 mg/L. The data presented allows for an extensive assessment of the tolerance of ureolytic bacteria in different wastewater types towards heavy metals. This analysis is critical for understanding the adaptability of these microorganisms in challenging environments. Key parameters, including urease activity, pH, and optical density (OD), were examined to assess the response of ureolytic bacteria to heavy metal stress. These parameters offer insights into the survival strategies of these microorganisms.

Table 1. Tolerance performance of ureolytic bacteria from restaurant and leachate wastewater for copper, cadmium and nickel heavy metals

	Urease activity (mM urea hydrolyzed/min)			pH			Optical Density (OD_{600})		
Control	7.40	8.1	7.7	7.95	9.03	9.05	0.32	0.68	0.23
Restaurant	7.83	9.2	8.8	7.87	9.16	9.30	0.26	0.89	0.43
Leachate	7.55	8.4	7.9	7.98	9.08	9.35	0.16	0.68	0.31

The comparison of tolerance between the ureolytic bacteria in control, restaurant wastewater, and leachate wastewater towards heavy metals is depicted in Table 1. It is evident that both sets of bacteria exhibit higher urease activity values compared to the control group, indicating their developed tolerance towards heavy metal concentrations. However, an interesting distinction emerges as the ureolytic bacteria in restaurant wastewater display slightly higher urease activity values compared to the leachate wastewater samples. This suggests a potential higher degree of tolerance towards heavy metals in the ureolytic bacteria of restaurant wastewater. The elevated urease activity in restaurant wastewater underscores their stronger capacity to adapt and withstand the presence of heavy metals, highlighting their remarkable resilience and survival capabilities in such challenging environments. Higher urease activity values in restaurant wastewater compared to leachate samples suggest an exceptional ability of ureolytic bacteria in restaurant wastewater to thrive under heavy metal conditions. As suggested by Omoregie *et al*. [30], the increased urease activity in restaurant wastewater likely indicates the presence of internal regulatory mechanisms within ureolytic bacteria, allowing them to maintain ionic balance despite heavy metal stress. This regulatory ability is pivotal for the maintenance of their cellular functions and successful adaptation to thrive under adverse conditions.

The data reveals that the ureolytic bacteria in both restaurant wastewater and leachate wastewater exhibit comparable levels of pH values observed in the samples. The slight variations in pH between the two wastewater types are within the normal range of variation, suggesting that the bacteria in both samples have the ability to regulate their internal pH despite the presence of heavy metals. This ability to maintain pH homeostasis reflects the bacteria's adaptive mechanisms to counteract the negative effects of heavy metal stress. Furthermore, a higher pH environment, as described by Ahmad *et al*. [34], can provide advantages to ureolytic bacteria in terms of enhancing the solubility and availability of heavy metals for uptake and metabolism. This increased availability allows the bacteria to effectively interact with and potentially detoxify the heavy metals. Thus, the observed similarities in pH values indicate that the ureolytic bacteria in both wastewater types possess robust mechanisms to tolerate heavy metals and sustain their survival and functionality. Regulating pH is crucial for ureolytic bacteria as it influences the solubility and bioavailability of heavy metals. This adaptive mechanism allows bacteria to effectively interact with and potentially detoxify these metals.

Additionally, Table 1 also demonstrates that the ureolytic bacteria in restaurant wastewater consistently exhibit higher OD_{600} values for copper, cadmium, and nickel compared to the control group. This indicates that the ureolytic bacteria in restaurant wastewater have a greater biomass and cellular growth in the presence of heavy metals. A particularly noteworthy finding is the significant increase in OD_{600} specifically for cadmium in the ureolytic bacteria of restaurant wastewater when compared to the leachate wastewater samples. This suggests a remarkable tolerance of the ureolytic bacteria in restaurant wastewater towards cadmium. One possible justification for the higher OD_{600} values in restaurant wastewater could be the presence of organic compounds and nutrients commonly found in restaurant wastewater, such as fats, proteins, and carbohydrates, which can serve as additional energy sources for the bacteria [35]. These organic compounds may enhance the metabolic activity and proliferation of the ureolytic bacteria, leading to the observed higher OD_{600} values. The significant increase in OD_{600} for cadmium in restaurant wastewater suggests a remarkable tolerance of these bacteria to cadmium toxicity. This could be due to the presence of organic compounds and nutrients in restaurant wastewater, which serve as additional energy sources. Furthermore, the ureolytic bacteria in restaurant wastewater may have developed specific mechanisms to counteract the toxic effects of cadmium, further contributing to their higher tolerance and growth in the presence of this heavy metal.

More so, the data strongly supports the notion that ureolytic bacteria in restaurant wastewater possess an exceptional level of tolerance towards heavy metals, particularly cadmium. The consistent findings of higher urease activity, pH, and optical density values in restaurant wastewater samples compared to the control and leachate wastewater samples underscore this remarkable phenomenon. The elevated tolerance can be attributed to several factors. The observed higher OD_{600} values in restaurant wastewater as shown in Table 1 may be attributed to the presence of organic compounds, such as fats and proteins, which provide additional energy sources. Additionally, ureolytic bacteria in restaurant wastewater may have developed specific mechanisms for detoxifying cadmium. This availability of diverse nutrients gives the ureolytic bacteria a competitive advantage and enhances their adaptability to challenging environments. Secondly, it is highly plausible that the ureolytic bacteria in restaurant wastewater have developed specialized mechanisms to combat the toxic effects of cadmium. These mechanisms may include the production of metal-binding proteins or the activation of detoxification pathways, allowing the bacteria to effectively neutralize and eliminate the harmful effects of cadmium. Furthermore, the selective pressures exerted by the consistent presence of heavy metals in restaurant wastewater might have led to the evolution of a more resilient and tolerant bacterial population. By understanding the underlying mechanisms behind the high tolerance of ureolytic bacteria in restaurant wastewater towards cadmium, we can gain valuable insights for the development of innovative wastewater treatment strategies and the mitigation of heavy metal pollution in various industrial sectors.

Analysis of Heavy Metal Removal

The removal of heavy metals via MICP is governed by two primary mechanisms, namely (i) physical entrapment and (ii) surface adsorption. Heavy metal ions can become physically entrapped within the growing calcium carbonate matrix, rendering them less mobile and therefore less bioavailable and toxic to the environment. Heavy metal ions can also adsorb onto the surfaces of calcium carbonate crystals. The adsorption affinity of heavy metal ions to calcium carbonate is influenced by various factors, including the physicochemical properties of the heavy metal ions, the pH, and the ionic composition of the surrounding environment [14]. Figure 6 presents a comparative analysis of heavy metal removal percentages by ureolytic bacteria through the MICP process in different wastewater types, namely restaurant wastewater and leachate wastewater. Overall, the ureolytic bacteria from restaurant wastewater recorded a better removal performance compared to leachate wastewater for the related heavy metals. Statistical analysis using one-way ANOVA indicates no significant difference between the removal performance of ureolytic bacteria from restaurant and leachate wastewater for copper, cadmium, and nickel heavy metals, F= 2.671, p (0.2156 > 0.0001), R^2 = 0.6404. The R-squared value of 0.6404 indicates that the model explains 64.04% 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 the average removal percentage of copper using ureolytic bacteria originated from restaurant wastewater is 12.3%. Meanwhile ureolytic bacteria in leachate wastewater achieved only 72%. The low removal performance of ureolytic bacteria in both restaurant wastewater and leachate wastewater can be attributed to a few factors such as tolerance of ureolytic bacteria towards copper and specific characteristic of copper. As discussed in section 3.4, the tolerance of ureolytic bacteria for both wastewater sources towards copper are low compared to cadmium and nickel. Notable observations were made regarding the pH value and OD_{600} of the ureolytic bacteria upon their mixture with copper. The pH value is a crucial parameter that affects the enzymatic activity and overall performance of ureolytic bacteria. Low pH levels are known to create an uncomfortable environment, which can significantly impact the metabolic activity of the bacteria [34]. In the case of copper mixture, the low pH might have resulted from the formation of copper complexes or the release of acidic byproducts during the microbial metabolism. This uncomfortable environment could potentially hinder the ureolytic bacteria's ability to carry out urea hydrolysis and subsequent precipitation, leading to a reduced removal percentage of copper. In addition to pH, the OD_{600} value provides insights into the bacterial growth and proliferation. OD_{600} is commonly used as an indirect measure of bacterial cell density. A higher OD_{600} value generally indicates a larger population of actively dividing bacteria. However, in the context of copper mixture, a low OD_{600} value suggests that the growth and proliferation of ureolytic bacteria might have been inhibited. Copper ions can have toxic effects on microbial cells, disrupting cellular processes and inhibiting bacterial growth [36]. The reduced metabolic activity and limited bacterial proliferation under copper stress conditions can contribute to the decreased removal percentage observed. The role of pH cannot be overstated in the context of heavy metal removal. The low pH conditions observed upon mixing ureolytic bacteria with copper suggest that the formation of copper complexes or the release of acidic byproducts during microbial metabolism may have contributed to the decline in removal percentages. These pH-induced unfavorable conditions potentially hinder ureolytic bacteria's ability to execute urea hydrolysis and subsequent heavy metal precipitation, ultimately affecting removal efficiency. Furthermore, the inhibitory effects of copper on bacterial growth illustrated in Figure 6, reflected by lower optical density (OD_{600}) values, highlight the toxic nature of copper ions to microbial cells. Copper has the capability to disrupt crucial cellular processes and inhibit bacterial growth. As a result, the reduced metabolic activity and limited proliferation of ureolytic bacteria under copper-induced stress conditions directly contribute to the decrease in copper removal percentages

In the case of nickel, the average removal percentage achieved by ureolytic bacteria in both restaurant and leachate wastewater shows a significant contrast. Ureolytic bacteria derived from restaurant wastewater demonstrate a remarkable removal efficiency of 71%, while those from leachate wastewater only manage to remove 17.5%. This discrepancy in performance can be attributed to several factors related to the characteristics of the ureolytic bacteria and the source wastewater composition. One crucial aspect to consider is the presence of distinct contaminants in each wastewater source [37]. Restaurant wastewater may contain specific co-existing pollutants that the ureolytic bacteria have encountered consistently in that environment. Over time, these bacteria have developed a higher tolerance to nickel due to continuous exposure, enabling them to adapt and perform more effectively in removing the metal. In contrast, leachate wastewater originating from landfills contains a diverse array of pollutants, presenting a different combination of contaminants to the ureolytic bacteria. This unfamiliar mix may challenge their ability to efficiently remove nickel, resulting in a lower removal efficiency compared to the bacteria from restaurant wastewater [38]. Moreover, the overall characteristics of the wastewater play a crucial role in the performance of ureolytic bacteria. Parameters such as urease activity, pH, and OD_{600}, which reflects bacterial growth and density, can significantly influence the metabolic activity and proliferation of ureolytic bacteria. It is plausible that the wastewater characteristics in restaurant wastewater create a more favorable environment for the ureolytic bacteria, facilitating their adaptation and optimal functioning in the presence of nickel. This adaptability contributes to their higher removal efficiency. Conversely, the wastewater characteristics in leachate wastewater may present challenges to the ureolytic bacteria, hindering their ability to effectively remove nickel. Leachate wastewater originating from landfills is known for its complex mixture of contaminants, which greatly differs from the composition of restaurant wastewater. The presence of diverse pollutants in leachate wastewater poses a unique challenge for ureolytic bacteria, as they encounter varying combinations of heavy metals and co-existing pollutants. This unfamiliar mix of contaminants may be a key factor contributing to the reduced efficiency of ureolytic bacteria in removing heavy metals like nickel.

Among all the heavy metals, cadmium stands out as the heavy metal with the highest removal percentage achieved by ureolytic bacteria in both waste sources. Ureolytic bacteria derived from restaurant wastewater demonstrate an impressive removal rate of 95%, significantly surpassing leachate wastewater, where only 53% removal is observed. The exceptional cadmium removal performance of ureolytic bacteria from restaurant wastewater can be attributed to their high tolerance towards cadmium, excelling in all tolerance parameters, including urease activity,, pH, and OD_{600}. The remarkable ability of ureolytic bacteria from restaurant wastewater to remove cadmium may be attributed to their advanced adaptation to high cadmium concentrations. The remarkable consistency in the removal trends of copper, cadmium, and nickel across restaurant and leachate wastewater samples suggests that ureolytic bacteria possess certain intrinsic characteristics that enable them to adapt to and address specific challenges posed by each heavy metal. These bacteria have likely encountered elevated levels of cadmium in the restaurant wastewater environment over time. This prolonged exposure has led to the development of specific tolerance mechanisms within the ureolytic bacteria, allowing them to efficiently metabolize and precipitate cadmium, resulting in the outstanding removal percentage observed [39]. Furthermore, the specific chelating agents present in restaurant wastewater contribute to the enhanced cadmium removal performance of ureolytic bacteria. Chelating agents are organic compounds with a strong affinity for metal ions, forming stable complexes [40]. In the case of cadmium, these chelating agents effectively sequester cadmium ions, reducing their mobility and bioavailability. This allows the ureolytic bacteria to more efficiently interact with and remove cadmium from the wastewater. In summary, cadmium exhibits the highest removal percentage by ureolytic bacteria among all the heavy metals in both restaurant and leachate wastewater. The presence of chelating agents in restaurant wastewater significantly contributes to the remarkable cadmium removal by ureolytic bacteria. These organic compounds possess a strong affinity for metal ions, forming stable complexes. In the case of cadmium, these chelating agents effectively sequester cadmium ions, reducing their mobility and bioavailability. This chemical interaction allows ureolytic bacteria to interact more efficiently with and remove cadmium from the wastewater, resulting in the observed outstanding removal percentage. Ureolytic bacteria derived from restaurant wastewater demonstrate exceptional cadmium removal rates due to their high tolerance towards cadmium and proficiency in various tolerance parameters. The exceptional performance of ureolytic bacteria in removing cadmium can be attributed to a series of factors. These may include the development of specific tolerance mechanisms within the bacteria [6]. It's plausible that these mechanisms involve the production of cadmium-binding proteins or the activation of detoxification pathways, allowing the bacteria to effectively neutralize and eliminate the harmful effects of cadmium. Furthermore, the consistent presence of high cadmium concentrations in restaurant wastewater, over time, might have driven the evolution of a more resilient and tolerant bacterial population.

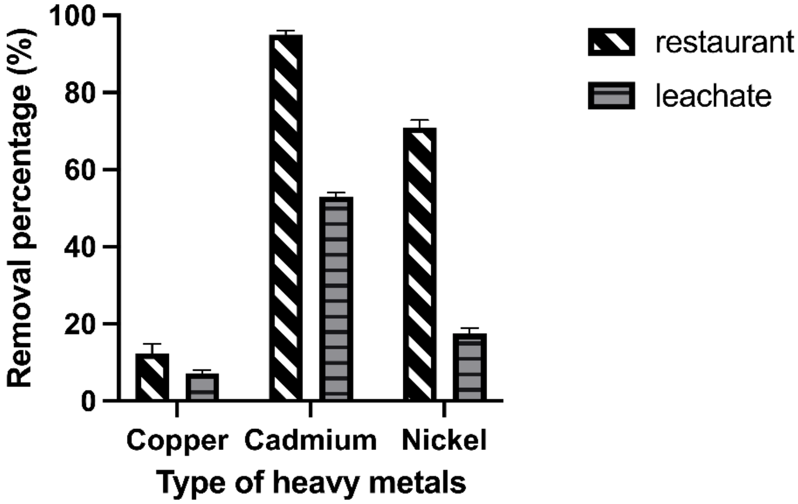

Figure 6. Removal percentage of ureolytic bacteria from restaurant and leachate wastewater for copper, cadmium and nickel heavy metals

Conclusions

In this study, leachate and restaurant wastewater were used as a waste sources of ureolytic bacteria for the purpose of CaCO3 precipitation and heavy metal immobilization. The results showed that a high reactive biomineralization process with precipitation mass of 1.7591 g and urease activity (10.095 mM urea hydrolyzed/min) were achieved when restaurant wastewater was used. Additionally, the results revealed ureolytic bacteria from restaurant wastewater exhibit superior tolerance to Cd^{2+} and Ni^{2+} compared to their leachate counterparts, manifesting optimum urease activity, pH, and optical density (OD). Moreover, AAS analysis demonstrates the restaurant wastewater-derived sample's remarkable proficiency in Cd^{2+} and Ni^{2+} removal, achieving a substantial 95% and 71%removal rate, significantly outperforming the leachate wastewater sample's removal rate of 53% and 18%. This indicates that restaurant wastewater has great potential as an alternative wastewater growth medium for MICP heavy metal application. However, the study also showed that for certain heavy metals such as Cu^{2+}, the tolerance and removal of ureolytic bacteria from both restaurant and leachate wastewater samples were below par (13 and 7%) respectively. This study highlights the potential of wastewater as valuable resources for using ureolytic bacteria to remediate heavy metals. It also underscores the importance of further research including microscopic and mineralogical analyses of post-MICP treatment precipitates for various heavy metals, and deeper insights into the microbial community within restaurant wastewater through metagenomic analysis.

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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