BACILLUS SUBTILIS MEDIATED CALCIUM CARBONATE PRODUCTION AND POLLUTANT ENTRAPMENT IN WASTEWATER USING PRECIPITATING AGENTS

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by

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LIST OF ABBREVIATIONS

MICP	Microbially Induced Calcium Carbonate Precipitation
$CaCO_3$	Calcium Carbonate
B. subtilis	*Bacillus subtilis*
UYE	Urea Yeast Extract (medium)
FTIR	Fourier – Transform Infrared Spectroscopy
SEM	Scanning Electron Microscopy
EDX	Energy – Dispersive X – ray Spectroscopy
XRD	X – ray Diffraction
COD	Chemical Oxygen Demand
AAS	Atomic Absorption Spectrometry
OFAT	One Factor at a Time (Experimental approach)
ACC	Amorphous Calcium Carbonate
HMs	Heavy Metals
IWK	Indah Water Konsortium (wastewater treatment facility)

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PENGHASILAN KALSIUM KARBONAT YANG DIMEDIASI OLEH *BACILLUS SUBTILIS* DAN PERANGKAP PENCEMAR DALAM AIR SISA MENGGUNAKAN AGEN PEMENDAKAN

ABSTRAK

Kalsium karbonat ($CaCO_3$) digunakan secara meluas dalam industri seperti pembinaan, kertas, farmaseutikal, dan bioperubatan disebabkan kelimpahannya dan sifat serbaguna. Namun, pengekstrakan dan penghasilan $CaCO_3$ konvensional memerlukan penggunaan tenaga yang tinggi dan memberi impak negatif kepada alam sekitar, mendorong keperluan kaedah pengeluaran yang lebih lestari. Presipitasi kalsium karbonat yang dipacu secara mikrob (MICP), yang dikendalikan oleh aktiviti urease mikrob, menawarkan kaedah mesra alam. Kajian ini menilai *Bacillus subtilis* untuk penghasilan $CaCO_3$ berasaskan MICP menggunakan kalsium klorida dan kalsium nitrat tetrahidrat, dengan penekanan pada air sisa domestik sebagai sumber lestari. Pengoptimuman sistematik menggunakan pendekatan Satu Faktor pada Masa (OFAT) dalam medium urea sintetik mengenal pasti keadaan optimum (20 g/L urea, pH 8, 30°C, 150 rpm), menghasilkan 2.47 g/L $CaCO_3$ dengan kalsium klorida dan 1.94 g/L dengan kalsium nitrat tetrahidrat. Penggunaan kaedah ini pada air sisa menggandakan hasil kepada 5.00 g/L dan 3.53 g/L masing-masing, meningkatkan kecekapan. Pencharakteran (FTIR, SEM-EDX, XRD) menunjukkan bahawa persekitaran mempengaruhi bentuk $CaCO_3$, dengan struktur amorfus dalam medium sintetik dan campuran kalasit-amorfus dalam air sisa. Analisis EDX mengesan logam berat (Pb, Cu, Cr, Zn) dalam $CaCO_3$ yang diperoleh dari air sisa, menunjukkan manfaat berganda untuk pemulihan sumber dan pengurusan pencemar. Keputusan ini

menunjukkan potensi MICP untuk aplikasi ekonomi kitaran lestari dengan menukar pencemar kepada bahan berguna.

BACILLUS SUBTILIS MEDIATED CALCIUM CARBONATE PRODUCTION AND POLLUTANT ENTRAPMENT IN WASTEWATER USING PRECIPITATING AGENTS

ABSTRACT

Calcium carbonate ($CaCO_3$) is widely used in industries like construction, paper, pharmaceuticals, and biomedicine due to its abundance and versatile properties. However, conventional $CaCO_3$ extraction and synthesis require high energy consumption and have negative environmental impacts, driving the need for more sustainable production methods. Microbially induced calcium carbonate precipitation (MICP), driven by microbial urease activity, offers an eco-friendly method. This study evaluates *Bacillus subtilis* for MICP-based $CaCO_3$ production using calcium chloride and calcium nitrate, focusing on domestic wastewater as a sustainable resource. Systematic optimization using the One Factor at a Time (OFAT) approach in synthetic urea medium identified optimal conditions (20 g/L urea, pH 8, 30°C, 150 rpm), yielding 2.47 g/L $CaCO_3$ with calcium chloride and 1.94 g/L with calcium nitrate tetrahydrate. Applying these to wastewater doubled yields to 5.00 g/L and 3.53 g/L, respectively, enhancing efficiency. Characterization (FTIR, SEM-EDX, XRD) showed that the environment influenced $CaCO_3$ forms, with amorphous structures in synthetic media and mixed calcite-amorphous in wastewater. EDX detected heavy metals (Pb, Cu, Cr, Zn) in wastewater-derived $CaCO_3$, indicating dual benefits for resource recovery and pollutant management. These results demonstrate MICP's promise for sustainable circular economy applications by converting pollutants into valuable materials.

CHAPTER 1

INTRODUCTION

1.1 Research Background

Nanomaterials have become integral to numerous industrial and technological sectors due to their unique physicochemical properties, such as high surface-area-to-volume ratios, tuneable reactivity, and enhanced catalytic efficiency (Comes et al., 2024). Their applications span a wide range of fields, including electronics, textiles, food and agriculture, environmental remediation, and healthcare, where they enable advances such as targeted drug delivery and advanced diagnostics (Fadia et al., 2021). Among these, calcium carbonate ($CaCO_3$) nanoparticles have attracted considerable research interest owing to their abundance, biocompatibility, biodegradability, and low-cost synthesis (Bewernitz et al., 2024). $CaCO_3$ is widely used in construction, paper manufacturing, pharmaceuticals, and biomedicine, especially for drug and gene delivery systems due to its safe interaction with biological environments (Manikandan et al., 2022; Xiang et al., 2022; Zheng et al., 2022).

Despite its abundance, conventional extraction and synthesis of $CaCO_3$ primarily through mining and chemical processing are energy-intensive and environmentally taxing, catalyzing interest in alternative, sustainable production methods (Dardau et al., 2021). One such promising approach is microbially induced calcium carbonate precipitation (MICP), which uses microbial metabolic activity to precipitate $CaCO_3$ in an environmentally benign manner, aligning with the objectives of green chemistry and sustainable industrial development (Comes et al., 2024).

MICP relies on the metabolic activity of microbes to manipulate the physicochemical conditions of their environment, facilitating the rapid formation of calcium carbonate through microbial enzyme activity in the presence of calcium and carbon sources (Ahmad et al., 2023). Several metabolic pathways can drive MICP, including photosynthesis, urea hydrolysis, sulfate reduction, and methane oxidation (Fang & Achal, 2024). However, the urea hydrolysis pathway is the most widely used and well-developed method for $CaCO_3$ precipitation (Addenan et al., 2023; Cuccurullo et al., 2024; Liu et al., 2024; Wang et al., 2024).

Among urease-producing bacteria, *Bacillus subtilis* demonstrates promising potential due to its environmental resilience (Javeed et al., 2024), non-pathogenic nature (risk group 1) (Yamasamit et al., 2023) and robust urease activity (Ezzat, 2023). For instance, a previous study reported the highest rate of calcium carbonate formation and urease activity recorded in *B. subtilis* with 24.15 mg/mL of calcium carbonate and 4.40 x 10^3 unit/L of urease, surpassing *B. mobilis* and *B. alkalinitrilicus,* which yielded 22.85 mg/mL and 3.93 x 10^3 unit/L, respectively (Fazelikia et al., 2023). In addition, the optimum conditions for *B. subtilis*-mediated MICP typically occur at 30–35°C and a pH of 7–9 (Rajasekar et al., 2021), making it particularly suitable for industrial and environmental applications (Thongchom et al., 2024). These attributes highlight the capacity of *B. subtilis* to provide a sustainable pathway for $CaCO_3$ production while enabling applications in environmental remediation and self-healing construction materials (Chen et al., 2024; Ibrahim et al., 2025).

Furthermore, this study aims to advance the principles of the circular economy by exploring the integration of MICP with real-world waste streams, particularly domestic wastewater, as a resource for biomineralization. This approach not only valorizes waste by converting it into valuable materials but also reduces environmental pollution and conserves natural resources, aligning with sustainable development goals. By coupling waste management with bio-based material synthesis, the research extends the environmental sustainability impact of MICP beyond simple calcium carbonate precipitation, fostering a closed-loop system that promotes resource efficiency and ecological balance. Nevertheless, despite well-established mechanistic insights into MICP, knowledge gaps remain regarding the fine-tuning of process parameters and, more importantly, the integration of MICP with complex real-world waste streams such as domestic wastewater.

1.2 Problem Statement

Despite the natural abundance of calcium carbonate ($CaCO_3$), Current industrial $CaCO_3$ production is fraught with environmental and economic challenges, including high energy consumption, greenhouse gas emissions, and the generation of hazardous byproducts (Dardau et al., 2021). These conventional processes are not only costly but also environmentally unsustainable, prompting increased interest in alternative, greener production methods. As a result, research has focused on sustainable approaches such as MICP, which offers the potential to induce $CaCO_3$ in an environmentally friendly and energy-efficient manner.

Among the different MICP pathways, such as denitrification, sulfate reduction, and urea hydrolysis, ureolysis is the most widely preferred. This is because it provides a faster reaction rate, higher efficiency in carbonate ion generation, and requires relatively simple substrates like urea, making it more suitable for controlled laboratory and practical applications. In this process, urea acts as the main substrate, and bacteria such as *B. subtilis* produce urease to catalyze its breakdown, ultimately leading to $CaCO_3$ precipitation. In this study, a synthetic medium composed of urea and yeast extract was used to promote microbial growth and activity. Additionally, domestic wastewater was investigated as an alternative substrate, as it contains organic matter and nutrients that can support microbial metabolism and urea hydrolysis, thus inducing $CaCO_3$ precipitation when a calcium source is present.

The use of domestic wastewater is particularly significant, as improper treatment and discharge of such effluents into natural water bodies can lead to serious environmental issues, including eutrophication, proliferation of harmful microorganisms, and the spread of waterborne diseases (Obaideen et al., 2022; Tajarudin et al., 2019). By utilizing domestic wastewater in the MICP process, this research not only aims to provide a cost-effective and sustainable substrate for $CaCO_3$ production but also addresses the urgent need for improved wastewater management.

Interestingly, $CaCO_3$ precipitated from wastewater has been shown to possess the ability to entrap heavy metals and other pollutants present in the effluent (Li et al., 2022; Liu et al., 2024; Rajasekar et al., 2021). This co-precipitation and lattice substitution mechanism not only reduces the concentration of hazardous contaminants in water but also results in the formation of a valuable product. However, it is important to note that ureolysis-based MICP has certain limitations, including the generation of ammonia byproducts that may pose secondary environmental concerns, as well as scale-up challenges in large systems that affect process control and efficiency.

Therefore, the approach presented in this study is both sustainable and novel, as it simultaneously addresses the challenges of green $CaCO_3$ production and effective wastewater remediation, while also considering the practical limitations of the ureolysis pathway. This contributes to advancing environmental sustainability and public health protection.

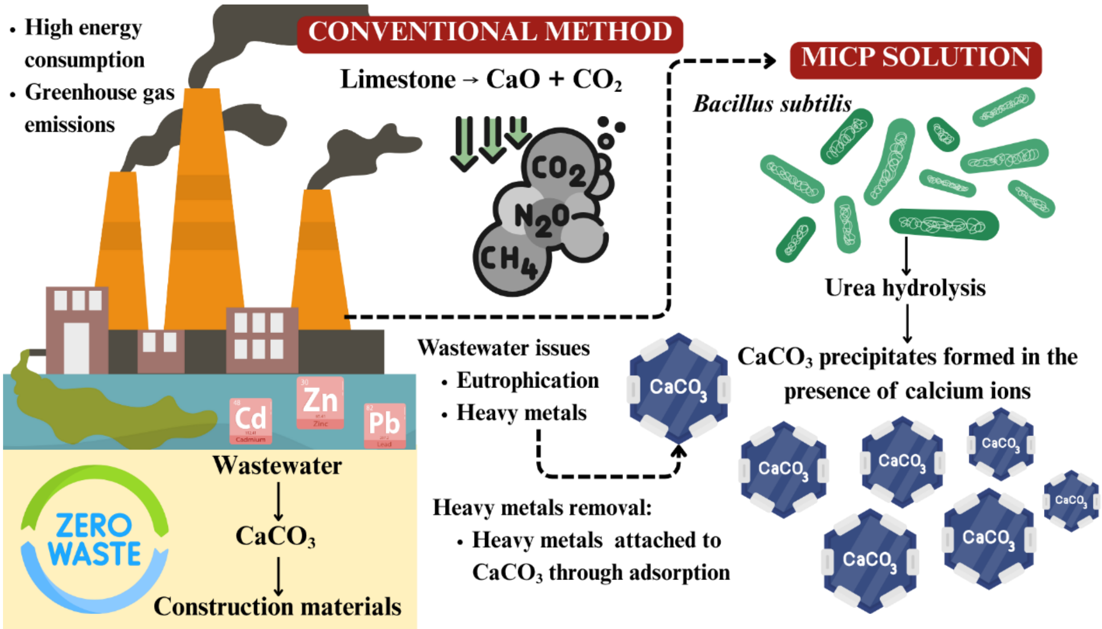

Figure 1.1 Sustainable $CaCO_3$ Production via MICP coupled with wastewater remediation

1.3 Research Scopes and Objectives

This research focuses on addressing the environmental and industrial challenges outlined in the problem statement by optimizing the MICP process using *Bacillus subtilis*. The scope encompasses both synthetic urea medium and domestic wastewater as substrates, with the dual goals of sustainable $CaCO_3$ production and wastewater remediation. By bridging the gap between laboratory-scale MICP optimization and real-world wastewater treatment, this study aims to advance eco-friendly alternatives to conventional $CaCO_3$ production while addressing urgent environmental challenges. This study focuses on the following objectives:

1.3.1 Objective 1: To investigate the optimal conditions for *Bacillus subtilis* in converting urea into ammonia and carbonic acid through urea hydrolysis, ultimately leading to the formation of calcium carbonate ($CaCO_3$).

This objective involves systematically optimizing key parameters, including pH, temperature, urea concentration, and agitation speed, to maximize urea hydrolysis efficiency and $CaCO_3$ yield. The synthetic urea medium, which consists of urea and yeast extract, serves as a controlled environment to establish baseline conditions, while domestic wastewater is tested as a cost-effective alternative substrate. Yeast extract provides essential nutrients to sustain bacterial growth and urease activity, critical for efficient urea hydrolysis.

1.3.2 Objective 2: To determine and compare the characteristics of the $CaCO_3$ polymorphs formed in both synthetic urea and domestic wastewater.

The second objective of this study focuses on examining the characteristics of $CaCO_3$ precipitates produced from both synthetic urea medium and domestic wastewater. To achieve this, several analytical techniques, including Fourier-transform infrared spectroscopy (FTIR), scanning electron microscopy (SEM), and X-ray diffraction (XRD), will be employed to observe and compare the crystal structure, morphology, and chemical composition of the $CaCO_3$ formed under different conditions.

Identifying $CaCO_3$ polymorphs such as calcite, aragonite, and vaterite is crucial because they differ in stability, solubility, and mechanical properties. Polymorph formation depends on factors such as substrate composition, impurities, microbial activity, pH, and temperature. Synthetic urea media yield more consistent polymorphs, whereas domestic wastewater's complexity causes variability. Nonetheless, controlled conditions can reproducibly form main polymorphs even in complex substrates, underscoring the importance of polymorph characterization to optimize biomineralization in practical applications.

1.3.3 Objective 3: To evaluate the potential of $CaCO_3$ formed in wastewater to entrap environmental pollutants.

This objective examines the ability of MICP-derived $CaCO_3$ to immobilize heavy metals and other pollutants through mechanisms such as co-precipitation, lattice substitution, and adsorption. The detection of heavy metals and other pollutants was observed through Energy Dispersive X-ray Analysis (EDX).

CHAPTER 2

LITERATURE REVIEW

2.1 Introduction to Calcium Carbonate ($CaCO_3$) and Its Industrial Significance

Nanomaterials have become integral to numerous industrial and technological sectors due to their unique physicochemical properties, such as high surface-area-to-volume ratios, tuneable reactivity, and enhanced catalytic efficiency. Their applications span a wide range of fields including electronics, textiles, food and agriculture, environmental remediation, and healthcare (Comes et al., 2024). For instance, in electronics, nanomaterials enable the development of smaller, faster, and more efficient devices, while in textiles, they impart water repellence, stain resistance, and durability to fabrics. Environmental applications include air and water purification technologies, and in healthcare, nanomaterials facilitate targeted drug delivery and advanced diagnostics (Fadia et al., 2021).

Among the various types of nanomaterials, metal oxide nanoparticles have gained significant prominence due to their versatility in catalysis, energy conversion, and industrial manufacturing processes. Calcium carbonate ($CaCO_3$) nanoparticles, in particular, have attracted considerable research interest owing to their distinctive physical and chemical characteristics. Calcium is one of the most abundant elements in the Earth's crust, with $CaCO_3$ constituting about 4% of it, making calcium carbonate a widely available and economically viable material (Bewernitz et al., 2024). The unique properties of $CaCO_3$ nanoparticles, such as biocompatibility, biodegradability, and low-cost synthesis, have facilitated their extensive use in industrial sectors including construction, paper manufacturing, pharmaceuticals, and biomedicine. In biomedical applications, $CaCO_3$ nanoparticles are especially valued for drug, gene, and enzyme delivery systems, leveraging their ability to safely interact with biological environments (Manikandan et al., 2022; Xiang et al., 2022; Zheng et al., 2022).

Despite its abundance, the conventional extraction and synthesis of calcium carbonate primarily through mining and chemical processing are often energy-intensive and environmentally taxing. This has catalysed growing interest in alternative, sustainable production methods. One such promising approach is through biomineralization by microbial-induced calcium carbonate precipitation (MICP), which offers a route to generate $CaCO_3$ in an environmentally benign manner, aligning with the broader objectives of green chemistry and sustainable industrial development (Comes et al., 2024).

2.2 Biomineralization through Microbially Induced Calcium Carbonate Precipitation (MICP)

Over the past several decades, MICP has garnered significant interest within the scientific community, resulting in a substantial volume of research dedicated to evaluating various methodologies and their outcomes (Rajasekar et al., 2021). This innovative technique leverages the metabolic processes of microorganisms by carefully adjusting the physicochemical conditions of their environment to promote the precipitation of specific biominerals. In essence, MICP enables the rapid synthesis of calcium carbonate through the catalytic action of microbial enzymes, which are activated in the presence of calcium ions and suitable carbon sources (Ahmad et al., 2023).

Several metabolic pathways can drive MICP, including photosynthesis, urea hydrolysis, sulphate reduction, and methane oxidation (Fang & Achal, 2024). Despite the diversity of available pathways, the precipitation of calcium carbonate via bacterial urea hydrolysis has become the predominant method employed in both research and practical applications (Addenan et al., 2023; Cuccurullo et al., 2024; Liu et al., 2024; Wang et al., 2024). This approach, favored for its efficiency and well-characterized mechanisms, commonly employs urease-producing bacteria such as *Sporosarcina pasteurii*, *B. subtilis*, *B. megaterium*, *B. cereus*, and *B. sphaericus*, among others, which have demonstrated high urease activity and robust calcium carbonate precipitation capabilities in various environmental and engineering contexts (Feng et al., 2021; Liu et al., 2024; Rajasekar et al., 2021; Su et al., 2020).

2.3 Mechanism of Urea Hydrolysis

Urea hydrolysis represents the most extensively researched and established process within geotechnical engineering, constituting approximately 43% of relevant metabolic pathways. This process is characterized by its relatively low complexity, ease of control, and capacity to precipitate up to 90% of the targeted calcium carbonate ($CaCO_3$) within less than 24 hours (Safaa et al., 2023). Effective microbially induced calcium carbonate precipitation (MICP) is a straightforward biochemical reaction, mediated by urease-producing bacteria when an adequate supply of urea and calcium ions (Ca^{2+}) is present.

The principal chemical reactions underpinning $CaCO_3$ crystallization via ureolysis are delineated in equations below (Prajapati et al., 2023). Initially, bacterial urease enzymes catalyze the hydrolysis of urea, yielding ammonia (NH_3) and carbamic acid (NH_2COOH) (1.1). Subsequently, carbamic acid spontaneously decomposes to form carbonic acid (H_2CO_3) and an additional mole of ammonia (NH_3) (1.2). Further hydrolysis of the generated ammonia leads to the production of ammonium ions (NH_4^+) and hydroxide ions (OH^-) (1.3). The resultant increase in hydroxide ion concentration elevates the pH of the surrounding environment, thereby facilitating the formation of bicarbonate (HCO_3^-) and carbonate (CO_3^{2-}) ions (1.4 and 1.5). Ultimately, the carbonate ions (CO_3^{2-}) combine with available calcium ions (Ca^{2+}) to precipitate as calcium carbonate ($CaCO_3$) crystals (1.6). The overall reaction governing ureolysis-driven MICP for $CaCO_3$ precipitation is summarized in Equation 1.7.

$$CO(NH_2)_2 + H_2O \rightarrow NH_2COOH + NH_3 \quad (1.1)$$

$$NH_2COOH + H_2O \rightarrow NH_3 + H_2CO_3 \quad (1.2)$$

$$NH_3 + H_2O \rightarrow NH_4^+ + OH^- \quad (1.3)$$

$$H_2CO_3 \leftrightarrow HCO_3^- + H^+ \quad (1.4)$$

$$HCO_3^- + H^+ + 2OH^- \leftrightarrow CO_2^{3-} + 2H_2O \quad (1.5)$$

$$Ca^{2+} + CO_2^{3-} \rightarrow CaCO_3 \text{ (S)} \quad (1.6)$$

$$CO(NH_2)_2 + 2H_2O + Ca^{2+} \rightarrow 2NH_4^+ + CaCO_3 \quad (1.7)$$

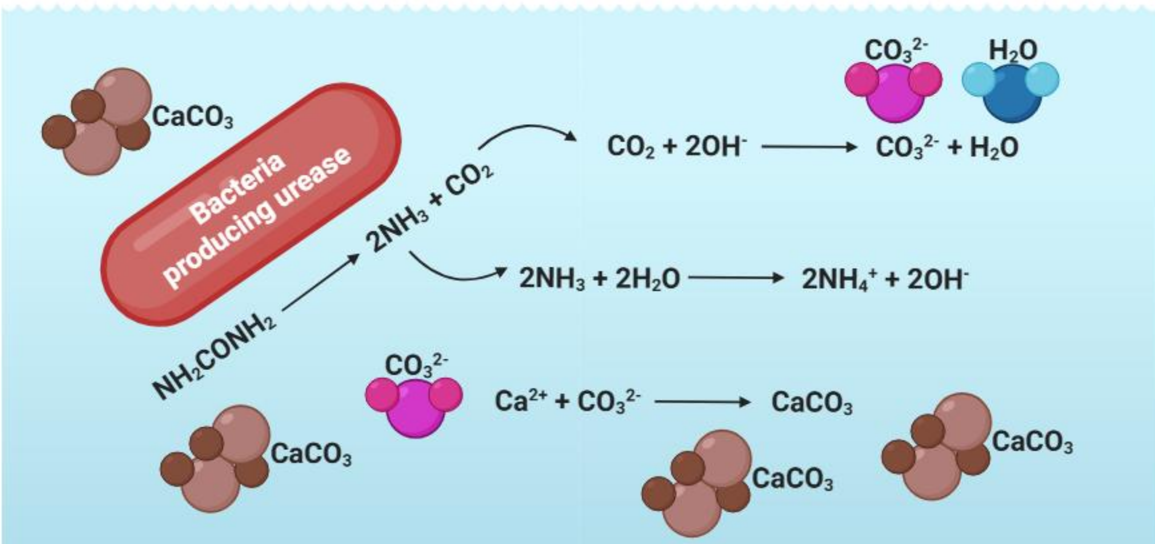

Figure 2.1 Mechanism of MICP.

2.4 Applications of MICP

2.4.1 Removal of heavy metals

Rapid industrialization has led to significant environmental contamination by heavy metals due to their toxicity, persistence, and resistance to biodegradation (Bayuo et al., 2022; Garg et al., 2023; Li et al., 2022; Wang et al., 2021). Furthermore, the accumulation of heavy metals and other hazardous contaminants in environmental matrices poses significant risks to human health and the well-being of various biological organisms. For instance, industrial waste discharge can release toxic heavy metals into aquatic systems, where they are absorbed by plants. Animals consuming these plants accumulate the metals, which ultimately enter the human food chain. Consumption of contaminated plant- and animal-derived foods is the main route of human exposure to elevated heavy metal levels (Kaur et al., 2023). The degree of toxicity depends on metal concentration, highlighting the urgent need for effective remediation of contaminated soils and wastewater (Tajarudin et al., 2019).

Common heavy metals, including cadmium, chromium, cobalt, copper, arsenic, lead, nickel, selenium, silver, zinc, and mercury, occur naturally but are concentrated through human activities (Rajasekar et al., 2021; Zhang et al., 2023). Conventional remediation methods such as adsorption, precipitation, electrochemical treatment, filtration, ion exchange, membrane separation, oxidation-reduction, and reverse osmosis are widely used (Saleh et al., 2022; Sathya et al., 2022; Shehata et al., 2023) but often show limited efficiency, high costs, and significant chemical and energy demands (Ahmed et al., 2021).

In recent years, bioremediation techniques utilizing microorganisms, including phytoremediation, bioaccumulation, bio-coagulation, bioleaching, biosorbents, and bio immobilization, have been implemented for heavy metal removal (Baltrocchi et al., 2024). However, these methods face significant limitations such as high operational costs, extended treatment durations, and risks of remobilization of immobilized or adsorbed metals into ecosystems. Consequently, MICP has emerged as a promising alternative, offering efficient, cost-effective, and environmentally sustainable remediation (Rajasekar et al., 2021; Zhang et al., 2023).

In MICP, calcite precipitates incorporate heavy metal ions not only through isomorphic substitution of divalent cations (Ca^{2+}) within the crystal lattice but also by co-precipitation. Both mechanisms transform soluble metal species into insoluble carbonate compounds, enhancing heavy metal removal from aqueous systems (Yu et al., 2023; Zeng et al., 2023). Supporting this, Li et al., (2022) demonstrated that *Bacillus* species, including *B. subtilis*, achieved removal efficiencies of up to 98.95% for heavy metals within 48 hours under optimized MICP conditions.

2.5 Factors Affecting MICP Efficiency

2.5.1 Bacterial Strain: The Case of *Bacillus subtilis*

The selection of bacterial species is a critical determinant in microbial–induced calcium carbonate precipitation (MICP) efficiency, as the ability to produce urease directly impacts calcite formation. Among the various genera, *Bacillus* species are commonly preferred due to the vigorous urease activity and proven ability to precipitate calcium carbonate (Wang et al., 2024). This is supported by many studies where the *Bacillus* species play a significant role in carbonate deposition in natural habitats. Many urease-producing bacteria have been investigated, including *B. megaterium*, *B. subtilis*, *Bacillus* sp. CR2, *B. thuringiensis*, *B. sphaericus*, *B. cereus,* and *B. pasteurii* (Chuo et al., 2020; Feng et al., 2021; Prajapati et al., 2023; Yamasamit et al., 2023; Thongchom et al., 2024). Different types of bacteria were found to produce various amounts of urease and calcium carbonate precipitation.

Among the various bacteria studied for MICP, *B. subtilis* has received significant attention since the 1970s due to its Gram-positive, rod-shaped, and non-pathogenic characteristics. This microorganism is particularly notable for its exceptional resilience in extreme environments, such as high pH and nutrient-deficient conditions, which are typical in concrete matrices (Rajasekar et al., 2021). Its ability to form durable endospores enables it to survive and remain metabolically active even under these challenging circumstances, making it a preferred candidate for the development of bacteria-based construction materials (Chuo et al., 2020). The effectiveness of *B. subtilis* in MICP has been demonstrated in multiple studies, including those by Fu et al. (2023) and Thongchom et al. (2024), which reported that *B. subtilis* can successfully precipitate calcium carbonate and contribute to enhanced self-healing and mechanical properties in mortar. In addition, Yamasamit et al. (2023)

reported that the *B. subtilis* strain was selected as the microbial agent in their MICP experiment due to its environmental safety and lack of adverse physiological effects on humans (bacteria risk group 1).

Owing to the established safety profile and absence of adverse physiological effects on humans and proven functional urease activity, *B. subtilis* was selected as the primary microbial agent in this research. As a facultative anaerobe, *B. subtilis* produces catalase and various hydrolytic enzymes, supporting its survival in diverse and challenging environments. It exhibits robust urease activity and demonstrates adequate calcium carbonate precipitation, with optimal performance observed across a broad pH range of approximately 4.8 to 9.1 (Javeed et al., 2024).

Temperature tolerance is crucial for MICP, ensuring bacterial survival and effectiveness. Most bacteria, including *Bacillus* species, thrive within a specific temperature range, typically 30–37 °C (Javeed et al., 2024). Comparative studies have shown that *B. subtilis* achieves superior growth within this range, outperforming species such as *B. licheniformis*, *B. cereus*, and *B. halodurans* (Durga et al., 2020). Additionally, *B. subtilis* can withstand extreme temperatures from 10 °C to 50 °C due to its spore-forming capability. This adaptability enhances its potential for MICP applications under varying environmental conditions (Reddy et al., 2020). Therefore, *B. subtilis* remains a strong candidate for MICP because of its urease activity, environmental tolerance, and capacity to promote calcium carbonate precipitation.

2.5.2 Calcium source

Calcium is an essential element in the precipitation of calcium carbonate, which is the primary mechanism of MICP treatment. The calcium ions in the surrounding solution react with the bicarbonate ions, produced by the carbon dioxide released during urea hydrolysis, forming calcium carbonate (Yin et al., 2024). Mori and Uday (2021) reported that the amount of $CaCO_3$ precipitation depends more on Ca^{2+} concentrations than on urea concentrations. These supported by Cuccurullo et al., (2020) investigated the importance of Ca^{2+} for urease activity and found that enzyme activity increased tenfold in the presence of Ca^{2+} when compared to the absence of Ca^{2+}.

Recent research underscores that the selection of calcium source is pivotal in determining the efficiency of the MICP process (Tao et al., 2025). Interestingly, Maston et al. (2025) note that different calcium sources could lead to various crystal morphologies and mineral compositions, which may affect the mechanical properties of the MICP-treated samples. Commonly used calcium sources include calcium chloride, calcium nitrate, and calcium oxide (Ahmad et al., 2023). Calcium chloride is widely favoured due to its high solubility and ability to deliver a substantial concentration of calcium ions, which enhances both urease activity and calcite production. Calcium nitrate is also frequently utilized and has demonstrated comparable effectiveness to calcium chloride in certain applications, with studies showing similar improvements in unconfined compressive strength and calcite formation in treated soils. However, calcium nitrate generally results in a lower reduction in permeability compared to calcium chloride. Calcium oxide, while natural and cost-effective, tends to induce slower calcium carbonate precipitation than calcium chloride and calcium nitrate (Ahmad et al., 2023; Qiu et al., 2024; Ren et al., 2024).

Comparative studies have consistently shown that calcium chloride outperforms other calcium sources in promoting MICP efficiency, followed by calcium nitrate, calcium acetate, and calcium oxide (Achal & Pan, 2014; Gorospe et al., 2013; Tao et al., 2025). For instance, Achal et al. (2014) and Gorospe et al. (2013) both identified calcium chloride as the most effective for inducing calcium carbonate precipitation, with calcium nitrate serving as a viable alternative. These studies collectively demonstrate that calcium chloride consistently enhances MICP performance more effectively than other commonly used calcium sources.

2.5.3 Carbon source

The carbon source is another critical chemical factor influencing the effectiveness of MICP treatment. Carbon plays an essential role in the precipitation of carbonate minerals, which is the fundamental mechanism driving the MICP process (Erdmann & Strieth, 2023). Urea is the most widely utilized carbon source in MICP applications due to its affordability and ready availability. Urease-producing bacteria hydrolyzed urea to generate carbon dioxide and ammonium ions. The carbon dioxide subsequently reacts with calcium ions in the environment to form calcium carbonate. However, the success of urea-based MICP treatments largely depends on the presence of active urease-producing bacteria and the solution's pH (Ahmad et al., 2023).

Alternative carbon sources such as acetate and lactate yield lower precipitation rates and require longer treatment times (Chuo et al., 2020). Furthermore, carbon source concentration critically impacts MICP efficiency, where higher levels enhance $CaCO_3$ formation but may compromise bacterial viability (Lai et al., 2024). This is supported by Azmi et al. (2018), who demonstrated that elevated urea concentrations result in higher ammonia production during hydrolysis, potentially to inhibit bacterial growth.

2.5.4 pH

The pH of the solution is a crucial parameter that significantly affects both the activity and viability of bacteria involved in MICP, as well as the rate and efficiency of calcium carbonate ($CaCO_3$) precipitation (Prajapati et al., 2023). The enzymatic hydrolysis of urea by urease, which catalyses $CaCO_3$ formation, is most effective within a specific pH range that favours this biochemical reaction. Typically, urease-producing bacteria exhibit optimal activity in mildly alkaline environments, with pH values between 8 and 9.5 being particularly conducive to $CaCO_3$ precipitation (Safaa et al., 2023). In this context, *B. subtilis* was selected for this study due to its ability to tolerate slightly alkaline conditions up to pH 9.1 (Javeed et al., 2024).

Liu et al. (2021) reported that *B. subtilis* has been demonstrated through MICP experiments to perform optimally at pH 8. Additional comparative studies have reported that *B. subtilis* can sustain growth in alkaline environments with pH values up to 9, exhibiting functionality comparable to *Sporosarcina pasteurii*, a widely used bacterium in MICP known to thrive at an optimum pH of 9.25 (Addenan et al., 2023; Feng et al., 2021; Thongchom et al., 2024). However, bacterial growth tends to cease when the pH reaches 10. At lower pH values, bacterial activity is often suppressed, resulting in slower carbonate precipitation. Conversely, excessively high pH levels reduce calcium ion solubility, thereby limiting bacterial access to these ions in solution. Maintaining an optimal pH not only promotes efficient $CaCO_3$ formation but also enhances CO_2 absorption in the solution, facilitated by the presence of hydroxide ions (OH^-) (Gebru et al., 2021).

2.5.5 Nutrient concentration

MICP relies on bacterial metabolic activity, necessitating essential nutrients such as carbon, nitrogen, and phosphorus to sustain growth and biochemical functions (Lapierre et al., 2020). Carbon, in particular, is vital for energy production and organic molecule synthesis, with the choice of carbon source directly influencing the efficiency and rate of calcium carbonate formation. Urea, calcium lactate, and sodium acetate are commonly used carbon sources, though urea stands out due to its dual role as a nitrogen source and its cost-effectiveness (Ahmad et al., 2023).

Research indicates that urea is a highly effective nitrogen source for enhancing urease activity in *B. subtilis*. Liu et al. (2024) demonstrated that cultures in urea yeast extract (UYE) medium achieved peak urease activity (11.43%), outperforming alternative nitrogen sources like ammonium chloride-YE, ammonium-YE, and sodium nitrate-YE. This superiority stems from urea's dual capacity to stimulate urease expression while maintaining cost efficiency. It solidifies its status as the optimal nitrogen source for MICP applications (Azmi et al., 2018). Rajasekar et al. (2021) corroborated these findings, emphasizing urea's economic advantage over alternatives such as ammonium chloride for large-scale MICP implementation.

Yeast extract further complements urea by providing a rich blend of amino acids, carbs, and organic acids, critical for bacterial metabolism. Pei et al. (2021) noted that yeast extract significantly boosts urease activity and biomineralization efficiency. Upon uptake, carbs from yeast extract were hydrolyzed in glycolysis, thus producing pyruvate. The amino acids will undergo deamination and transamination, yielding intermediates such as glutamate, α-keto acids, and α-ketoglutarate (Chen et al., 2022). These compounds will be fed into TCA cycle. Pyruvate from glycolysis is decarboxylated to acetyl-CoA, a key substrate for the TCA cycle, while organic acids are directly converted to acetyl-CoA, enhancing metabolic flux.

The TCA cycle oxidizes these intermediates, generating NADH, $FADH_2$, and CO_2 as byproducts. CO_2 diffuses into the surrounding environment, where it reacts with water to form carbonic acid (H_2CO_3). This acid dissociates into bicarbonate ions (HCO_3^-), which, in the presence of calcium ions, precipitate as calcium carbonate ($CaCO_3$), driving biomineralization (Bu et al., 2022; Fowler et al., 2024). Thus, the UYE medium not only supports *B. subtilis* growth through nutrient-rich yeast extract but also connects metabolic pathways to carbonate ion production, enhancing MICP efficiency. The remarkable metabolic adaptability of *B. subtilis*, exemplified by its ability to utilize diverse pathways such as glycolysis, the TCA cycle, amino acid metabolism, and other related processes, makes it an exceptionally promising agent for effective and robust MICP across various environments, as evidenced by extensive prior research (Akinsemolu et al., 2024; Hernández-Sancho et al., 2024).

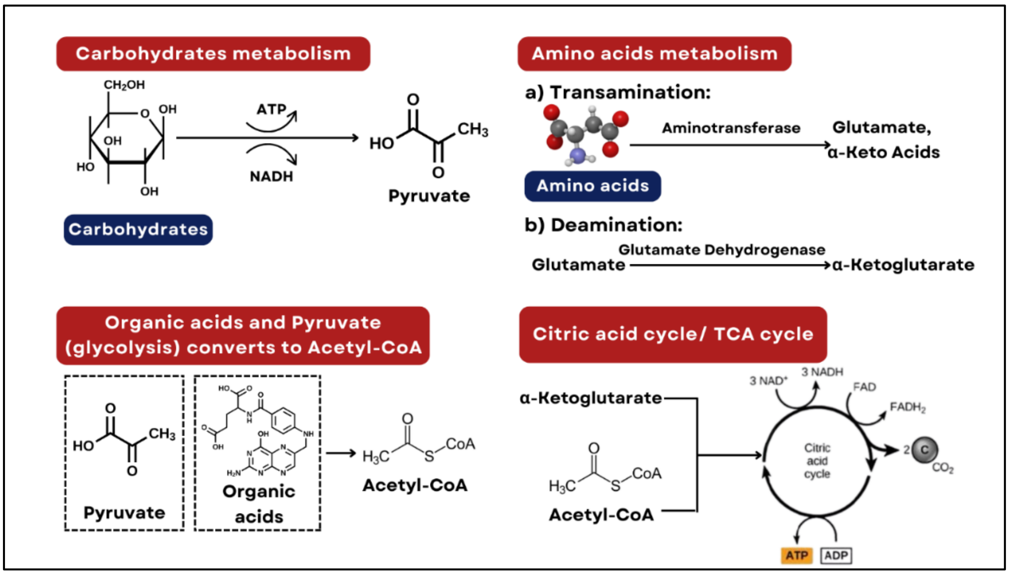

Figure 2.2 Integrated metabolic pathway involved by *B. subtilis* from glycolysis, through transamination, deamination and the TCA cycle, finally linking CO_2 production to the MICP process.

2.5.6 Temperature

Every enzyme-related mechanism relies heavily on the temperature of its environment. Since bacteria are also sensitive to temperature, optimizing the temperature for MICP is crucial when it comes to its application in the ecosystem. Multiple studies have shown that the ideal temperature for MICP involving urease-secreting bacteria (USB) is 30–35°C (Song et al., 2024; Yuze & Charalampos, 2024), as the enzyme concentration is superior due to rapid exponential bacterial growth. This temperature range is also employed in fermentation processes for *B. subtilis* when examining its self-healing properties in mortar and its MICP efficiency, as reported by Feng et al. (2021) and Thongchom et al. (2024). In addition, Hu et al., (2023) also observed that urease had maximum activity at 30°C.

Wang, et al. (2023) investigated the effects of temperatures (4–50°C) on urease activity and calcium carbonate precipitation, and found that low temperature (4°C) did not reduce urease activity, but limited the final amount of cementation. This is because low temperature reduced bacterial growth and attachment ratio, as well as calcium carbonate precipitation rate. At high temperature (50 °C), the reduction in bacterial urease activity with time was due to the reduction in both bacterial density and bacterial-specific urease activity (Lai et al., 2025). Garg et al. (2023) reviewed the effects of temperature on pure free enzyme activity and found that urease activity decreases with time at different temperatures, with higher temperatures reducing the activity more quickly than lower temperatures. Neha et al. (2021) studied the urease production ability of bacterial strains at different temperatures and found that urease production rates increase with temperature until the temperature reaches 30 °C, after which urease activity starts decreasing until 50 °C.

2.5.7 Agitation

Agitation is another factor that influences the effectiveness of microbial-induced calcium carbonate precipitation (MICP) treatment on fine aggregates. It involves the mechanical mixing or stirring of the MICP solution and fine aggregates, which promotes nutrient diffusion and facilitates the removal of reaction byproducts, thereby enhancing bacterial activity and calcium carbonate precipitation. Increased agitation can improve mixing efficiency and accelerate nutrient diffusion and byproduct removal, resulting in more extensive calcium carbonate precipitation (Arpajirakul et al., 2021). Several methods are available to introduce agitation during MICP treatment, including shaking, stirring, and sonication. Mechanical agitation is the basis for shaking and stirring, whereas sonication uses ultrasonic waves to promote mixing and create cavitation. The choice of agitation method depends on the particular needs of the MICP process, since each approach can influence bacterial activity and the effectiveness of precipitation in a distinct way.

2.6 Significance of $CaCO_3$ Polymorphs: Properties and Applications

Calcium carbonate ($CaCO_3$) has garnered significant attention due to its polymorphic nature and wide-ranging applications in fields such as bio-cementation, medicine, and soil reinforcement. As a polymorphic material, $CaCO_3$ exists in several structural forms, including calcite, aragonite, vaterite, and amorphous calcium carbonate (ACC). Among these, calcite is the most stable polymorph under standard temperature and pressure conditions (Khanjani et al., 2021; Liu et al., 2021; Rautela & Rawat, 2020). The crystallization process of $CaCO_3$ in natural systems typically begins with the formation of ACC, which subsequently transforms into other polymorphs depending on environmental factors. Each polymorph exhibits distinct physical and chemical properties, influencing its suitability for specific applications.

Aragonite is characterized by its needle-like crystalline structure, which imparts higher hardness and brittleness compared to other polymorphs. This makes aragonite ideal for applications requiring mechanical strength and wear resistance, such as abrasives and polishing compounds. On the other hand, calcite's relatively low hardness and good cleavage properties make it suitable for applications requiring flexibility and ease of processing. Vaterite, the least stable polymorph, offers unique morphological features such as spherical shapes with high specific surface areas and abundant pore structures (Rizky et al., 2024; Stolarski et al., 2025). These features make vaterite particularly useful in advanced technologies, including drug delivery systems and heavy metal adsorption. Thus, the structural characterization of $CaCO_3$ is very important so that the desired material criteria can be obtained and the application is more precise.

2.7 Alternative Substrate Approach: Wastewater

Most MICP-USB processes require a nitrogen and calcium source (Ahmad et al., 2023; Lapierre et al., 2020; Rajasekar et al., 2021). The substrate source must be cost-effective since MICP is currently being proposed as an alternative to conventional remediation techniques. From industrial, domestic, and agricultural sources, wastewater has been proposed as an ideal solution due to its high organic matter content (Omoregie et al., 2024). The wastewater usually contains high amounts of calcium, which could serve as a calcium source for mineral precipitation. Senfter et al. (2021) claimed that effluents of those different sectors contain organic carbon, nitrogenous organics, inorganics, suspended or dissolved solids, and heavy metals. This observation is consistent with findings by Liu et al. (2024) who highlighted that wastewater effluents contribute significantly to water pollution by discharging a variety of contaminants, including ammonia nitrogen (NH_4^+-N), nitrate (NO_3^--N), fluoride (F^-), heavy metals, phosphate (PO_4^{3-}), and organic pollutants such as phenol, into aquatic environments. These pollutants pose severe risks to environmental sustainability, ecosystem stability, and human health, with long-term exposure leading to methemoglobinemia and cancer (Kaur et al., 2023).

Tajarudin et al. (2019) highlighted the importance of wastewater management, and this statement is strongly agreed upon by Wang et al. (2024), where improperly treated wastewater is discharged into natural water bodies, which can cause irreversible damage, necessitating the development of effective remediation technologies. Biological treatments like microbial-induced calcium precipitation (MICP) offer eco-friendly, cost-effective alternatives with minimal secondary pollution. Interestingly, under the action of MICP, the efficient removal of the aforementioned pollutants can be achieved through reaction mechanisms such as co-precipitation and lattice replacement (Chuo et al., 2020; Rajasekar et al., 2021, Wang & Konstantinou, et al., 2023; Zhang et al., 2023). For instance, Li et al., (2022) investigated using MICP technology to combine heavy metals with carbonate ions generated by microorganisms to form metal carbonate. They found that it reduces the bioavailability of heavy metals with a removal efficiency of up to 98.95% (Li et al., 2020). Apart from that, the innovative potential of MICP technology for carbon sequestration exceeds theoretical expectations, and practical applications are already underway (Fang & Achal, 2024).