Research Article

Exploration of Microbial Culture Techniques For Microbial-Induced Calcite Precipitation in Civil Engineering Applications

Sheikh Abbas Muhammad[1,2], Mohd Aaqib[1], Mir Aijaz[1], Malik Nowsheen[3], Ishfaq ul Abass[2]

[1]IUST, Kashmir, India.
[2]MNIT, Jaipur, India.
[3]SKUAST, Kashmir, India.
Corresponding Author: Sheikh Abbas Muhammad, E-mail: sheikh.abbas@iust.ac.in

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Abstract

Microbial-induced calcite precipitation (MICP) holds promise for revolutionizing civil engineering practices, particularly in soil stabilization and ground improvement. This paper presents a detailed exploration of microbial culture techniques crucial for understanding and replicating MICP experiments, catering to civil engineers unfamiliar with biological methodologies. Bacillus subtilis, chosen for its non-hazardous nature, serves as the primary organism, cultured under controlled conditions. The preparation of Luria Agar (LA) and Luria Broth (LB) media is meticulously outlined, along with bacterial colony cultivation through streaking and spreading techniques. Spectrophotometry facilitates precise cell density quantification, while urease tests differentiate urease-positive organisms. Microscopic examination provides visual insights into bacterial morphology. Centrifugation enables efficient cell harvesting, while stock preparation ensures long-term preservation. Common challenges in bacterial culture are addressed, emphasizing the importance of quality control measures. Through interdisciplinary collaboration, this study bridges microbiology with civil engineering, fostering innovation and sustainable practices.

Keywords: Microbial culture techniques, Bacillus subtilis, Luria Agar (LA) and Luria Broth (LB) media, Microbial-induced calcite precipitation (MICP), Civil engineering applications.

INTRODUCTION

Microbiological culture, a fundamental technique in microbiology, involves the controlled reproduction of microbial organisms within predetermined culture media in laboratory conditions. This method serves pivotal roles in identifying microbial types, quantifying their abundance in tested samples, and, notably, isolating pure cultures. A pure culture, devoid of other species or types, is indispensable for various applications, especially in the context of biologically influenced civil engineering innovations.

The gel medium used for microbial culture is typically agarose gel (agar), a gelatinous substance derived from seaweed. While agar is widely employed, a cost-effective alternative is guar gum, facilitating isolation and maintenance processes Neidhardt et al. (1974). Essential to microbial growth, culture media comprise nutrients and physical parameters Young, E. W., & Beebe, D. J. (2010). However, not all microorganisms thrive in a single medium, necessitating diverse formulations Jeanson, S. et al. (2015) Muhammad, S. A et al (2025) .

Key constituents of culture media include inorganic salts, crucial for maintaining osmotic balance and regulating membrane potential. Carbohydrates, such as glucose and galactose, serve as the primary energy source, while amino acids, the building blocks of proteins, must be supplemented, especially essential amino acids that cells cannot synthesize. Vitamins, proteins, and peptides, often sourced from serum or enriched in media, contribute to co-factors and support cell growth Rouf, A. et al. (2017). Additionally, fatty acids, lipids, and trace elements play vital roles in serum-free media.

Obligate parasites, organisms unable to grow in artificial culture media, underscore the diversity of microbial culture requirements. Bacterial culture media are classified based on composition, consistency, and purpose Rouf, A. et al. (2017) . Solid media, containing agar, enable informative growth patterns, while softer variants serve specific purposes. Liquid (broth) media lack gelling agents and find applications in large-scale organism propagation, fermentation studies, and diverse tests.

The bacterial growth curve delineates the typical progression of bacterial population dynamics within a closed system over time Steward, K. (2022). It encompasses four distinct phases as shown in figure 1. Firstly lag phase denotes a duration of minimal growth as bacteria acclimate to their environment, synthesize essential enzymes, and

prepare for replication. Following this, the exponential (log) phase ensues, characterized by a rapid and exponential increase in population size as conditions become optimal for growth Buchanan, R. E. (1918). Subsequently, the growth curve plateaus during the stationary time phase, where the rate of cell division becomes equal to the rate of cell death, resulting in no net increase in the population of cells Maier, R. M., & Pepper, I. L. (2015). Finally, the death phase signifies a decline in bacterial viability because of resource depletion and to accumulation of waste products, leading to a rapid decrease in population size. Graphically, the growth curve illustrates these phases, with time plotted against the logarithm of bacterial population size, displaying distinct patterns of growth, plateau, and decline Monod, J. (1949). This representation offers valuable insight into the active changes in bacterial populations over time within a controlled environment.



Figure 1. Bacterial Growth Curve

The utilization of Microbial-Induced Calcite Precipitation (MICP) in civil engineering has garnered significant attention as a novel approach for soil improvement and ground stabilization. While previous studies have focused on the mechanical effects and effectiveness of MICP treatments, there remains a notable gap in understanding the underlying microbial processes, particularly for civil engineers who may lack familiarity with cell culture techniques. Kim and Park (2019) and Kim et al. (2020) provided valuable insights into the outcomes of bacterial treatments, including cementation and reduction in permeability. However, to bridge this gap, our paper offers a detailed exploration of the cell culture process involved in MICP treatments. By elucidating step-by-step cultivation procedures, we aim to empower future researchers, particularly those from non-biological backgrounds, with the necessary knowledge to understand and replicate MICP experiments effectively. This contribution not only enhances comprehension but also facilitates the broader adoption and advancement of MICP techniques in civil engineering practices. Ivanov and Chu (2021) highlighted the promising role of microbial biotechnology, particularly bioclogging and biocementation, in improving soil mechanics for construction purposes. Van Paassen et al. (2022) demonstrated the scalability of MICP through large-scale sandbox experiments, achieving impressive compressive strengths akin to practical applications. Meanwhile, Mir and Wani (2023) explored the use of boulder crusher dust for stabilizing dredged soil, showcasing improvements in California Bearing Ratio (CBR) values and Unified Classification (UC) strength. Furthermore, Wani and Mir (2024) introduced Bacillus microbes into weak soils, demonstrating significant enhancements in soil properties and UCS strength, thus affirming MICP as an environmentally friendly soil stabilization method. The collective findings underscore MICP's potential as a sustainable and effective approach for bolstering soil mechanics and advancing geotechnical engineering practices.

2. Material and Methods:

2.1 Cell culture

In the pursuit of a thorough examination of cell culture processes, Bacillus subtilis bacteria were deliberately selected as the primary organism. The Bacillus Subtilis Microbial Culture Tube Figure 2, identified by the National Collection of Industrial Microorganisms (NCIM) strain number 2480, was acquired from the prestigious National Chemical Laboratory in Pune, India. Under precisely controlled conditions, the propagation of Bacillus subtilis occurred at a constant temperature of 30 degrees Celsius. Certification from the National Chemical Laboratory affirmed the microbial strain's non-hazardous, non-flammable, and non-corrosive nature. In a further commitment to the study's

reliability, NCL issued a guarantee, attesting to the purity, viability, and identity/authenticity of the Bacillus subtilis strain. These stringent quality control measures, combined with the careful selection of Bacillus subtilis, establish a robust foundation for the cell culture process, ensuring the integrity and reproducibility of the experimental methodologies.



Figure 2. Microbial culture strain of Bacillus Subtilis

2.2 Preparation of Luria Agar (LA) The composition of both solid and liquid components essential for the formulation of Luria Agar (LA) medium is detailed in Table 1. The preparation process involved adding the specified solid components to distilled water to achieve a total volume of 1 L, followed by thorough mixing Figure 3a. The mixture was gently heated until reaching the boiling point, after which it underwent autoclaving Figure 3b for 15 minutes at 15 psi pressure and 121°C. Post-autoclaving, the medium was carefully cooled to a temperature between 50-55°C. The nutrient-rich agar medium was then meticulously poured into approximately 15 sterile Petri plates within the confines of a laminar flow hood Figure 3c. This controlled environment, providing filtered air, was crucial in minimizing the risk of contamination in the culture growth media. The finalized LA medium was prepared, ensuring sterility, and served as the optimal substrate for the introduction of Bacillus subtilis culture using streaking and spreading techniques, thereby establishing a pristine and supportive environment for bacterial growth.



(a) (b) (c)

Figure 3. a) LA mixed with distilled water. b) Autoclaving of LA in process. c) LA poured into Petre plates.

Table 1. Media contents of Luria Agar.

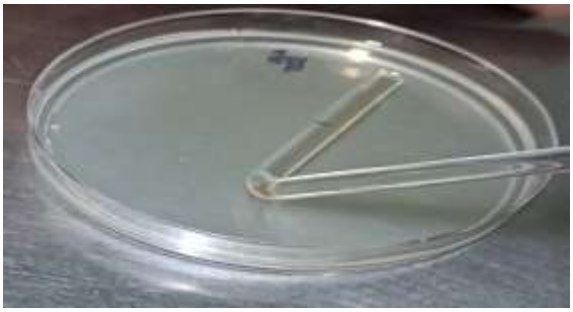
Composition	gm/l
Peptone	20
Agar	15
Tryptone	20
Yeast extract	10

2.3 Cultivation of Bacterial Colonies through Streaking and Spreading

The cultivation of bacterial colonies was accomplished through two distinct methodologies: streaking and spreading. In the streaking process, a bacterial loop, laden with bacterial colonies obtained from a pure culture tube procured from NCL Pune, was gently scratched onto Petri plates filled with agar. This facilitated the proliferation of multiple bacterial colonies upon subsequent growth, achieved by incubating the plates at 30 degrees Celsius for 12 hours. Conversely, the spreading method involved depositing drops of bacterial solution onto agar-filled Petri plates Figure 4a. The dispersion of these drops was meticulously executed using an L-shaped loop Figure 4b. The objective was to yield individual bacterial colonies following growth, which was achieved by incubating the plates at 30 degrees Celsius for 12 hours Figure 4c. This dual approach, incorporating streaking and spreading techniques, ensured the generation of diverse bacterial colonies Figure 4d while maintaining optimal conditions for their growth during the incubation period.



(a)



(b)



©



(d)

Figure 4. a) Putting 1 ml of bacterial inoculum on LA using pivot. b) spreading in the process using an L-shaped Loop c) Under incubation in the incubator. d) Growth colonies visible on Petre plate.

2.4 Preparation of LB(Luria broth) media and Inoculation

Preparation of Luria Broth (LB) media involved combining the solid components listed in Table 2 with distilled water to achieve a total volume of 1 litre. The mixture was thoroughly mixed and gently heated until it reached boiling temperature. Subsequently, it underwent autoclaving at 121°C and 15 psi pressure for 15 minutes to ensure sterilization. After autoclaving, the media was allowed to cool Figure 5a. To minimize the risk of contamination during inoculation, a laminar flow hood was employed to provide filtered air. Bacillus subtilis culture was then inoculated into the LB solution Figure 5b. The inoculated LB solution was transferred to an orbital shaker incubator set at 30°C and 180 rpm for 12 hours to facilitate growth Figure 5c.

Table 2. Media contents of Luria Broth.

Composition	gm/l
Yeast Extract	5

Sodium Chloride	10
Tryptone	10







©

(b)

(a)

Figure 5. a) Autoclaved LB, b) Inoculation of LB in process, c) Orbital shaking Incubation of LB for growth.

3. Results And Discussions

3.1 Spectrophotometry

In this study, spectrophotometry played a pivotal role in accurately quantifying cell density within the culture medium. Following the preparation of cell suspensions through centrifugation and subsequent resuspension in a suitable buffer solution, samples were meticulously analyzed using spectrophotometry. Concurrently, blank solutions, typically composed of distilled water or buffer, were prepared to correct for any background absorbance. Each sample was then introduced into a cuvette and subjected to analysis within the spectrophotometer, with absorbance measurements taken at a specific wavelength of 600 nm. By carefully subtracting the absorbance of the blank from that of the sample and utilizing established calibration curves or reference values, precise estimations of cell density were derived. This calculated cell density provided invaluable insights into monitoring the progression of cell growth, assessing cell viability, and ensuring standardized cell seeding densities for subsequent experiments. The attainment of reliable and reproducible results was ensured through rigorous adherence to calibration protocols and meticulous handling of equipment. Additionally, to calculate bacterial cell concentrations at various time points during the growth period, we employed the expression proposed by Ramachandran, Ramakrishnan, and Bang (2001):

$Y = 8.59 \times 10^{7} \times Z^{1.3627}$

Where, Y = Concentration (Cells . mL^{-1}) & Z = reading at OD_{600}

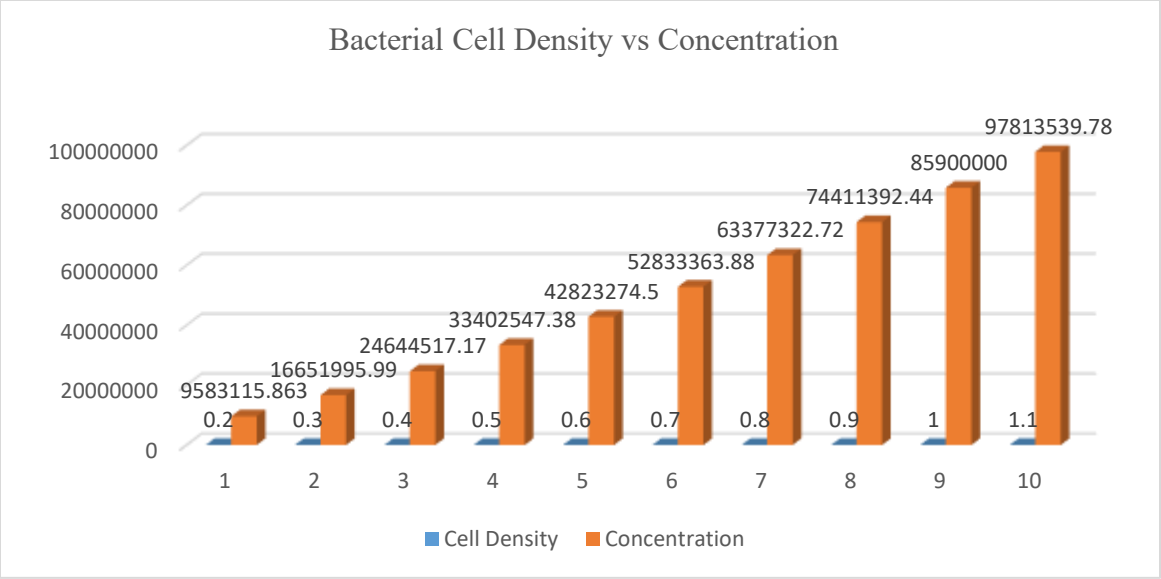


Figure 6. Concentration of bacterial cells at different cell densities.

3.2 Urease Test (As per Urease Protocol Brink, B. (2010))

In various civil engineering applications, microorganisms must exhibit a urease positivity test. The urease test serves to differentiate organisms based on their capacity to hydrolyze urea using the enzyme urease.

The test was initiated by preparing a bacteriological agar solution by mixing 1.5 g of agar with 90 ml of distilled water in a flask. Simultaneously, a Falcon tube containing 10 ml of distilled water was prepared, and its contents, as outlined in Table 3, were added and vortexed to ensure uniformity of the solution. The agar solution flask was then autoclaved, while the Falcon tube solution was filtered using a syringe and filter before combining both solutions. Slants were formed by allowing the solution to solidify, followed by streaking bacterial colonies onto the slants. The inoculated slants were then incubated at 30°C in an incubator. After growth, the slants were examined for any colour changes Figure 7(a-d). A distinct change to pink coloration validated the urease test, confirming the hydrolysis of urea by the organisms, as depicted in the figure below.



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

Figure 7. Colour change observed from day 1 to day 4 (a-d) due to incubation of slant for growth.

Table 3. Media contents**.**

Contents	Amount (g)
urea	2
NaCl	0.5
KH_2PO_4	0.2
Peptone	0.1

Dextrose	0.1
Phenol Red	0.002

3.3 Microscopy

In this study, we employed a fluorescence microscope Figure 8a to investigate bacterial samples prepared through a meticulously executed protocol. Initially, a 1 ml sample was extracted from cultured broth, followed by centrifugation at 5000 rpm for 5 minutes using a mini centrifuge. The cells were then diluted in Milli-Q water, a process repeated thrice to ensure optimal dispersion. Subsequently, the prepared sample (20 µl) was carefully deposited onto a clean slide glass, ensuring the absence of air bubbles, which could distort image clarity. Immersion oil was applied to the glass surface to enhance microscopic visualization. The microscope scale was set to 10 µm for accurate visualization. Finally, images were captured in Figure 8b using the fluorescence microscope and stored for further analysis. This methodological approach ensured the preparation of high-quality bacterial samples for detailed microscopic examination, facilitating robust data acquisition and analysis.



(a) **(b)**

Figure 8. (a) Fluorescence Microscope. (b) Bacillus subtilis bacterial cells on 10 µm scale.

3.4 Centrifuge (Harvesting of cells)

In the process of cell harvesting, bacterial solutions were carefully transferred into Falcon tubes, ensuring appropriate load balancing to maintain stability during centrifugation Figure 9a. The Falcon tubes were then placed in a centrifuge Figure 9b and spun at 5000 rpm for 15 minutes to facilitate the sedimentation of bacterial pellets Figure 9c. Following centrifugation and discarding LB, the resulting pellets Figure 9d were promptly stored in Falcon tubes within a 4°C refrigerator until their subsequent use. It is noteworthy that these pellets were utilized within a timeframe of 48 hours for further experimentation, as dictated by the research protocols across various domains. This standardized procedure ensured the efficient retrieval and preservation of bacterial samples for downstream analyses and experimental applications.



(a) (b)



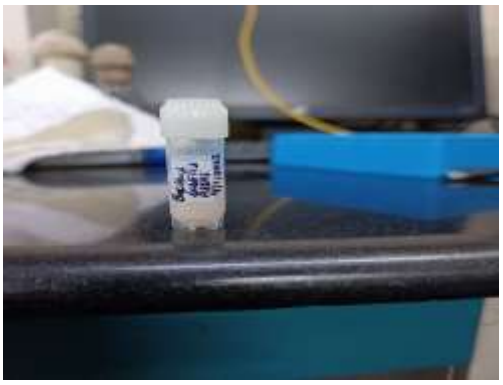
© (d)

Figure 9. (a) Falcon tube put for centrifuge. (b) Centrifuge run @ 5000 rpm for 15 mins. (c) Pellets settled at the bottom of the falcon tube. (d) LB was discarded to get bacterial cells.

3.5 Stock Preparation

In the stock preparation process, we utilized the Laminar chamber, along with a few essential items: a glass of ice, pre-made stock (obtained from a Petri plate), and a burning stove Figure 10a. For storage, we opted for cryovial containers. To prevent freezing at extremely low temperatures, we added 1 ml of 20% glycerol to each cryovial. Using a loop, a bacterial colony was carefully extracted from a Petri plate and transferred into the cryovial. After sealing the cryovial with its cap Figure 10b, we vortexed its contents for 5 seconds to ensure uniform dispersion throughout the glycerol solution. For optimal preservation over an extended period, we recommend an initial storage temperature of -20 degrees Celsius, followed by a transfer to -80 degrees Celsius after one day.





(a) (b)

Figure 10. (a) Stock preparation in the Laminar chamber. (b) Stock ready to store.

4. Common Challenges in Bacterial Culture:

4.1. Contamination: Contamination is a pervasive issue in bacterial culture, potentially leading to the need for isolating pure cultures anew or necessitating costly corrective actions, especially in food or production environments Chen, J. et al. Muhammad, S. A et al. . Sources of contamination vary, ranging from the original sample to the processes of culturing and storage. Adhering to rigorous aseptic techniques is vital to mitigate contamination risks effectively.

4.2. Overgrowth of Robust Species: Some bacterial species exhibit vigorous growth, which can overshadow the presence of slower-growing target species when isolating from mixed samples. Strategies to address this challenge include employing selective media and optimizing growth conditions tailored to the target species. Prompt culturing of samples post-collection enhances their representativeness.

4.3. Influence of Prior Antibiotic Treatment: The administration of antibiotics before sampling can complicate diagnostic efforts. The failure to culture a specific species does not necessarily discount its role in infection. It is crucial to ascertain the antibiotic treatment history to facilitate accurate diagnosis and inform appropriate treatment decisions.

4.4. Incorrect Growth Conditions: Inappropriate or suboptimal growth conditions can hinder or entirely inhibit the growth of target strains. Ensuring adherence to accurate growth requirements, such as selecting appropriate antibiotics based on resistance gene presence, is essential for achieving successful culture outcomes.

4.5. Challenges with Non-Culturable and Slow-Growing Organisms: Certain bacterial species remain unculturable in laboratory settings, presenting significant challenges in both diagnosis and research. Moreover, slow-growing organisms like mycobacteria may require extended culture periods, sometimes spanning months, further complicating timely diagnosis and treatment interventions.

5. Conclusion:

In conclusion, this interdisciplinary study provides a comprehensive guide for civil engineers seeking to integrate biological processes into innovative advancements such as self-healing concrete and bio-cementation. By demystifying the complexities of cell culture cultivation, the research bridges the gap between microbiology and civil engineering, offering practical insights and methodologies for seamless integration. Through meticulous attention to detail in the preparation of culture media, cultivation techniques, and analytical methods like spectrophotometry and microscopy, the study ensures reproducibility and reliability in experimental outcomes. Additionally, the identification and mitigation of common challenges in bacterial culture underscore the importance of rigorous quality control measures. Ultimately, this work not only facilitates the incorporation of bio-inspired materials into civil engineering practices but also fosters interdisciplinary collaboration and advances in biotechnological innovation.

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