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# BIOCEMENT PRODUCTION UTILIZING UREOLYTIC BACTERIA ISOLATES FROM CAVE PREMISES SOIL

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**Abstract–** Biocement is a microbial induced calcite precipitation (MICP) process that occurs due to biochemical activities of Ureolytic bacteria. In present work, from caves premises soil sample, ten ureolytic bacteria were isolated at 37°C after 24h incubation on nutrients agar supplemented with 5% urea. Six ureolytic bacterial isolates (S2, S3, S4,W2, W3 and W4) has shown highest calcium carbonate precipitation range from 0.14g-1.2g in 25 ml medium after one week incubation,$CaCO_3$ precipitation was confirmed by analytical test. Most efficient isolates (S2)was identified by 16SrRNA sequencing as *Bacillus* cereus BC1 and remaining isolates were identified as *Bacillus tequilensis, Bacillus amyloliquificians, Paenibacillus thiaminolytics, Bacillus lincheniformis,* and *Bacillus subtilis*, as per Bergey's manual of a systemic bacteriology and ABIS software. The isolate *Bacillus cereus* BC1, showed 173.30 U/ml urease activity by using standard ammonia graph. Biocement cube was significantly produced faster, time effective and Eco-friendly to overcome harmful impact of cement on environment by using these isolates.

## INTRODUCTION

Cave ecosystems host a diverse array of microorganisms with exceptional characteristics that enable them to thrive under challenging conditions. These environments are characterized by limited nutrients, low or no light, relatively low temperatures, and high humidity, presenting a unique set of challenges for organisms (Simmons *et al*., 2008). Nutrient recycling within caves is primarily dependent on the activities of bacteria and fungi, which play essential roles in maintaining ecosystem balance (Prabhavathi *et al*., 2012). Caves are regarded as extreme environments that provide niches for highly specialized microorganisms, which have evolved enzymes capable of functioning under such harsh conditions (Burg *et al*., 2003).

Microorganisms possessing specific metabolic abilities, including denitrifying, iron-reducing, sulfate-reducing, and ureolytic bacteria, play crucial roles in carbonate biomineralization (Dhami *et al*., 2012). Urease, an enzyme produced by ureolytic bacteria, hydrolyzes urea to generate ammonium and carbonate. The resulting carbonate spontaneously decomposes, producing ammonium and carbonic acid. The equilibrium between deprotonated and protonated forms of ammonium and carbonic acid in aqueous solution leads to an increase in pH, inducing ion precipitation (Anbu *et al*., 2016).

The precipitation of $CaCO_3$ occurs when the concentration of calcium or carbonate in solution increases or their solubility decreases (Castanier *et al*., 2000). The rate of microbiological $CaCO_3$ precipitation is closely correlated with cell growth. Concrete, a primary building material valued for its strength and cost-efficiency, is a major contributor to worldwide CO2 emissions. The cement manufacturing process entails high-temperature fossil fuel combustion and chemical reactions that release CO2 and convert calcium carbonate into calcium oxide. This current approach makes cement production environmentally unsustainable, necessitating the exploration of alternative methods. (Worrell *et al*., 2001). To mitigate the harmful impact on the natural environment, an innovative approach involves utilizing microorganisms to produce sustainable materials with reduced environmental effects (Camere and Karana, 2018). Biocement production through microbially induced calcium carbonate precipitation (MICP) represents a promising process that bio-geochemically triggers the precipitation of calcium carbonate (Simkiss, 1964). Biocement serves as a viable substitute for

traditional cement, offering a solution to the harmful impacts associated with cement production (Mortensen *et al.*, 2011).

Microbially induced calcium carbonate precipitation (MICP) has demonstrated numerous potential applications, including the prevention of concrete corrosion and remediation of cracks (Achal *et al*., 2014). MICP, offering expedited and enhanced calcite deposition, stands as the focal point of our current research. Our primary objective involves the isolation of ureolytic bacteria from soil samples collected in cave environments, with the intent to incorporate them into biocement production.

## MATERIALS AND METHODS

### Materials

Christensen's urea agar (Hi-media) and all chemicals use are of analytical grade.

### Sample Collection

Water samples were collected from cave pools, drip water from stalactites, and soil from Aurangabad caves in Maharashtra using sterile containers and transported them to the laboratory in an icebox.

### Isolation, Screening, and Identification of Ureolytic Bacteria

Ureolytic bacteria initially isolated by employing a 5% urea solution on nutrient agar plates and incubating them at 37 ºC for 24 hours. The isolated strains, exhibiting different morphologies, were screened on Christensen's urea agar plate (pH 6.8) at 37 ºC for 48-72 hours, identified by a color change in the medium from pale yellow to pink-red (Mekonnen *et al*., 2021). To pinpoint efficient urease-producing bacteria, we followed the identification procedures outlined in Bergey's Manual of Systemic Bacteriology and ABIS software. The most efficient isolate was confirmed through 16S rRNA molecular sequencing and deposited in Gene Bank under accession OR091341.

### Urease assay

Quantitative estimation of urease production was carried out using a modified $NH_3$ Nessler's assay method. The most effective isolate identified during the initial screening was cultured in 100 ml of sterile nutrient broth supplemented with 2% urea and incubated at 37 °C for 3 days with continuous agitation. Following incubation, the enriched broth underwent centrifugation at 5000 rpm for 20 minutes, yielding the urease-containing supernatant. The assay involved a substrate solution comprising 3% urea buffered with 1 ml of 0.2M phosphate buffer (pH 7). The reaction was initiated with the addition of 1 ml of enzyme extract, followed by a 15-minute incubation at 55 °C. To halt the reaction, 1 ml of 0.66N H2SO4 was introduced, and 1 ml of 1M sodium tungstate solution was added to precipitate the protein. After removing the precipitate by filtration from the supernatant, portions of the filtrate were assessed for NH3 using Nessler's reagent at 500 nm. Enzyme activity was quantified in μg/ml, with one urease unit defined as the amount of enzyme generating ammonia (NH3) (μg) per 1 ml of enzyme within a 15-minute period (http://www.biochem Den.com).

### Estimation of $CaCO_3$ precipitation

The capacity of ureolytic bacteria to precipitate calcium carbonate ($CaCO_3$) in a broth medium was evaluated as per method described by Li Wei *et al.* (2010). The nutrient broth was supplemented with 2% urea (w/v), 2% calcium chloride solution (w/v), and inoculated with 2% (v/v) overnight grown bacterial cultures. The culture flasks were incubated under shaking conditions (130 rpm) at 37 °C for 168 hours. The bacterial cultures were then suspended by centrifugation (10000 rpm for 10mins) and the supernatants were discarded. The bacterial pellets were washed with distilled water (pH 8.5) in the centrifuge tubes, followed by air-drying at 37 °C for 24 hours. The resulting dried pellets were then weighed to determine the quantity of precipitated calcium carbonate and its confirmation was by HCl acid method, which involved the reaction of $CaCO_3$ powder with 2N HCl, resulting in the release of three carbon dioxide gas bubbles, indicating the presence of carbonate ions (Sinha and Gupta, 2016).

### Application of Ureolytic Bacteria in Biocement formation

In this study, biocement cubes were prepared using a blend of sand, urea, CaCl2, and ureolytic bacterial isolates, using plastic waste bottles as molds. Process started by covering the bottom of the bottle mold with a perforated sieve. Sand was poured into the bottle mold, with 10 mm increments, and then added 10 ml of *Bacillus cereus BC1* and *B. subtilis* solution (OD 600) on top of the sand, ensuring even bacterial distribution, then poured 10 ml of CaCl2 solution on top of the sand and mixed it with the sand at every 10 mm increment, building a 60 mm

high sand column. Once the sand reached 60 mm, added 10 ml of cementation solution (containing urea, CaCl2, NaHCO3, 150mM NH4Cl, and meat yeast extract) on top, allowing it to drain out. The mold is covered with a perforated plate and rotated it 180 º from top to bottom. The cementation solution was poured on top one final time. The cementation solution was applied twice a day for 15 days, until it could no longer pass through the coagulated surface. After 15 days, the biocement sample was rinsed twice with distilled water and then dried at 60 ºC (Omoregie *et al.,* 2016).

## RESULTS AND DISCUSSION

### Isolation, Screening, and Identification of Ureolytic bacteria

Ten bacterial isolates with different sizes and shapes were obtained from soil and water samples collected from the pools in the cave premises, stalactite drip water, and Aurangabad caves in Aurangabad, Maharashtra. These isolates were obtained by plating the samples on nutrient agar supplemented with 5% urea and incubating them at 37 °C for 24 hours. Further screening for ureolytic activity was performed on Christensen's urea agar, where all ten isolates exhibited a color change from pale yellow to pink-red, confirming their ureolytic activity (Fig. 1). The most efficient isolate (S2) was identified as *Bacillus cereus* BC1 through 16S rRNA sequencing deposited to gene Bank under accession OR091341, while the remaining isolates were identified as *Bacillus tequilensis, Bacillus amyloliquificians, paenibacillus thiaminolytics, Bacillus lincheniformis,* and *Bacillus subtilis*, as per Bergey's Manual of Systemic Bacteriology (Table 1) and ABIS software. Previous studies by Mekonnen *et al.* (2021) have also reported the isolation of urea-hydrolyzing bacteria such as *Bacillus, Citrobacter*, and *Enterobacter* from soil samples.

### Urease assay

Among the ten isolates, six showed the urease activity, such as *Bacillus tequilensis* 23.78 U/ml, *Bacillus amyloliquificians* 38.48 U/mL, *paenibacillus thiaminolytics* 55.95 U/ml, *Bacillus licheniformis* 58.48 U/mL, *Bacillus subtilis* 71.13 U/ml and *Bacillus cereus* BC1 173.30 U/ml based on the standard ammonia graph (Fig No.3) and were chosen for $CaCO_3$ precipitation experiments. Urease activity of 575 U/mll and 670 U/ml after 120 hours of incubation was reported for *Bacillus cereus* (CT2) and *Bacillus fusiformis* (CT5), respectively, by Achal *et al.* (2010).

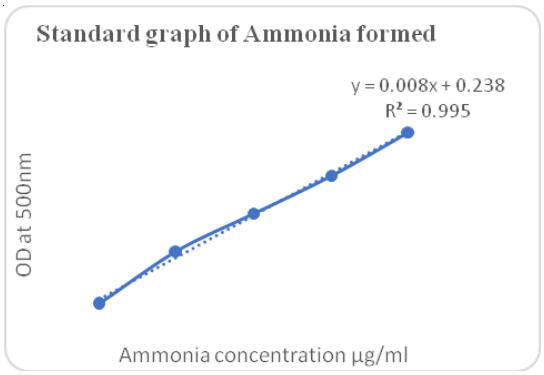


**Fig. 2.** Standard ammonia graph

### $CaCO_3$ precipitation by bacterial isolates

In this study, all ten bacterial isolates showed varying degrees of $CaCO_3$ precipitation (Table 1 and Fig. 2) in a 2% (w/v) calcium chloride medium after one week of incubation. The confirmation of $CaCO_3$ precipitation was carried out using 2N HCl tests, which demonstrated effervescence (Fig. 3). Among the six bacterial isolates, S2 and W3 exhibited the highest levels of $CaCO_3$ precipitation and were selected for further biocement production. Similar findings were reported by Tanvi Patil (2020), where $CaCO_3$ precipitation was observed after 7 days of incubation using *Bacillus megaterium* tara 2.

### Biocement Formation Using Ureolytic Bacteria

Biocement cubes measuring 7.5 cm were

**Fig. 1.** Ureolytic activity by Bacterial

**Fig. 3.** Confirmatory test

**Table 1.** Morphological and Biochemical Characteristic of bacterial isolates

| | S2 | | S3 | | S4 | | W2 | | W3 | | W4 | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Size | 4um | | 1.8-3.0 um | | 1.5 um | | 4.3um | | 0.5-1.0 um | | 2-6 um | |
| Shape | Cocci, long rod | | Rod long | | Cocci, short rod | | Short long rod | | Diploid cocci, short rod | | Short rod | |
| Margin | smooth | | undulate | | Erose,smooth | | viscus | | undulate | | Irregular | |
| Opacity | opaque | | opaque | | opaque | | opaque | | opaque | | Opaque | |
| Colour | Creamy white | | Fuzzy white | | Creamy white | | Creamy white | | Creamy white | | Creamy white | |
| Consistency | Moist | | mucoid | | rough | | Moist | | Moist | | Moist | |
| Grams Staining | +ve | | +ve | | +ve | | +ve | | +ve | | +ve | |
| Motility | +ve | | +ve | | +ve | | +ve | | +ve | | +ve | |
| Indole | +ve | | +ve | | +ve | | +ve | | +ve | | +ve | |
| MR | +ve | | -ve | | +ve | | +ve | | +ve | | -ve | |
| VP | +ve | | +ve | | +ve | | -ve | | -ve | | -ve | |
| Citrate | +ve | | -ve | | -ve | | +ve | | +ve | | +ve | |
| Catalase | +ve | | +ve | | +ve | | +ve | | +ve | | +ve | |
| Oxidase | +ve | | +ve | | +ve | | +ve | | +ve | | -ve | |
| Urease | +ve | | +ve | | +ve | | +ve | | +ve | | +ve | |
| Nitrate Reduction | +ve | | +ve | | +ve | | +ve | | +ve | | +ve | |
| Casein Production | +ve | | +ve | | +ve | | +ve | | +ve | | +ve | |
| Sugar Fermentation Test | Acid | Gas | Acid | Gas | Acid | Gas | Acid | Gas | Acid | Gas | Acid | Gas |
| Glucose | -ve | -ve | +ve | -ve | +ve | -ve | +ve | -ve | +ve | -ve | +ve | -ve |
| Lactose | -ve | -ve | -ve | -ve | -ve | -ve | -ve | -ve | -ve | -ve | -ve | -ve |
| Mannitol | -ve | -ve | +ve | +ve | +ve | -ve | +ve | -ve | -ve | -ve | -ve | -ve |

**Table 2.** $CaCO_3$ precipitation by bacterial isolates

| Bacterial Isolates ⇨ | S2 | S3 | S4 | W2 | W3 | W4 |
|---|---|---|---|---|---|---|
| $CaCO_3$ precipitation (g) | 1.2 | 0.16 | 0.14 | 0.23 | 0.35 | 0.24 |

successfully developed within 15 days at room temperature using a biocement mixture consisting of sand, urea, $CaCl_2$, and ureolytic bacterial isolates, specifically *Bacillus cereus* and *B. subtilis*, in plastic waste bottles. This eco-friendly and cost-effective method has potential applications in the prevention of concrete corrosion and remediation of cracks. A study by Zaghloul *et al.* (2021) produced 7 cm biocement cubes using marine isolates of *S. epidermidis* after incubation for 10 days at 37 °C.


**Fig. 4.** Biocement formations

## CONCLUSION

Ten ureolytic bacteria isolated and identified, namely *Bacillus tequilensis*, *Bacillus cereus BC1*, *Bacillus amyloliquificans*, *Paenibacillus thiaminolyticus*, *Bacillus licheniformis*, and *Bacillus subtilis*. Among these isolates, *Bacillus cereus* exhibited the highest urease activity, measuring 173.30 U/ml based on the standard ammonia graph. Furthermore, *Bacillus cereus BC1* and *Bacillus subtilis* demonstrated the greatest capacity for CaCO3 precipitation, making them promising candidates for biocement production. Our study successfully produced an eco-friendly, cost-effective, biobased, and durable 7.5 cm biocement cube in a 15-day timeframe using the developed biocement mixture. In summary, these research findings underscore the potential of

ureolytic bacteria in biocement production, offering a promising and environmentally friendly alternative for preventing concrete corrosion and addressing cracks, all while being economically viable.

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