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Harnessing Native Ureolytic Bacteria from the Hilly Region for Soil Strength Improvement: Investigating the Effect of Urea-$CaCl_2$ Concentration

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Abstract **–** Mitigating geo-hazards such as soil erosion and landslides is a great challenge in hilly regions worldwide**.** The bio-cementation of soil using native ureolytic bacteria is an environmentally friendly approach to improve the strength of hilly region soil. This study presents the biological investigations and soil improvement potential of the urease-producing bacteria isolated from the slope failure region of Uttarakhand, India. To this end, the bio-mineralization test and quantitative urease assay were used to screen potential ureolytic bacteria among the six bacterial strains isolated from the soil sample. Out of them, three bacterial strains showed high pH values (8.45 - 8.78) and $CaCO_3$ precipitation (160.96 - 175.30 mg/10ml) in the biomineralization test and high urease activity values (1912.7 – 3494.8 µM/ml). The 16S rRNA gene sequencing analysis of the outperforming bacterial strain revealed that the strain belongs to *Cytobacillus sp*. Later, the effect of different urea-$CaCl_2$ concentrations on the soil improvement potential was evaluated through unconfined compressive strength tests, calcium carbonate precipitation in soil, pH of outflow, and dry density values of the MICP-treated soil using outperforming bacterial strain. A maximum unconfined compressive strength of 1 MPa was achieved using 500 mM urea-$CaCl_2$ concentration, and the trend of test results matched with pH and dry density values. The FESEM images of soil samples confirmed that the high unconfined compressive strength is due to a high amount of rhombohedral-shaped calcium carbonate crystals at the particle contact and soil surface.

Keywords**:** MICP, Urea-$CaCl_2$, Hilly region, Native bacteria, Soil improvement

1. Introduction

Hilly regions of Uttarakhand are prone to frequent geo-hazards such as soil erosion and landslides [1]. The high porosity and low shear strength due to different natural and anthropogenic factors are the major cause of slope failures in hilly regions [2]. The existing chemical stabilizers are used to enhance the shear strength properties of soil, such as lime [3] and cement [4] but at the cost of environmental pollution and depletion of natural resources [5]. Thus, sustainable and eco-friendly soil improvement techniques must be explored to reduce our dependency on them. Microbiologists and geotechnical experts recently explored the concept of nature's biology and using the living phase of soil for bio-cementation of soil [6]–[8]. Microbes are considered geologic agent [9] that plays an essential role in the formation of mineral structures such as limestones [10] and dolomite [11] and are considered natural lithification or natural ground improvement [12]. However, mineral and rock formation is a time-consuming process in the natural environment. However, microbial-induced calcite precipitation (MICP) is a bio-geo-chemical process that utilizes metabolic activities (urease) of the ureolytic bacteria such as *Sporosarcina pasteurii*[12] to precipitate calcium carbonate crystals within the soil pores, leading to bio-cementation and bio-clogging of pore spaces and alter the engineering properties of soil [7]. MICP has been explored for different soil improvement applications such as river bank erosion [8], wind erosion control [13], [14], and landslide mitigation [15]. However, the adaptability of the foreign ureolytic bacteria to the ecosystem and the resistance of the soil microbiome are significant concerns regarding their performance. Thus, researchers are exploring native bacteria for MICP application because they are naturally adapted to the specific environmental conditions, soil properties, and microbial communities of the region [16]. This adaption enhances their survival and activity in the soil, increasing the effectiveness of MICP. The

native bacteria are more likely to coexist and interact with the existing microbial communities in the soil. This reduces the risk of disrupting the natural microbial balance and minimizes the potential ecological impacts. Native bacteria are adapted to the local environment, which can enhance their efficiency in promoting calcite precipitation and may result in higher rates of calcite formation compared to non-native strains. However, most studies have explored non-native bacterial strains for MICP, and limited studies focused on using indigenous bacterial strains such as *Lysinibacillus xylanilyticus* and *Psychrobacillus sp.* for slope stabilization[17], *Pseudogracilibacillus Auburnensis* for river bank erosion[14] and are found effective. The strength improvement in soil depends upon the $CaCO_3$ precipitation. The concentration of cementation solution i.e. urea and $CaCl_2$ is considered as an important parameter that contributes to the calcium carbonate precipitation and soil strength improvement and therefore needs to be carefully studied, when designing MICP protocols [7]. Many authors have studied the effect of cementation solution usually for < 2000 mM concentration to find the optimal concentration for their experimental conditions[18]. A concentration of cementation solution > 1000 mM is not preferred for MICP due to its inhibiting effect on the calcite precipitation because of enzyme amount that give limited urea hydrolysis rate, and influences the MICP efficiency[19]. Authors have discussed that microbes responsible for biocementation are living organisms and it is likely possible that the response to concentration of cementation solution varies due to their adaptability in different environments[20].

The current study aimed to investigate the potential of urease-producing bacteria found in hilly regions of Uttarakhand for soil improvement. To this end, the isolation of ureolytic bacteria was carried out from the native soil, and their potential for urease production and calcium carbonate precipitation was investigated. The performance of the outperforming bacterial isolate on soil improvement was examined at different urea-$CaCl_2$ concentrations through unconfined compressive strength testing and calcium carbonate precipitation and was confirmed with the pH, dry density values, and Field emission scanning electron microscopy (FESEM) investigations.

2. Materials and Methods

2.1. Bacterial isolation and biological investigations

For bacterial isolation, a soil sample was collected from the slope failure location in Rudraprayag district of Uttarakhand, India. Initially, samples were enriched with autoclaved Nutrient Broth-Urea medium and incubated at 200 rpm at 30ºC for 168 h. Then, the serial dilution technique was used to isolate the bacterial strains. The bacterial strains were streaked onto Christensen urea agar base (UAB) plates and kept in an incubator at 30ºC for pink color development as per the procedure suggested by [21] to screen ureolytic bacterial strains qualitatively. The gelatine tube method was adopted to check the gelatinase production, i.e., the pathogenicity of the bacterial strains. The gelatin medium (gelatin 120 g/l, peptone 5 g/l, beef extract 3 g/l) was prepared, and 24 h grown isolated colonies were stabbed into the autoclaved medium and kept in an incubator at 37ºC for 48 h. Later, the tubes were assessed for partial or complete liquefaction.

The potential urease-positive bacterial strains were tested for in-vitro calcium carbonate precipitation in 10 ml of calcium carbonate precipitation (CPM) medium that consisted of 2% of urea and calcium chloride each and 1% bacterial suspension of (OD_{600} = 0.8-1.0), and incubated at 30ºC for 168 h, as suggested by [22]. After the incubation period, the CPM medium was centrifuged, the supernatant pH was measured, and the $CaCO_3$ pellets suspended at the bottom of the falcon tubes were washed to separate cell debris from precipitated $CaCO_3$. The weight difference between tubes with $CaCO_3$ precipitation and the empty weight of the tubes is used to estimate $CaCO_3$ precipitation.

The bacterial strains selected after the bio-mineralization test were further quantified for urease production using the phenol-hypochlorite method [23]. For this, 250 µl of bacterial culture (OD_{600} = 0.8-1.0) was added to a mixture of 100 mM potassium phosphate buffer and 2.5 ml of 100 mM urea. The mixture was incubated at 37ºC for 25-30 min. Then, 1 ml each of phenol nitroprusside and alkaline hypochlorite was added to the mix and further incubated for another 25-30 min. Then, the optical density (OD) of the solution was measured in a spectrophotometer at 626 nm. The measured OD values were converted to parts per million (ppm) developed with NH_4Cl and reported in µmole urea/min.

The 16S rRNA gene sequencing of the best-performing bacterial strain was carried out, and the sequencing result was compared with the available ones in the GenBank database using National Center for Biotechnology Information (NCBI) BLAST analysis.

2.2. Soil properties and sample preparation

A cohesionless soil was used to study the performance of ureolytic bacteria for soil strength improvement. The soil characteristics, such as specific gravity, Minimum and maximum dry density, were determined as per ASTM standards (ASTM D854-14, 2014, ASTM D4254-16, 2016) and are added in Table 1. The acid washing technique was used to determine $CaCO_3$ precipitation of initial or untreated soil sample [24]. The grain size distribution of the soil is presented in Fig. 1.

Table 1: Properties of soil

Soil property	Value
Specific gravity	2.66
D_{50} (mm)	0.22
Minimum dry density (kN/m^3)	13.1
Maximum dry density (kN/m^3)	15.3
$CaCO_3$ (%)	1.12

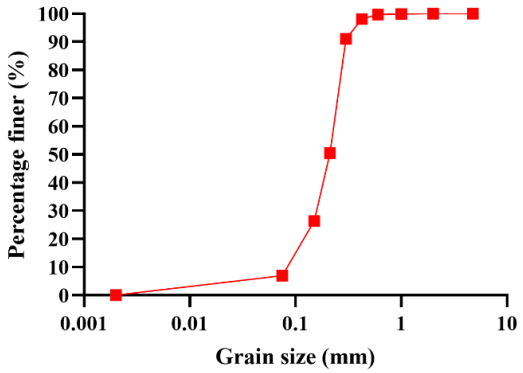


Fig 1: Grain size distribution of soil

A split set mould of size 38 mm × 76 mm was used to prepare soil samples for treatment. The mould has an open top to add soil and treatment solution, whereas the bottom part has a small opening to facilitate drainage and outflow collection. A piece of filter paper was placed at the bottom to avoid the migration of soil particles during treatment. A piece of sponge was placed at the top to avoid the disturbance of the soil surface during the addition of the treatment solution. The soil samples for treatment were prepared at 60% relative density using the air pluviation method followed by the tamping technique.

2.3. MICP-treatment of soil

The soil treatment involves the addition of one pore volume of solution through the surface percolation method at each stage, as adopted by [17]. Initially, soil samples were flushed with water to remove air voids and measure initial pH. The treatment sequence TS6 from the different treatment sequence methods discussed by [25] was adopted in the study. A pre-treatment solution called as fixation solution consisting of $NaHCO_3$ and NH4Cl, was prepared and added to the soil. Then, the selected bacterial strain grown in autoclaved nutrient broth (NB) (13 g/l) at 170 rpm for 18-24 h at 30ºC was added to the soil and left undisturbed for 12 h. Later, a cementation solution (CS) consisting of urea-$CaCl_2$, $NaHCO_3$, and NH_4Cl was prepared and added. After all the sequential steps, NB and CS were added consecutively for 14 days. The details of the

concentrations of the different treatment ingredients used for the treatment are mentioned in Table 2. After 14 days, soil samples were rinsed with distilled water to wash out the residual salt precipitates, and pH of the outflow was noted.

Table 2: Treatment solution ingredients

Bacterial solution	Nutrient broth (g/l)	Urea-$CaCl_2$ conc. (mM)	$NaHCO_3$ (g/l)	NH_4Cl (g/l)
OD = 0.8-1.0	13	250	2.12	10
		500		
		1000		

2.4. Testing

The outflow was collected after each treatment for 14 days, and the pH of the outflow was measured using a pH meter (HI2550, Hanna instruments). The soil samples after the 14 days of treatment were oven-dried at 105-110 ºC for 24 h, and their dry weights were measured to determine the dry density values. The unconfined compression testing of the treated soil samples was carried out as per (IS:2720 (Part 10). 1991). The vertical displacement and load values were continuously recorded during the test, and the axial stress corresponding to sample failure is called unconfined compressive strength (UCS). The testing of the samples was carried out in triplicates, and average shear strength and the variance among the replicates are reported. The $CaCO_3$ precipitation in different treated soil samples was estimated using acid washing [24]. The soil samples from the middle portions of the UCS-tested samples were extracted, and the weight difference before and after the acid washing was used to determine the calcium carbonate precipitation produced with different urea-$CaCl_2$ concentrations.

The effect of urea-$CaCl_2$ concentrations on the $CaCO_3$ precipitation and binding of soil particles were confirmed through Field emission scanning electron microscopy (FESEM) images at different magnifications. For this, soil specimens were prepared in powdered form and were fixed on the aluminium stub, followed by gold coating.

2.5. Statistical Analysis

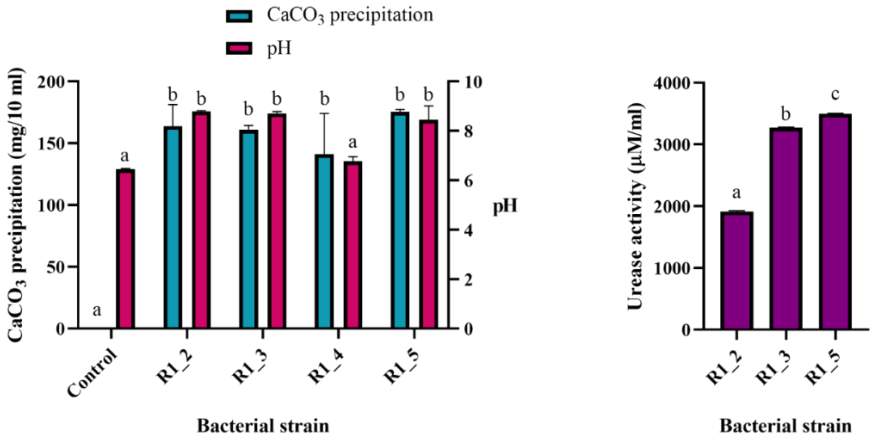
The analysis of variance (ANOVA) was performed between different samples of the same test, and the means were compared by Tukey's test ($p < 0.05$) using IBM SPSS statistics 23.

3. Results and Discussions

3.1. Biological characteristics of ureolytic bacteria

A total of 6 ureolytic bacterial strains were obtained after the serial dilution of the enrichment solution, out of which 4 bacterial strains were found gelatinase negative (non-pathogenic), which also changed the color of the UAB plate from yellow to pink in 12 – 24 h indicating that the isolated bacterial strains are highly urease active in nature compared to other strains and are therefore taken into further consideration.

The invitro-$CaCO_3$ precipitation test results exhibited that the $CaCO_3$ precipitation and pH values of the selected strains, i.e., R1_2, R1_3, R1_4, and R1_5, were high compared to the control sample, but varied with the bacterial strains, the results of which are shown in Fig. 2(a). The bacterial strains with high precipitation potential were quantified for urease activity, the results of which are also shown in Fig. 2(b). It was evident from Figs. 2(a) and 2(b) that the bacterial strain R1_5 outperformed the rest of the bacterial strains. The 16S rRNA gene sequence followed by the NCBI blast analysis identifies that the R1_5 bacterial strain is related to the *Cytobacillus* family.



(a) (b)

Fig 2: (a) In-vitro $CaCO_3$ precipitation (b) Urease activity of bacterial isolates

3.2. Effect of different urea-$CaCl_2$ concentrations on soil improvement using ureolytic bacterial isolate

3.2.1. pH of the outflow and Dry density of MICP-treated samples

The ureolytic bacteria capable of producing urease enzyme catalyze the urea hydrolysis process and lead to an increase in the pH of the environment due to the production of ammonia that favors calcium carbonate precipitation on the surface of the particles as well as at particle contacts in the presence of calcium ions. Therefore, the increase in pH of the environment can act as an indicator to monitor ureolytic activities of the bacteria under different urea-$CaCl_2$ concentrations during MICP treatment, as suggested by [26]. It can be deduced from Fig. 3(a), representing the average pH values, that the initial pH of the outflow was 6.8, which increased to 7.7 and 8.0 for 250 mM and 500 mM concentrations, respectively, on the 14^{th} day of treatment. An increasing trend in the pH of the outflow can also be observed for all the treatment, which is also represented in Fig. 3(a); however, the increase in pH values was high in treatments 500 mM followed by 250 mM, demonstrating a high rate of urea hydrolysis process. Whereas, a lesser increase in pH values can be seen for 1000 mM treated samples, which could be possibly related to the inhibition of the production of the enzymes at higher concentrations of cementation solution and was also discussed by [27]. [7] also reported that the pH value between 7-9 develops favorable alkaline conditions to trigger the urease hydrolysis process by the urease enzyme.

Fig. 3(b) represents the dry density values of the soil samples that increased due to MICP treatment and are in the range of 17.9 – 19.8 kN/m^3 compared to untreated samples with a value of 17.4 kN/m^3. The increase in dry density values demonstrates the potential of bacteria to produce $CaCO_3$ precipitation, which can be affected by the urea-$CaCl_2$ concentration used [7]. The increase in urea-$CaCl_2$ concentration can lead to an increase in the dry density of MICP-treated soil. This is because a higher treatment concentration provides more nutrients and calcium ions for the bacteria, promoting their metabolic activity and precipitation of calcite. As a result, more calcite crystals are formed, filling the pore spaces and increasing the density of soil. However, increasing the urea-$CaCl_2$ concentration can lead to an increase in the dry density of MICP-treated soil up to a certain point, and there is an optimal concentration range of urea-$CaCl_2$ solution beyond which further increase may not significantly affect the dry density or may even have decreasing trend. This is because excessively high urea or calcium chloride concentrations can negatively affect bacterial activity or induce osmotic stress, inhibiting their growth and metabolic processes [19]. Consequently, the amount of calcite precipitation and the resulting increase in dry density may plateau or decrease at very high concentrations. Thus, the highest dry density was achieved with a 500 mM concentration, indicating the possibility of highest $CaCO_3$ precipitation.

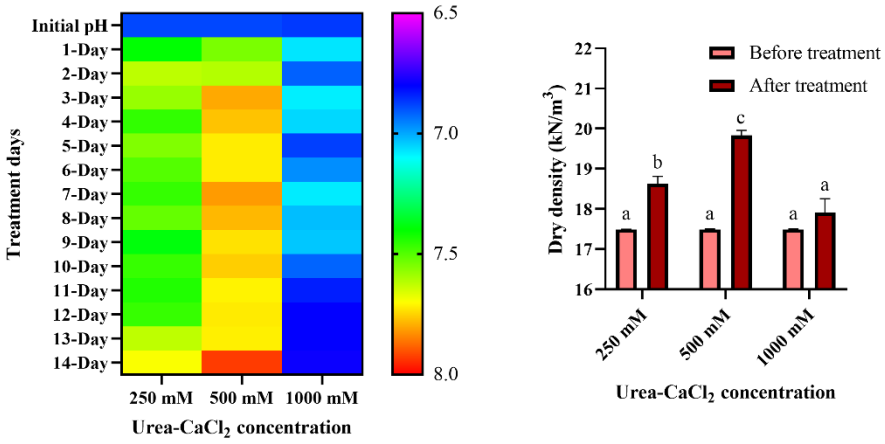


Fig 3: (a) pH of the outflow and (b) dry density of MICP-treated samples under different concentration of urea-$CaCl_2$

3.2.2. Unconfined compressive strength and average $CaCO_3$ precipitation

The unconfined compressive strength (UCS) result demonstrates differences in strength enhancement in MICP-treated soil due to differences in urea-$CaCl_2$ concentration. The UCS value increased from 136 kPa to 1069 kPa, with an increase in the urea-$CaCl_2$ from 250 mM to 500 mM. However, an average UCS value of 93 kPa was obtained for soil samples treated with 1000 mM. The strength improvement in MICP-treated soil is the result of $CaCO_3$ precipitation, which seems to vary with the urea-$CaCl_2$ concentration and was also highest with 500 mM, as shown in Fig. 4. The differences in the strength improvement are attributed to the amount of calcium carbonate precipitation under different treatments and can be confirmed with the $CaCO_3$ precipitation results. The concentration of urea-$CaCl_2$ affects the shape and amount of calcium carbonate crystal precipitation. Thus, microscopic investigations on the treated soils were carried out.

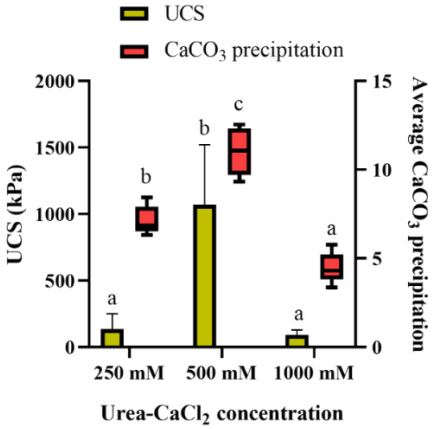


Fig 4: Unconfined compressive strength (UCS) and average $CaCO_3$ precipitation of MICP-treated soil samples

3.2.3. Microscopic investigations

The precipitation of calcium carbonate crystals and soil strength improvement due to the binding of soil particles were established through FESEM images at different magnifications in Fig. 5. At 2000X, the soil particles covered with calcium carbonate crystals and bridging of soil particles can be observed in 250 mM and 500 mM treated samples in Fig. 5 and is result of the accumulation of crystals because of continuous soil treatment for 14-days. A high amount of

calcium carbonate crystals precipitation can be observed in the 500 mM sample, demonstrating the highest strength and $CaCO_3$ precipitation values. However, uncovered soil particles and low precipitation of crystals can be seen in 1000 mM treated samples, due to which low strength and $CaCO_3$ values were attained after 14-day soil treatment. At 4000X, the formation of rhombohedral-shaped calcite crystals can be observed in all the treated samples, and no change in the shape of of crystal formation due to different urea-$CaCl_2$ concentrations was observed.

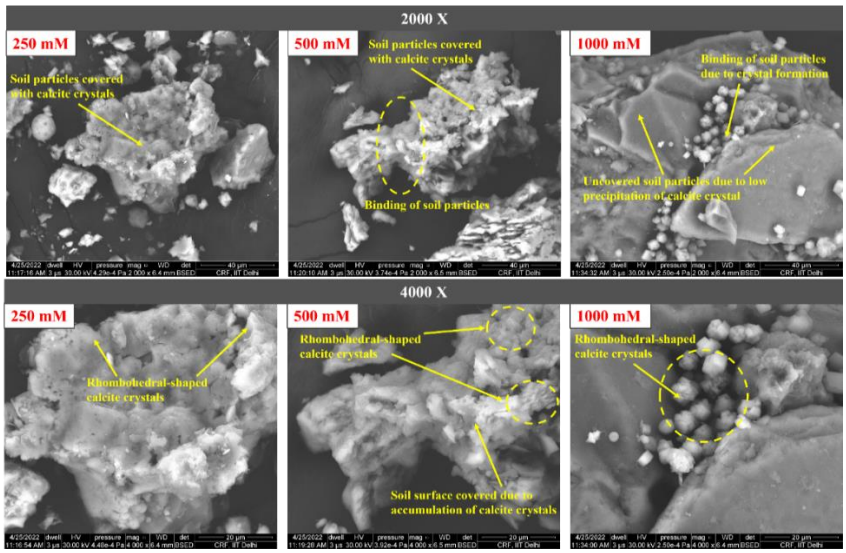


Fig 5: Field emission scanning electron microscopy (FESEM) images of MICP-treated soil treated with different urea-$CaCl_2$ concentrations under different magnifications.

4. Conclusion

The current study aimed to investigate the ureolytic bacterial strain isolated from the slope failure region of Uttarakhand, India, and establish its potential for soil strength improvement. Further, the effect of urea-$CaCl_2$ concentration on strength improvement and calcium carbonate precipitation was investigated in this study. The major conclusions drawn from the study are as follows:

- The bacterial strain R1_5 of *Cytobacillus* sp., demonstrated the highest in-vitro $CaCO_3$ precipitation, pH, and urease activity values.
- Maximum unconfined compressive strength of 1 MPa was achieved using the surface percolation method with 500 mM urea-$CaCl_2$ concentration, demonstrating rock-like behavior and is found suitable for soil strength improvement compared to 250 mM and 1000 mM concentrations. The increase in the average calcium carbonate precipitation with 500 mM improved the soil strength.
- The FESEM images showcased that the effect of urea-$CaCl_2$ concentration is more on the amount of calcium carbonate precipitation than on the shape of crystal formation. The precipitation of $CaCO_3$ evidently increased with the weight of cemented soil, manifested through increased dry density values and pH of the outflow of the soil samples.

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