Improving "Shrinkage-Swelling" Response of Expansive Soil Using Bio-calcite and Exopolysaccharide Produced by Bacillus sp.

V. Guru Krishna Kumar, Kaling Taki, Sharad Gupta and Ajanta Sachan

Abstract Biological phenomena standout as a key towards green method for improving the properties of engineering construction material. The present study investigates the effect of Microbial Induced Calcite Precipitation (MICP) and Extracellular Polymeric Substance (EPS) produced by *Bacillus cereus* (*B. cereus*) SG4 on "shrinkage-swelling" behavior of expansive soil. The soil used for the study was commercially available Bentonite cohesive soil. The critical soil parameters such as Liquid Limit (LL), Plastic Limit (PL), and Differential Free Swell Index (DFSI) were found to be very high (LL = 608%, PL = 50%, and DFSI = 661%) due to the presence of Montmorillonite mineral. The results showed that treatment of Bentonite expansive soil with bio-calcite and EPS containing *B. cereus* SG4 culture media worked effectively. Bentonite soil was treated with bacteria along with culture medium for 5 and 10 days. It was observed that there was no significant reduction in geotechnical properties after 10th day of treatment. Maximum effect was observed at the end of 5th day exhibiting the efficiency and strong capability of proposed soil treatment method. After 5th day, LL, PL, and DFSI values were observed to be decreased; 177%, 39%, and 371% for EPS, respectively. The similar response was observed for Bio-calcite technique, which exhibited a significant reduction in LL, PL, and DFSI values (158%, 39%, and 271%), respectively. Both the treatment techniques worked successfully in improving the shrinkage-swelling response of Bentonite soil, but bio-calcite treatment was observed to be more effective than EPS treatment to control the shrinkage-swelling response.

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1 Introduction

Last decade has seen the emergence of Construction Biotechnology as a new area of Science and Engineering and a significant contribution has come from the development of microbial products or processes for the enhancement of the properties of construction materials [12, 19, 29]. A wide variety of microorganisms in the environment exhibit the phenomenon of mineralization of diverse nature and chemical composition, including carbonates, silicates, and iron and manganese oxides. Metabolites from microbes using specific substrates often contribute to products such as insoluble organic compounds and salts which have cementation and clogging properties [17, 35]. Biocalcification also known as Microbial Induced Calcite Precipitation (MICP) is dependent upon the ability of a microorganism to secrete the urease enzyme leading to the production of insoluble calcium carbonate [4]. Certain microorganisms occur in the form of microbial aggregates made of structural elements called Extracellular Polysaccharides (EPS) [14, 34]. The EPS, secreted by microorganisms into the surrounding for their survival are widely used as emulsifiers, gelling agents, binders, coagulants, and thickening agents [24]. Studies on the application of biocalcification produced by several microorganisms have been extensively carried out in concretes, bricks, cement mortar, and sand [1, 11, 16, 25, 28, 33]. Furthermore, there are reports of augmentation of functional properties and geotechnical aspects of soil by treatment with EPS biofilms produced by bacteria [5, 9].

Bentonite clay used in the current study has very high shrinkage and swelling response owing to the presence of large amounts of montmorillonite mineral [8]. Expansive soil such as black cotton soil (Gujarat, India) has nearly 30–60% of montmorillonite mineral which swells on the absorption of water and shrinks when dried out. Cycles of swelling-shrinkage place repetitive stress on structures resulting in a huge commercial loss. The present research focuses on two aspects (i) isolation and characterization of a unique *Bacillus cereus* strain which could produce bio-calcite and EPS (ii) a soil treatment technique which could significantly lower swelling-shrinkage response of expansive soil. In this study, Bentonite cohesive soil has been treated using bio-calcite and EPS produced from the same microbe *Bacillus cereus* SG4 to control the said response. The proposed method has excellent potential for effective treatment of expansive soil.

2 Experimental Program

2.1 Material Properties

For the study Bentonite soil was taken the soil is classified as clay with high compressibility (CH) type. The properties of Bentonite soils have been given in Table 1.

2.2 Soil Collection for Bacterial Isolation

Soil samples were collected from twelve different locations in Gujarat (India) where the nature of the soil was cohesive with significant variations in clay content. The soil was sampled into a clean plastic box and stored in 4 °C before use.

2.3 Bacterial Isolation

Bacterial isolation was carried out by first mixing 1 g of soil sample into 10 mL ddH_2O and the supernatant was further diluted 10-fold, 100-fold, and 1000-fold. A 100 µL aliquot from each dilution was spread onto nutrient agar plates and incubated at 37 °C for 24 h. All grown colonies were screened for urease and EPS production by using selective media such as Ammonium-Yeast extract agar plates for urease and nutrient broth (NB) supplemented with glucose media for EPS production. Microorganisms which yielded both urease and EPS were further characterized.

Table 1 Basic properties of Bentonite soil

Particulars	Specifications
Material used	Bentonite (Brown color)
Liquid limit (%)	608
Plastic limit (%)	50
Plasticity index (%)	558
Soil classified	Highly compressible
Differential free swell index (DFSI) (%)	661

2.4 Microbial Characterization

Gram staining was performed using Grams Stain-Kit (HiMedia). Biochemical tests such as Malonate, Citrate, Arginine, Sucrose, Mannitol, Glucose, Arabinose, Trehalose, Vogue's Proskauer, Catalase, Nitrate reduction and ONPG were performed by using HiBacillus identification kit (HiMedia). The changes in the color of API strips were used to characterize the strains based upon their response towards different tests. HiChrome bacillus agar (HiMedia) was used for species identification based on colony color.

2.5 Testing Procedure

2.5.1 Procedure for MICP and EPS Production

NB supplemented with 2% urea and 0.3% $CaCl_2$ was used as growth media to produce MICP. In brief, the strain was inoculated in sterile media and incubated at 37 °C and 250 rpm for one week. Baffled Erlenmeyer flasks were used to provide extra aeration and agitation to the bacterial culture. A switch in media to NB supplemented with 5% glucose was used for EPS production under similar growth conditions for three days.

2.5.2 EPS Extraction and Quantification

Extraction of EPS was done by using cold ethanol method [3]. In brief, three days old culture media was centrifuged at 7,000 g for 15 min at 4 °C to pelletize the bacterial cells. The supernatant was mixed with four volumes of ice cold ethanol and the suspension was stored at −20 °C overnight. Precipitated EPS was separated by centrifugation at 12,000 g for 30 min at 4 °C and lyophilized. Carbohydrates were quantified by the phenol-sulfuric acid method as reported by Dubios et al. [13]. Protein content was estimated by Bradford's method using BSA as standard.

2.5.3 Scanning Electron Microscopy

Precipitated calcite crystals were collected on Whatman No. 1 filter by filtration, washed with sterile distilled water, and air-dried at 37 °C for 48 h. Dried crystals were sputter coated with platinum and FE-SEM analysis was carried out using JEOL JSM-7600F at accelerating voltage of 35 kV and working distance of 9 mm. Energy Dispersive Spectroscopy (EDS) was performed to identify the composition of elements and their relative proportions in the precipitated bio-calcite.

2.5.4 Genomic and In Silico Analysis

Genomic DNA extraction was performed using DNeasy Blood & Tissue Kit (Qiagen). The amplification of 16S gene was carried out using the universal primers 27f and 1492r [15]. The reaction was performed in 50 µL reaction volume and the PCR conditions were as follows: primary denaturation at 94 °C for 5 min, followed by 30 cycles of 94 °C for 1 min, 55 °C for 1 min, 72 °C for 1.2 min, and final extension at 72 °C for 5 min. The PCR amplicons were analyzed on a 1% agarose gel along with a DNA molecular weight marker. Sequencing of 16S rDNA gene was performed on an ABI PRISM 377 genetic analyzer (Applied Biosystems, USA) by Eurofins Scientific, Bangalore India. NCBI's BLAST program (www.blast.ncbi.nlm.nih.gov/Blast.cgi) was used to identify the sequences that are homologous to extracted 16S rDNA sequence. Multiple sequence alignment was performed using the CLUSTALW program [20]. Phylogenetic analyses were carried out in MEGA v6.0 using the neighbor joining algorithm with 1000 bootstrap replicates [30]. Sequence information was submitted to NCBI GenBank (Accession number: KX817272).

2.5.5 Urease and Protease Activity

Urease activity was determined for the bacterial isolate in nutrient broth (NB) containing 2% urea and 0.3% $CaCl_2$ by measuring the amount of ammonia released on hydrolysis of urea using phenol-hypochlorite method [23]. One unit of urease activity is defined as the amount of enzyme hydrolyzing one µmole of urea per minute. Protease activity was determined according to the method of Tsuru et al. [31] using casein as a substrate and L-tyrosine (1.1 mM) as the standard [31]. One unit of protease activity is defined as tyrosine equivalents in µmoles released from casein per minute.

2.5.6 Geotechnical Test on Bacteria Treated Bentonite Soil

The following geotechnical tests were carried out on treated and untreated Bentonite clay.

(a) *Liquid Limit (LL) test*: [IS: 2720 (Part 5)—1985 (Reaffirmed 2006)].
(b) *Plastic limit (PL) test*: [IS: 2720 (Part 5)—1985 (Reaffirmed 2006)].
(c) *Differential free swell index (DFSI) test*: [IS: 2720 (Part XL)—1977 (Reaffirmed 2002)].

3 Results and Discussion

3.1 Screening of Bacterial Strains and Strain Identification

Soil samples were collected from twelve parts of Gujarat, India to isolate EPS and urease producing microorganism. Soil samples were serially diluted in ddH_2O and the extract was spread on NB agar containing glucose and isolated 33 different strains based on the colony morphology (Fig. 1 and Table 2).

Bacteria which produced mucoid secretion were identified and further tested for urease production by their ability to grow in Ammonium-Yeast extract media. Four strains namely Strain 1, Strain 2, Strain 3, and Strain 4 were screened for urease production form which strain 4 had maximum urease activity, which corresponds to 829.6 U/min (Fig. 2). Comparison of strain 4 with previously reported urease positive strain *B. megaterium* CT-5 was also performed and the urease activity was found to be qualitatively similar (Fig. 3).

Gram staining was performed to differentiate bacteria based on their cell wall constituent (Fig. 4). Isolated Strain 4 (hereafter referred to as SG4) which gave the highest urease activity was found to be Gram positive and rod-shaped bacteria. To identify whether the screened strains 1–4 were of Bacillus sp they were grown in HiCrome Bacillus agar. SG4 showed a luxuriant growth in this media exhibiting large colonies with blue centered morphology, which was a characteristic response for *Bacillus cereus*. HiBacillus identification kit was used to further characterize the bacteria based on its biochemical properties (Table 3). Positive test results suggested that the isolate SG4 can potentially be *Bacillus cereus*. To study the phylogeny and taxonomy of SG4 at genome level, 16s rRNA sequencing was performed. In silico sequence analysis of the 16S rDNA gene revealed that there was a significant level of similarity (>99%) between the sequence obtained in this

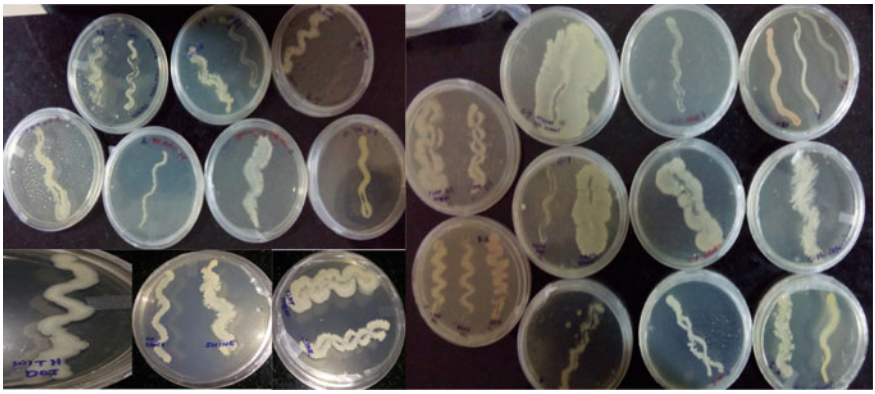

Fig. 1 Isolated strains from 12 different soil samples were tested on NB-Glucose agar for EPS production * (*Note* * Images have been taken by the authors during the experiment at Biological Engineering Lab IITGN)

Table 2 Bacteria screened on NB-glucose agar plates based on the colony morphology

Strain No.	Morphology	Obtained from Soil
1	With dot	S-III
2	With yellow sheen	S-VII
3	Light spread	S-VII
4	Full spread	S-XI
5	Dark	S-VIII
6	With flagella	S-IX
7	Radiating	S-VIII
8	Thick dot	S-VII
9	Crystal	S-XII
10	Round	S-XI
11	Zone from side	S-V
12	Light yellow	S-II
13	Dark yellow	S-II
14	Red	S-II
15	Spread yellow sheen	S-VII
16	Flat spread	S-II
17	Star	S-5
18	Small	S-II
19	Big	S-II
20	With sheen	S-VII
21	Like film from center	S-I
22	Double layer	S-VI
23	Yellow	S-XI
24	Outgrowth	S-II
25	Shine	S-II
26	Criss cross spread	S-X
27	Like film from side	S-I
28	Zone from center	S-V
29	Crystal center	S-VI
30	Crystal side	S-VI
31	White small dot	S-XII
32	Bluish yellow	S-III
33	Middle dark with translucent edge	S-III

study with the sequences reported earlier from *Bacillus cereus*. The phylogenetic tree analysis further divulged the closeness of the bacterial isolate SG4 with *Bacillus cereus* strains (Fig. 5). The tree also indicated that *Bacillus anthracis* and *Bacillus thuringienisis* formed a separate clade altogether. These results further affirmed that the bacterial isolate SG4 belonged to genus: *Bacillus* and species: *cereus*.

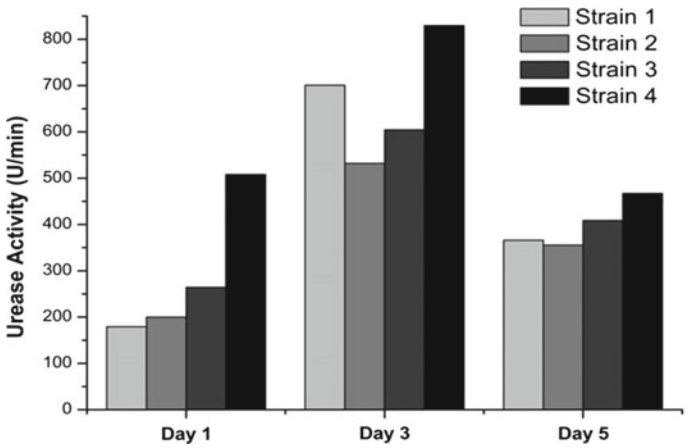

Fig. 2 Isolated bacterial strains tested for urease production in NB-Urea-$CaCl_2$ media

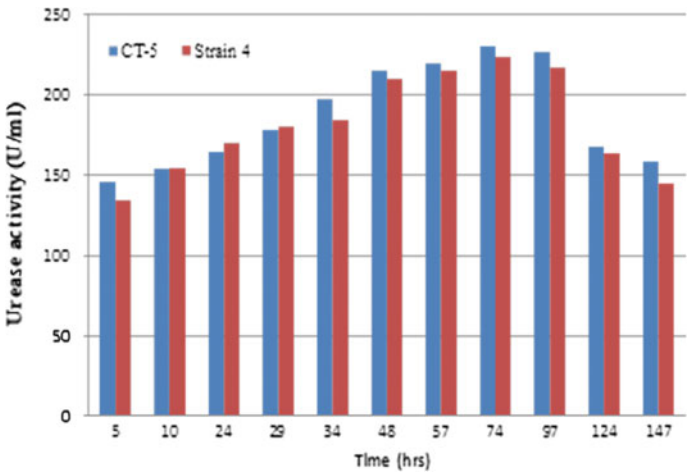

Fig. 3 Comparison of urease activity from *B. megaterium* CT-5 and Strain 4

3.2 Urease/Protease Activity and EPS Production

Bacillus cereus SG4 showed maximum urease productivity corresponding to 206 U/mL on the third day, in nutrient broth containing Urea-$CaCl_2$. Protease activity increased with time and showed maximum productivity on the fifth day corresponding to 1.4 U/ml (Fig. 6). It was also noted that the urease production gradually reduced after the third day, which is in agreement with increased protease concentration leading to the degradation of urease in culture media. *B. cereus* SG4 yielded a total of 1.8 g/L of dried calcium carbonate in NB-urea-$CaCl_2$ media and

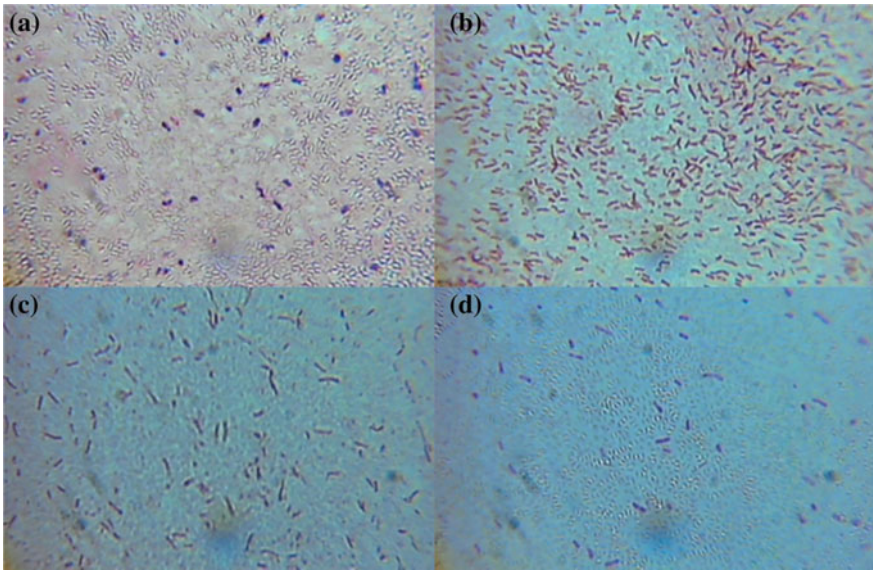

Fig. 4 Gram staining performed to differentiate bacteria based on their cell wall constituent, **a** Strain 1, **b** Strain 2, **c** Strain 3, and **d** Strain 4

Table 3 Comparison of distinctive biochemical characteristics of bacterial strains which tested positive for urease and EPS production

Characteristics	Strain 1	Strain 2	Strain 3	Strain 4
Malonate	−	+	−	−
VP	−	−	+	+
Citrate	−	+	+	−
ONPG	−	+	+	−
Nitrate reduction	+	−	−	+
Catalase	+	+	+	+
Arginine	−	+	−	
Sucrose	+	−	+	+
Mannitol	−	−	+	−
Glucose	+	−	+	+
Arabinose	−	−	−	−
Trihalose	+	+	+	+

300 mg/L of dried EPS in NB-glucose media. Crude EPS consisted of 85% carbohydrates and 6% proteins Fig. 7. Bio-calcite was visualized by SEM as shown in Fig. 8. The size of calcite crystals formed by *B. cereus* SG4 was about 10–50 μm in diameter. The composition of thus examined crystals was determined with EDS which verified the presence of calcium carbonate based on weight %.

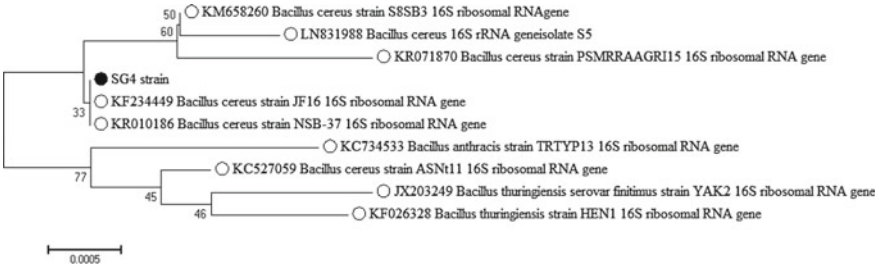

Fig. 5 Phylogenetic tree based on 16S rDNA sequence using neighbor joining algorithm with 1000 bootstrap replicates for *Bacillus cereus* SG4 strain. Branch distances represent nucleotide substitution rate and the scale bar represents the number of changes per nucleotide position

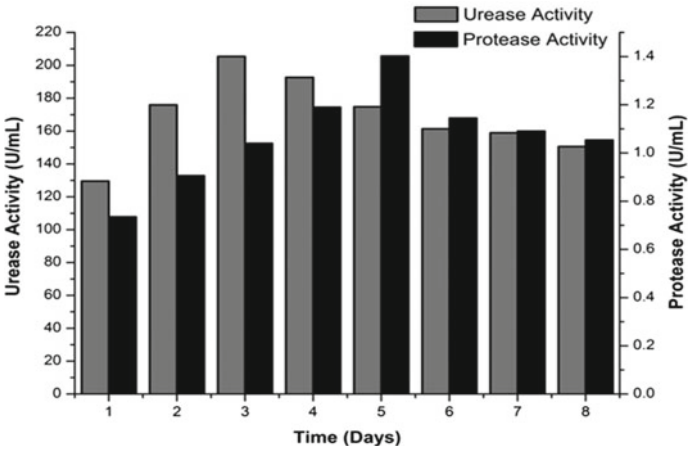

Fig. 6 Urease and Protease production by *Bacillus cereus* SG4

3.3 *Improvement in the Geotechnical Properties of Bentonite Soil*

Geotechnical properties of soil can be enhanced by the treatment with microbial-mediated processes or microbial products, e.g., microbial induced calcite precipitation and extracellular polysaccharides. The process of calcification involves decomposition of urea into ammonia and CO_2 by extracellular urease enzyme produced by bacteria [22]. Earlier studies state that bacteria can hydrolyze urea and utilize resulting ammonia as the nitrogen source for its metabolism [18]. Presence of ammonia raises the pH of media, thus increasing the concentration of CO_3^{2-} which reacts with supplemented Ca^{2+} ions to form insoluble calcium carbonate precipitate (Fig. 9). To achieve a higher yield of bio-calcite an organism exhibiting enhanced urease activity is required. EPS is an extracellular polymeric metabolite produced by bacteria utilizing sugars present in the culture media [7].

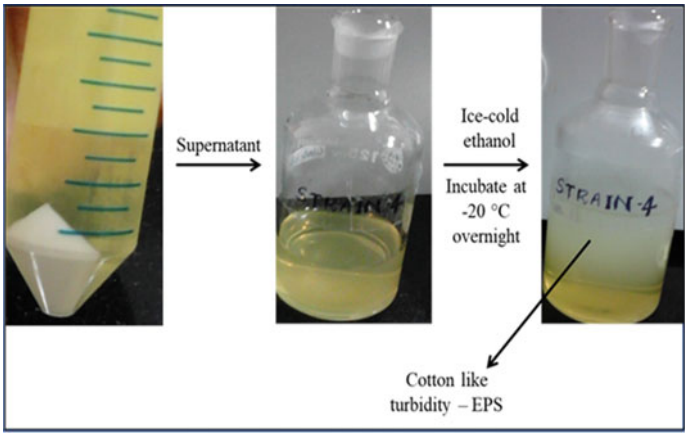

Fig. 7 Extraction of Extracellular Polysaccharide by cold ethanol method* (*Note* * Images have been taken by the authors during the experiment at Biological Engineering Lab IITGN)

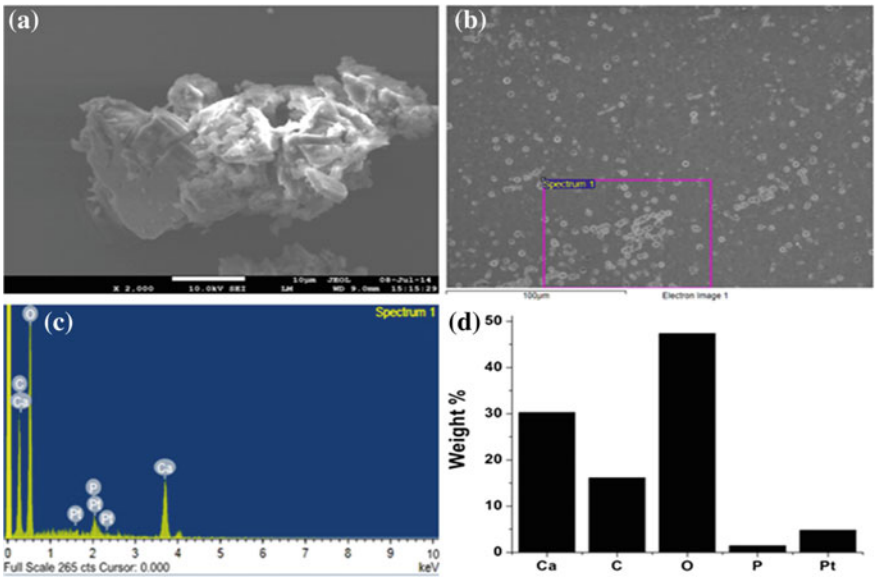

Fig. 8 Analysis of precipitated Bio-calcite using electron microscopy, **a** SEM of Bio-calcite precipitated by *Bacillus cereus* SG4 in NB-Urea-$CaCl_2$ media, **b** selection area for EDS analysis, **c** elemental analysis plot by EDS, **d** prevalence of calcium, carbon, and oxygen in weight%

Microbial polysaccharides have found wide applications as admixture for concrete and grout but have not been used for soil properties enhancement. Thus, we set out to extract urease or EPS producing bacteria which could potentially be used for soil treatment. We screened 33 bacteria isolated from 11 soil samples across Gujarat,

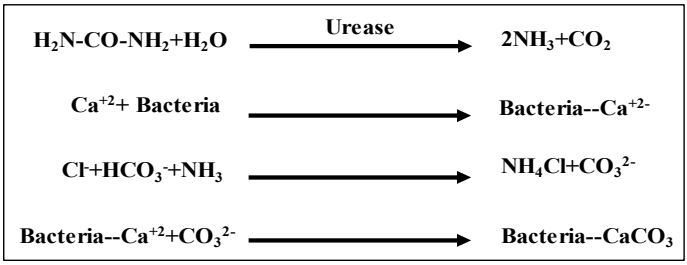

Fig. 9 Mechanism of calcite precipitation by bacteria [27]

India, and successfully identified as bacterial strain *Bacillus cereus* SG4 which not only exhibited significantly higher urease activity than previously isolated bio-calcite producing bacteria but also produced EPS when a suitable culture media with glucose as substrate was used.

Lian and co-workers have shown that the bacterial cell surface induces mineral deposition by providing nucleation sites [21]. Calcium carbonate crystal formation by ureolytic bacteria involved the nucleation of calcite on the microbial cell wall. Bacterial metabolic processes do not utilize Ca^{2+} ions, and thus they start accumulating outside the cell. Meanwhile, enzymatic reversible hydration of CO_2 leads to the formation of HCO_3^- or the dissolved CO_2 transformation to CO_2^{3-} commences the growth of $CaCO_3$ around the cell [2]. A similar mechanism can also be predicted for bio-calcite from *Bacillus cereus* SG4 where the analysis of a precipitated crystal by SEM and EDS confirmed the composition to be $CaCO_3$. EPS forms a surface film which reduces the absorption properties of soil resulting in bioclogging of soil. As *Bacillus cereus* SG4 could produce substantial quantities of EPS using glucose as a substrate. It was hypothesized that this property could be utilized to enhance the soil properties. After characterizing the extracellular metabolites, viz., bio-calcite and EPS, culture media containing bacteria was tested on expansive bentonite clay which has drastic shrinkage and swelling properties rendering it highly unusable for civil constructions.

Geotechnical properties of soil can be enhanced by the treatment with microbial-mediated processes or microbial products, e.g., microbial induced calcite precipitation and extracellular polysaccharides. When media containing bio-calcite was added to Bentonite the soil exhibited a large decrease in LL, PL, and DFSI. The calcium ion from calcite is released into the pores of soil and as a result divalent Ca^{2+} ions replace the monovalent Na^+ ions present in Bentonite clay. This replacement of ions causes an increase in electrolytic concentration, which may reduce the thickness of "diffuse double layer" (an ionic layer found around the clay particle). Reduction in thickness will lessen water absorption, retention, and swelling properties of soil leading to decrease in LL, PL, and DFSI [10]. In addition, $CaCO_3$ forms an aggregated structure (0.02–0.05 mm in diameter) which is larger than the size of clay particle (0.002 mm in diameter). These particles behave as silt sealing the pores and further enhance the clay properties [6, 32].

Treatment of Bentonite clay with culture media containing EPS also leads to a significant reduction in LL, PL, and DFSI. EPS was found to decrease the pore size of soil thus hampering the permeability of water [9]. When the culture containing EPS was added to Bentonite, it gave rise to tiny clumps of ~3–5 mm diameter after five days of curing as shown in Fig. 10. This is owing to the binding and encapsulating nature of EPS which holds the soil particles together. EPS coating to soil might stop the inflow of water thereby reducing the swelling property of Bentonite. Moreover, it is reported that biofilm formed by EPS has water retaining capacity [26]. When water was added to soil, EPS retained water and only a small portion may get exposed to soil particles. Thus, a low permeability nature of biofilm acts as a barrier for water molecules to interact with the soil which results in the minimal swelling index.

The results showed that treatment of expansive soil with bio-calcite and EPS containing *B. cereus* SG4 culture media worked effectively. As shown in Tables 4 and 5, these treatment strategies have considerably reduced LL, PL, and DFSI values of Bentonite clay. It was interesting to note that there was no significant reduction in geotechnical values after the tenth day of treatment showing the quickness and efficiency of proposed method. Both treatment strategies worked successfully in improving shrinkage-swelling response of Bentonite clay; with bio-calcite treatment proving more beneficial than EPS treatment.

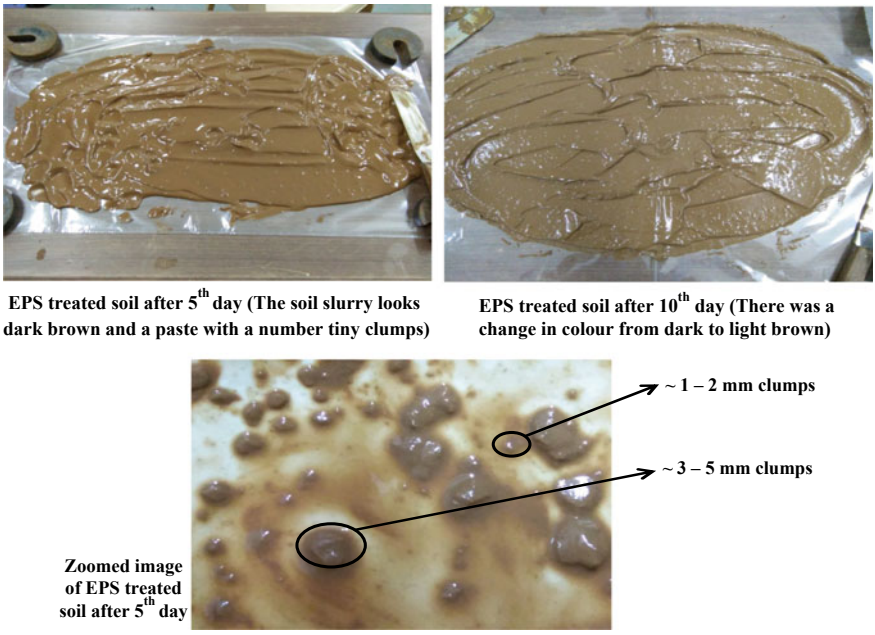

Fig. 10 EPS treatment on Bentonite cohesive soil* (*Note* * Images have been taken by the authors during the experiment at Geotechnical Engineering Lab IITGN)

Table 4 Bentonite clay treated with EPS produced by *B. cereus* SG4

S. No.	Properties	Control (%)	5 days		10 days	
			Treatment (%)	Percentage reduction (%)	Treatment (%)	Percentage reduction (%)
1	LL	608	177	70	171	72
2	DFSI	661	371	43	357	46
3	PL	50	39	22	37	27

Table 5 Bentonite clay treated with Bio-Calcite produced by *B. cereus* SG4

S. No.	Properties	Control	5 days		10 days	
			Treatment (%)	Percentage reduction (%)	Treatment (%)	Percentage reduction (%)
1	LL	608	158	73	150	75
2	DFSI	661	271	58	243	63
3	PL	50	39	22	36	29

4 Conclusions

In summary, *Bacillus cereus* SG4 was isolated and characterized for urease and EPS production. *B. cereus* SG4 effectively produced bio-calcite and EPS in Urea-$CaCl_2$ and glucose substituted nutrient broth, respectively. Culture media containing bio-calcite and EPS was used to treat expansive Bentonite clay, which had a severe shrinkage-swelling problem. The results showed that both bio-calcite and EPS treatments have capabilities to effectively reduce the geotechnical properties such as LL, PL, and DFSI. The most interesting aspect of the identified bacteria is that it can switch the metabolic pathway as per the availability of the medium and produce bio-calcite as well as EPS and both strategies can be applied to expansive soil to effectively control its shrinkage-swelling response.

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