Screening of Calcite Precipitation Inducing Bacillus Strains from Different Geologic Sources for Bio-Mediated Lateritic Soil Improvement

***DUROJAYE Adeyemi J.[1], DUROJAYE Omolola T[2]., ALLI Praise A[3]. and OLAPOJU Peter O[4]. ADERINTO Sunday. J[5]**

[1]Polytechnic Ibadan, Department of Civil Engineering, Ibadan, Oyo-State, Nigeria

[2]Dominion University, Department of Microbiology and Biotechnology, Ibadan, Oyo-State

[3]Lead City University Ibadan, Department of Civil Engineering, Ibadan, Oyo-State

[4]The Polytechnic Ibadan, Department of Civil Engineering, Ibadan, Oyo-State, Nigeria

[5]Department of Civil Engineering, Adeseun Ogundoyin Polytechnic Eruwa

***Corresponding Author**

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ABSTRACT

Bio-cementation is a new approach used in the stabilization of soil, in which eco-friendly microorganisms are manipulated to improve the geotechnical properties of soil. This study focused on screening for Bacillus strains with the highest induced calcite precipitation potential from different geologic sources. Thirty-two Bacillus strains were screened for urease, an enzyme which initiates calcite precipitation and six strains were observed to produce urease enzyme reasonably. Bacillus sp-CE11 had the highest urease enzyme production. Bacillus strains with a significant amount of urease enzyme production were employed forthe bio-cementation process using lateritic soil. The Unconfined Compressive Strength property shows that the soil samples treated with urease producing strains (UPS) had more strength compared with the untreated lateritic soil sample (control). Bacillus mycoides-CE8 at pH 7 had the highest UCS value (329.6 kPa) and the least coefficient of permeability (1.01x10-7 cm/s) while Bacillus subtilis- KA71 had the least UCS value (223.9 kPa) at pH 7. The Highest coefficient of permeability (8.92×10-7 cm/s) at pH 6 was observed in the soil specimen treated with Bacillus subtilis-KA7. Bacillus strains isolated from Celica soil sample were found to induce calcite precipitation more than other strains from other soil samples. Hence, the best isolate (Bacillus mycoides) has the capacity of calcite production. It provides basic geotechnical characteristics and specifications for the bio-cementation of tropical lateritic soil.

Keywords: Bio-cementation; Bacillus strains; Urease; Stabilization; Unconfined Compressive Strength.

INTRODUCTION

Minerals are deposited in sediments as a result of chemical, physical, and biological processes that lead to natural lithification. These minerals cause cementation by compressing the sediments tightly, which decreases pore space and removes water permeability. (Lee et al., 2018). Bio-cementation is a new approach that is currently employed in soil stability (Kishan et al., 2024). This method involves biologically mediated (He at al., 2023) and induced on site granular soils cementation by urea hydrolysis, which is very rich in calcium (Gomez et al., 2019). Bio-cementation involves the use of microorganisms that are eco-friendly which brings about improvement in the soil properties (Iqbal, et al, 2021). The affected properties include particle size, moisture-density distribution and Atterberg's limits.

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Most industrial processes of producing soil stabilizers release huge pollutants in the environment which are liable in depleting the ozone layer and constituting other environmental nuisance. This informs the decision of engineers to term up with the environmentalist to finding more effective and productive methods of improving soil stability by employing ecofriendly microbes. The use of microbes is an uncommon alternative for improving properties of soils. It is less toxic and ecofriendly when compared with conventional soil treatment methods (Wath and Pusadkar 2016, Sharma, 2024). Microbial-Induced Calcite Precipitate is a biological process that occur in nature, this biological process is achieved by inoculating the soil matrix with large concentration microorganisms with greater affinity to produce urease enzyme and the cementation reagents results in a cementing compound and this brings about improvement in the geotechnical properties of the soil. Urease is an enzyme produced by various bacteria (e.g., Sporosarcina pasteurii) that catalyzes the hydrolysis of urea into ammonia and carbon dioxide:

(NH2)2CO +H2O urease 2NH3 + CO3 The released ammonia reacts with water to form ammonium and hydroxide ions respectively.

NH3 +H2O NH4+ + OH- thus, leads to increase in the pH of the surrounding environment, making it more alkaline which results into conversion of carbon dioxide into carbonate ions.

CO2 + OH- HCO3- CO32- The presence of calcium ions in the environment, react with carbonate ions to precipitate calcium carbonate (calcite).

Ca 2+ + CO3 CaCO3 The resulting calcite the binds with soil particles or fill cracks in concrete. According to Hanjiang et al. (2024). This process is extremely complex, biological, physical and chemical and as well affected by several factors which include temperature, composition and concentration of the cementation material, pH, inoculum size and soil properties etc. The effect of urease producing microorganisms posed no harm on the environment however, the technique is relatively new with a handful of scholarly reports (Ehrlich, 1999 and Harkes et al., 2010). This study focused on screening for Bacillus strains with the highest induced calcite precipitation potential from different soil types.

MATERIALS AND METHODS

Collection of Samples: Four different geological samples used for bacterial isolation were collected from different sites within Ibadan metropolis. The location and description of the used soil samples for this study.is represented in Table 1 and figure 1 below

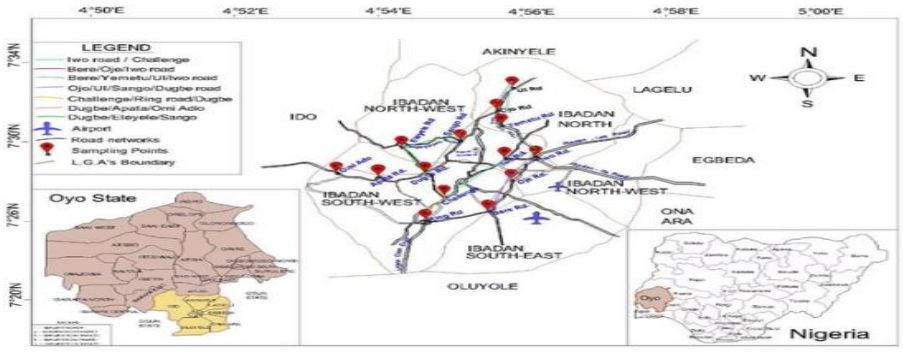

Figure 1: Map of Ibadan city showing the collection sites

Table 1: Location and Description of the samples used

S/N	Soil type	Location
1	Lateritic soil: wet, acidic, taken beside the roadside	07°22′49.72″ N, 04°00′3.28″ E
2	Celica soil: wet, moderately acidic, peaty	07°22′56.81″ N, 04°00′12.85″ E

ISSN No. 2454-6194 | DOI: 10.51584/IJRIAS |Volume X Issue VII July 2025

3	Kara soil: wet, moderately acidic, peaty	07°26′01.73″ N, 03°54′ 55.99″ E
4	Quarry dust: moist, alkaline, taken beside the roadside	07°31′10.82″ N, 03°54′45.59″ E

Isolation and Characterization: The culture was enriched by inoculating 1 g of the soil sample into 50 mL of Tryptic Soy Medium supplemented with 6% urea and incubated at the appropriate cultural conditions.

Isolation of microorganisms from the soil sample: One milliliter of the enriched culture samples was serially diluted and plated on Tryptic Soy Agar (TSA) containing 6% urea. 0.1 mL of the diluent was plated using spread plate techniques and the plates were incubated appropriately.

Culture Preservation: The selected urease producing Bacillus strains were preserved in glycerol broth and were kept at -20oC according to the method of Whiffin (2004).

Screening and production of microbial induced calcite precipitate (CaCO3).

Qualitative Method (Urease Agar Base Test): The screening and assay of urease enzyme was done using Urease agar media. Urea agar base was used for screening and assay of urease enzyme. 4% w/v of urea was sterilized by filtration and 10 mL of the sterilized solution was added to 990 mL of the screening medium under aseptic condition. The sterilized medium was inoculated with the isolates and then incubated under aerobically for 48 h at 35 °C to test for production of urease enzyme. The isolates were watched out for color change

Quantitative Method: Urease Activity was measured using conductivity method (Harkes et al. 2010).

Phenotypic characterization: The best urease enzyme producing Bacillus spp were identified and characterized phenotypically. (Cathcart, 2011; Olufunke et al., 2014)

Inoculum preparation: Bacillus strains that have high capacity of producing urease were cultivated under aerobic conditions in a medium containing 30 g/l of Tryptic Soy Broth and 20 g of urea per litre of deionized water. The pH of the medium was adjusted between pH 6-10 and was sterilized at 121oC and 15 psi for 15 min. Before sterilization, the medium pH was adjusted with 1M HCL for acidic and 1M NaOH for alkaline. The flask was incubated for 24 -36 h on a rotary shaker at 150 rpm and 30oC. Duplicate flasks were used according to Durojaye et al., 2022. After incubation the bacterial suspension is centrifuged at 8000 rpm for 10 min. The supernatant was decanted and the pellet was used for treating the lateritic soil samples.

Soil Treatment: The soil was treated by introducing the bacteria inoculum alongside with 0.5 M solution of calcium chloride. This was done manually and the mixed was compacted into the polyvinyl tube using the according to BS1377-6:1990 3.3.6.5

Testing for Unconfined Compression Strength (UCS): The UCS was carried out using BS1377-1990 Part 7:7 Code. BS1377-1990 Part 7:7 Code was followed in carrying out the UCS test on the bio-treated lateritic soil specimens. An unconfined compression apparatus, proving ring type was used. The prepared specimens were placed carefully in the device. Axial compressive load was applied to cause an axial deformation at the rate of 0.5 to 2% per minute, and thereafter load and deformation dial readings were noted at every 20 to 50 divisions on the dial. The test continued until an axial strain of 20% was reached.

Determination of Permeability Test of Soil: Permeability of the soil samples were using falling head method. Soil sample of 2.5 kg by weight was mixed with known water content, empty mold was weighed with the extension collar attached. The inside of the mold and collar were greased and kept the assembly on a firm base. The collar was removed and the excess soil scrapped off. The baseplate was weighed; the mould, the drainage base and the cap were coupled together with the porous disc. Water was allowed to flow through the specimen with sufficient head. While bottom outlet was kept open, the time taken t for the head difference from h1 to h2 was recorded. This was repeated in three trials by refilling the standpipe, and recording the time interval taken for water level to fall.

ISSN No. 2454-6194 | DOI: 10.51584/IJRIAS |Volume X Issue VII July 2025

Molecular characterization: 16S rRNA gene of the selected Bacillus strains were amplified using forward and reverse primer. Primers 27F: 5'-AGAGTTTGATCMTGGCTCAG-3' and 1525R (5'-AAGGAGGTGATCCAGCC-3') were used for forward and reverse primer respectively (Muramatsu, et al., 2003). The MEGA (Molecular Evolutionary Genetic Analysis) version 6 was used to analysis the Phylogenetic relationship of the organisms (Tamura and Stecher, 2013).

Urea content determination: The residual urea molecule was determined using spectrophotometry (ICP-OES) Knorst et al., (1997). 0.5 ml of the solution (95% ethanol with 4% (w/v) of p-dimethylaminobenzaldehyde and 4% (v/v) H2SO4) was added into 2 g of the samples (filtered through 0.45 µm filter) and properly mixing together for 10 minutes. Reaction of p-dimethylaminobenzaldehyde and urea molecules resulted in formation of yellowish compound which was determined by measuring the absorbance at 422 nm. Knorst et al., (1997).

Calcium content determination: Coupled plasma atomic emission spectroscopy (ICP-OES) test was used to determine calcium ion. 2.00 g of this oven-dried soil sample was measured into a 50 ml volumetric flask. 50mL of 2.5% acetic acid was added into the flask and was shaking at 121 rpm for 3 hours. The mixture was then centrifuged, and supernatant was tested for.

Data analysis: The data obtained were subjected to analysis of variance using one-way ANOVA

RESULTS AND DISCUSSION

Sample Collection and Isolation of Bacteria

The soil classification standard was done in accordance to British Standards (BS1377:1990) and the results are presented in Table 2 below; the lateritic soil used in this study belongs to the SM Group in the Unified Soil Classification System and the A-2-4 (7) soil group of the AASHTO soil classification system. The quarry dust has anaverage particle size of 45 microns (fine powder), specific gravity of 2.8 and pH of 8.34. The physical properties of the peat soil used presented an organic matter of 42.52%, which is responsible for its physical and geotechnical properties, a specific gravity 2.34 and with a fairly acidic pH value 6.16. Organic matter composition of the lateritic soil used was 3.92% which is more than less than 2% which is the required value of engineering soil (Al Rawi and Assaf , 2017). Increase in organic matter consistent of soil is said to relatively affects soil quality and the engineering properties. Thus, high organic content of soil has possibility of increasing soil permeability which will result in reduction of the soil strength.

Table 2: Properties of Geologic materials used for microbial isolation

Parameter	Properties			
	Lateritic soil	Quarry dust	Peaty soil-1	Peaty soil-2
Colour	Reddish brown	Whitish blue	Black	Black
pH	4.65	8.34	6.16	6.05
Specific gravity	2.58	2.8	2.34	2.1
Average particle size (microns)	-	45	-	-
Particle shape	-	Fine powder	-	-
Water absorption (%)	-	30	-	-
Fineness modulus (%)	-	22.9	-	-
Surface texture	-	Rough	-	-

ISSN No. 2454-6194 | DOI: 10.51584/IJRIAS |Volume X Issue VII July 2025

AASHTO classification	A-2-4 (7)	-	-	-
Plasticity index, PI (%)	5.2	-	40.2	39.02
Plastic limit, PL (%)	35.1	-	0	0
Liquid limit, LL (%)	40.3	-	40.2	39.02
USCS classification	SM	-	CL	CL
Maximum dry density, MDD (kg/m^3)	1700	-	1600	1560
Optimum moisture content, OMC (%)	16.5	-	13.5	14.45
Organic moisture content, (%)	3.92	-	42.52	50.25

Out of the Thirty-two Bacillus strains isolated from the soil samples, fourteen isolates (represented in Table 3) which were observed to change turn the medium from yellow to pink positive were further used in this study Change in color, signifies positive urease activity. Ureaseproducing Bacillus strains, had the capacity of turning the urea agar base medium pink from yellow, while non-urease producing isolates remained yellow. Hydrolysis of urea, results in accumulation of ammonia which increases the pH of the environment as reported by Armstrong et al. (2016). positive result, indicates a urea hydrolysis. From previous results, urea agar base media is preferred for qualitative assay of urease and to differentiate of microorganisms with ureolytic activity (Hammes et al., 2003; Achal, (2010); Achal and Pan (2011); Burbank et al. (2011) and Dhami 2013

Table 3: Qualitative screening of the isolated Bacillus strains for urease enzyme

S/N	Isolate code	Urease reaction	S/N	Isolate code	Urease reaction	S/N	Isolate code	Urease reaction
1	AD7	-	12	KA41	-	23	AD8	-
2	KA5	-	13	KA3	-	24	LD2	-
3	KA72	+	14	LD16	-	25	CE17	-
4	KA1	-	15	CE13	-	26	LD15	-
5	KA71	-	16	CE1	-	27	CE10	-
6	KA3	+	17	CE11	+	28	KA6	-
7	KA2	+	18	CE6	+	29	KA42	-
8	AD5	+	19	CE18	+	30	CE7	-
9	AD6	+	20	CE3	+	31	CE4	-
10	AD7	+	21	CE9	+	32	CE8	+
11	KA8	-	22	LD	+			

Key: - negative, + positive KA- Kara soil sample, AD- Adegbayi soil sample, CE- Celica soil, LD- Limestone Deposit

ISSN No. 2454-6194 | DOI: 10.51584/IJRIAS |Volume X Issue VII July 2025

The amount of urease enzyme produced by the selected Bacillus strains was determined by measuring the conductivity at the end of incubation period (24 h). The conductivity variation rate (mS/cm/min) of each isolate was measured and converted to specific urease activity, and this is presented in figure 2. For six selected Bacillus strain gotten from Celica soil sample. gotten form Celica soil sample. Armstrong et al., 2016 reported that bacterial isolates from limestone cave were positive for urease enzyme. Five isolates were gotten from peat soil while the remaining three Bacillus strains were gotten from lateritic soil. Bacillus-CE9 had the highest urease enzyme production (6.6 mM) while the control had the lowest production (0.44 mM) of urease enzyme. Also, this is in line with the report Al-Thawadi and Cord-Ruwisch (2012) and Stabnikov et al., 2013 who reported that the isolated Bacillus strains in their studies had 3.3 to 8.8 mM urease activities. For the morphological characterization of the Bacillus strains, standard methods were used. It was observed that the isolates were positive to the following tests: Gram reaction, oxidase positive, motile, catalase and were all rod-shaped, this is presented in Table 4.

Six Bacillus strains with probable identity were identified as: B. subtilis (KA71), B. pumilus (AD6), B. mycoides (CE8), B. subtilis (CE6), B. amyloliquefaciens (CE9) and Bacillussp (CE11) with significant amount of urease enzyme production were further employed for bio-cementation process in lateritic soil. The soil belongs to the SM group in the Unified Soil Classification System [d41] ASTM, 1992 or A-2-4(7)

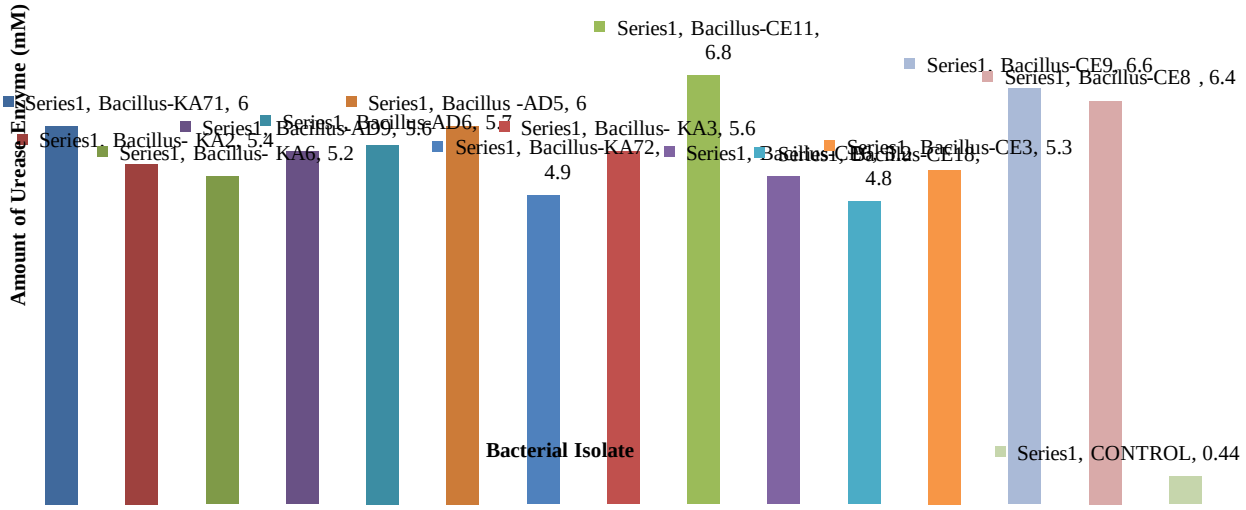

Figure 2: Quantitative screening of the isolated *Bacillus* strains for urease enzyme.
Key: KA-peat soil, AD-Lateritic soil and CE-Celica soil

Table 4: Physiological characteristics of urease producing bacterial isolate

Isolate	Characteristic	Size (mm)	Chromogenesis	Gram stain	Shape	Endosore staining	Catalase	Oxidase	Mobility	Probable organism
KA7[1]	Circular	4	white	+	R	+	+	+	+	*B.subtilis*
KA2	Irregular	6	white	+	R	+	+	+	+	*B.megaterium*
KA6	Circular	4	Brown	+	R	+	+	+	+	*B.pumilus*
AD9	Circular	3	Brown	+	R	+	+	+	+	*B. megaterium*
AD6	Circular	6	White	+	R	+	+	+	+	*B. pumilus*
AD5	Circular	3	White	+	R	+	+	+	+	*B.subtilis*

ISSN No. 2454-6194 | DOI: 10.51584/IJRIAS |Volume X Issue VII July 2025

KA7[2]	Irregular	8	White	+	R	+	+	+	+	*B. cereus*
KA3	Irregular	4	creamy	+	R	+	+	+	+	*B.polymyxa*
CE11	Circular	4	creamy	+	R	+	+	+	+	*Bacillus sp*
CE6	Circular	6	creamy	+	R	+	+	+	+	*B. subtilis*
CE18	Irregular	6	Brown	+	R	+	+	+	+	*B. cereus*
CE9	Circular	8	creamy	+	R	+	+	+	+	*B.amyloliquefaciens*
CE3	Irregular	6	creamy	+	R	+	+	+	+	*B. megaterium*
CE8	Circular	3	Brown	+	R	+	+	+	+	*B. mycoides*

Key;- negative, + positive, R- Rod

soil group of the AASHTO soil classification system [42] AASHTO, 1986.

The Result of X-ray Diffraction

Minerals constituents of the untreated lateritic soil were determined by X-ray powder diffraction. Table 5 shows the pattern of the distribution of the elements present in the lateritic soil sample used. Four major minerals were found present in the soil samples, which are quartz, muscovite, kaolin and goethite. Quartz (47%) was found to be the dominant mineral present while the least was in Geothite (8%). Tsozue and Yogue-Fouateu, 2012 and Kamtcheueng et al., 2015 reported the same trend in their previous findings highest peak was observed in quartz and muscovite with intensity count at 2θ position of Bragg's angle and 27o with corresponding d-value of 0.33 nm. The maximum peak for kaoline was observed at 2θ position 12.5o with corresponding d-value of 0.71 nm, while the maximum intensity counts for geothite occur at 2θ position 34o with d-value of 0.26 nm.

Table 5: Pattern List of X-ray diffractogram of the used Lateritic Soil

Visible					
Reference Code	Score	Compound	Displ.[°2Th]	Scale Factor.	Formula
96-900-5018	47	Quartz	0	0.143	Si6.00 O6.00
96-900-9235	21	Kaolinite	0	0.065	Al_2.00 Si2.00 O9.0.
96-900-6329	13	Muscovite	0	0.064	K2.40 Na1.48 Al11...
96-900-2159	8	Goethite	0	0.032	Fe4.00 H4.00 O8.00

The result of UCS shows that the tested soil samples (treated with urease producing strains) had more strength compared with the untreated lateritic soil sample (control). Bacillus mycoides-CE8 at pH 7 had the highest UCS (329.6 kPa) and with the highest strength increase of 35.08% while Bacillus subtilis-KA7 had the least UCS (223.9 kPa) at pH 7 with strength decrease of -8.23% as shown in figure 3a and figure 3b and the percentage increase in strength. The stress-strain curve for Bacillus mycoides-CE8 at pH 7 shows an increase in the axial stress increases as the axial strain increases until peak value of 329.6 KPa strength was reached at 6.7% strain and stiffness of 4919.40 KPa. Bacillus subtilis- KA7 at pH 7 produced the lowest stiffness of 3014.94 KPa. Increased in the strength of the tested lateritic soil samples can be attributed to the formation of urease enzyme which initiated the reaction of MICP leading to hydrolysis of urea and the ammonium (NH4+) and finally resulted in the increase of pH. Precipitation of the bicarbonate (HCO3−) with calcium ion (Ca2+) from the calcium chloride also resulted and hence resulted in the formation of the calcium calcite (CaCO3). The produced calcite helps in binding and clogging of the soil grains together. The formed calcium calcite binds the soil grains,

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which reduced the percolation level of the soil grains, and hence enhance the strength and stiffness characteristics of the lateritic soil matrix Ivanov and Chu (2008) and Kolawole et al. (2017).

Table 6.0 shows the coefficient of permeability for soil specimens treated with various isolates at varying pH Bacillus subtilis-KA7 had the highest coefficient of permeability (8.92×10-7cm/s) pH 6 with minimum percentage reduction in hydraulic conductivity of -6.20%. Meanwhile, Bacillus mycoides-CE8 had the least coefficient of permeability (1.01×10-7 cm/s) at pH 7 with maximum percentage reduction in hydraulic conductivity of -88.9% when juxtaposed with the value obtainable by the control specimen. It was observed that the decrease in permeability among the Bacillus strains used had a P-value of 0.033459 which indicated that there was significance difference between pH values and the isolates employed in this study. But the permeability coefficient of the Bacillus strains in relation to different pH has P- value of 2.65 which indicate that there is no significance difference (P-value 0.05).

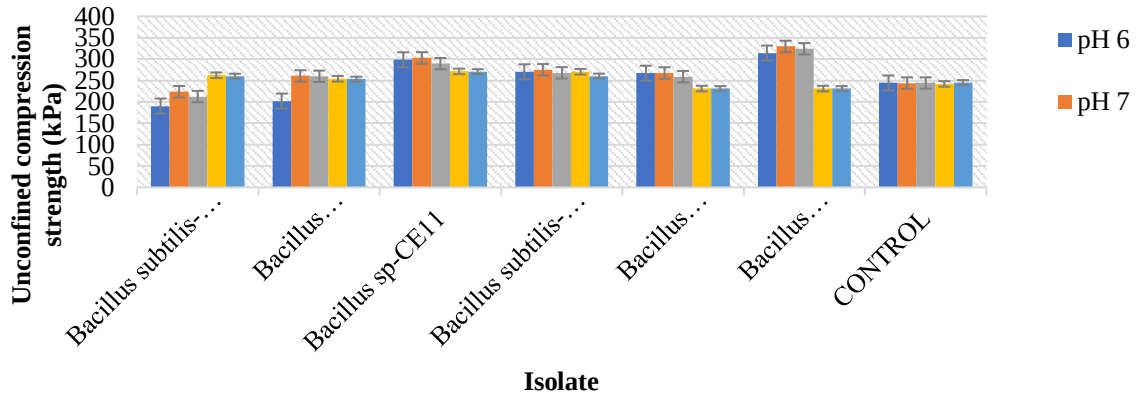

Figure 3a: Unconfined compression strength (kPa) of Bio-cemented Lateritic soil samples at varying pH

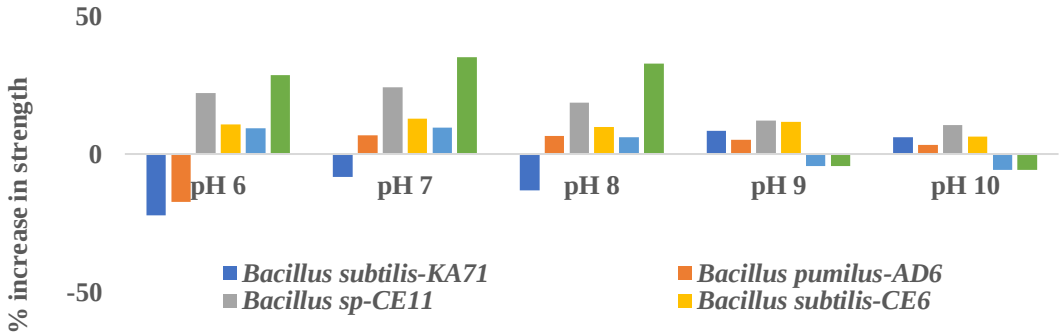

Figure 3b: Percentage Unconfined compression strength of Bio-cemented Lateritic soil samples at varying pH

Table 6.0: Permeability Coefficient (cm/s) of Bio-cemented Lateritic soil samples at varying pH

Isolate code	pH 6		pH 7		pH 8		pH 9		pH 10	
	Permeability coefficient	% decrease in permeability	Permeability coefficient	% decrease in permeability	Permeability coefficient	% decrease in permeability	Permeability coefficient	% decrease in permeability	Permeability coefficient	% decrease in permeability
***Bacillus subtilis*-KA7[1]**	**8.92×10-7**	**-6.2**	**7.93×10^{-7}**	**-13.14**	**8.91×10-7**	**-7.28**	**8.99×10-7**	**-6.74**	**9.01×10-7**	**-8.71**
***Bacilluspumilus*-AD6**	**8.43×10^{-7}**	**-11.36**	**8.08×10^{-7}**	**-11.5**	**8.49×10^{-7}**	**-11.65**	**9.01×10^{-7}**	**-6.54**	**8.89×10^{-7}**	**-9.93**

***Bacillus* sp-CE11**	**3.32×10^{-7}**	**-65.09**	**2.11×10^{-7}**	**-76.89**	**3.48×10^{-7}**	**-63.79**	**5.01×10^{-7}**	**-48.02**	**6.67×10^{-7}**	**-32.42**
***Bacillus subtilis*-CE6**	**6.62×10^{-7}**	**-30.39**	**6.51×10^{-7}**	**-28.7**	**6.92×10^{-7}**	**-27.99**	**7.40×10^{-7}**	**-23.24**	**7.45×10^{-7}**	**-24.52**
***Bacillus amyloliquefaciens*-CE9**	**6.65×10^{-7}**	**-30.07**	**5.58×10^{-7}**	**-38.88**	**5.60×10^{-7}**	**-41.73**	**$6.21/\times10^{-7}$**	**-35.58**	**8.01×10^{-7}**	**-18.84**
***Bacillus mycoides*-CE8**	**3.01×10^{-7}**	**-68.35**	**1.01×10^{-7}**	**-88.94**	**1.61×10^{-7}**	**-83.25**	**2.40×10^{-7}**	**-75.1**	**6.81×10^{-7}**	**-31**
CONTROL	**9.51×10^{-7}**		**9.13×10^{-7}**		**9.61×10^{-7}**		**9.64×10^{-7}**		**9.87×10^{-7}**	

The Bacillus strain with best performance was subjected to 16S rRNA and based on DNA-DNA relatedness as shown in figure 4. The phylogenetic relationship of isolate was constructed with Molecular Evolution Genetics Analysis (MEGA) version 6.0. The phylogenetic analysis of the Bacillus strains using the neighbor joining method based on maximum composite like hood revealed that the isolates as Bacillus mycoides strain ORE 1 with accession number MTO47265 and was then submitted in National Center for Biotechnology Information (NCBI), India gene.

The amount of urease in (Ao) and calcium ion in part per billion present in the treated lateritic soil is shown in Table 7. specimen treated with Bacillus mycoides had the highest urea (0.278 Ao) formation while the control sample had the least formation of urea ion (0.278 Ao). The amount of calcium ion formation in the specimens treated with Bacillus mycoides is 32.2082 ppm.

Table 7: Amount of urease and calcium ion production

Specimen	Urease (Å)	Calcium (ppb)
Bacillus mycoides-CE8	0.347	32.208
Control	0.278	21.667

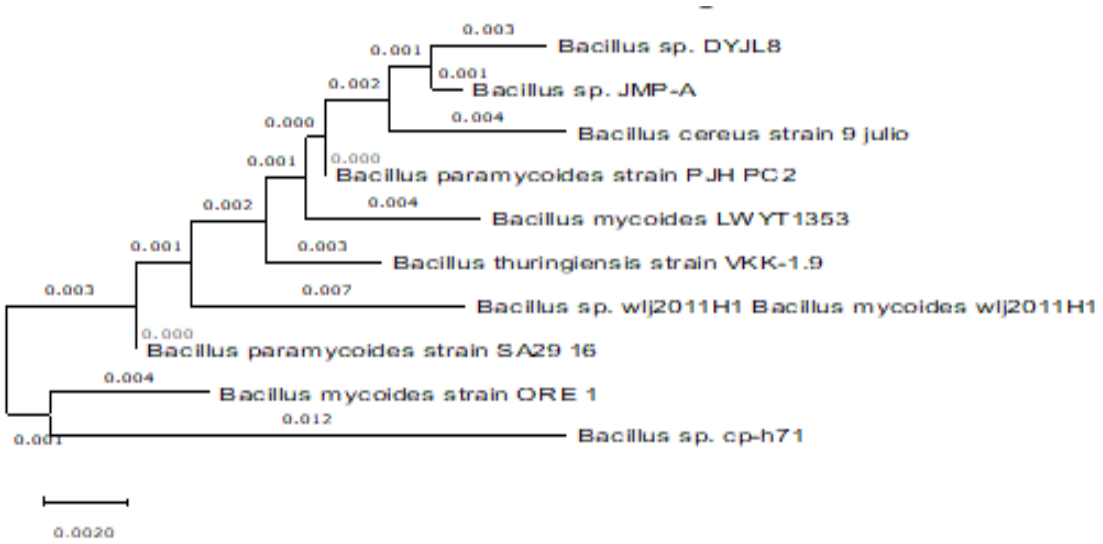

Figure 4: Phylogenetic relationship of the best urease producing Bacillus strain.

CONCLUSION

The findings in this study suggest that Bacillus isolates from Celica lateritic soil samples were capable of inducing calcite precipitation and serve as alternative microbial ureolytic agents. The research has been able to provide very vital insight to the use of Bacillus mycoides MTO47265 for treating lateritic soil, upon treatment

with Bacillus mycoides MTO47265, an increased strength and reduced permeability were observed. In cnclusion, this bacterial isolate (Bacillus mycoides MTO47265) has ability to produce urease enzyme which is responsible for calcite production. Also, basic geotechnical characteristics and specifications for the bio cementation of tropical lateritic soils were meant by this bacterial isolate.

Conflict of interest

The authors declare no competing interest.

Credit authorship contribution statement

XYZ: Conceptualization, Investigation, Writing; XZY: Conceptualization, Validation, and Supervision; XYZ: Conceptualization, Validation, and Supervision; XZY: Conceptualization, Validation, and Supervision.

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