Calcite Precipitation & Its Effect on the Stability of Earthen Bricks

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ABSTRACT

The calcite precipitation producing *streptomyces sp.* KRA01 was isolated from termite gut inhabiting red loamysoil in Kannur District Kerala, India. The isolate was confirmed through biochemical tests and molecular sequencing and was designated *Streptomyces* KRA01, the nucleotide sequence was submitted in GenBank with accession no. MW186183. The application of microbiological methods to improve the mechanical properties of the soil is known as microbial geo-technology. In this research, microbes were isolated from the termite gut and analyzed and identified as *Streptomyces sps. KRA01*. The objective of this study was to demonstrate the soil stabilization capacity of *Streptomyces sps. KRA01* through microbial geo-technology. The microbes grew well in the nutrient broth supplemented with carbon, nitrogen, and inorganic sources acting as their nutrient sources. Microbial Stabilized Mud Blocks (MSMB) were prepared using red loamy soil and microbial culture and cured by spraying an optimized nutrient medium for 28 days. Compressive strength of a microbially-treated mud block was found to be higher than that of the conventional brick. It was discovered that biomineralized calcium carbonate in bacterial cells contributed to the improvement of strength. Results showed that the rate of water absorption was significantly reduced in the brick stabilized with microbes and the results suggested that microbial activities can significantly improve the mechanical properties of MSMB. The calcite precipitation by *Streptomyces sps. KRA01* significantly reduced water absorption by the earthen brick. Hence, microbial isolates can be used as additives to stabilize the soil. If the technology is tested and found to be successful, it might lead to longer maintenance-free periods, reduced material demand, increased level of service, and preservation of nature.

Key words: *Streptomyces sps. KRA01*, soil stabilization, calcite precipitation, optimization of pH, temperature, termite gut.

INTRODUCTION

Construction with earthen bricks is a common practice among communities in developing countries as it is affordable, economic land has high strength. Production of earthen bricks compared to other bricks requires less energy. Apart from these benefits, adobe stores heat in winter and transmits heat in summer, which helps maintain the indoor temperature. These benefits fulfil the requirement of an adequate house, which is why it has been adopted by 50% of the population of the world (Smith *et al*., 1989). Cement plants contribute 5% of the world's carbon dioxide emissions, which are the primary factor in global warming. Since there is no practical way to recycle cement, every new structure and road requires brand-new cement (Andres *et al*., 1997). Thus, earthen bricks have more advantages over cement bricks as it is the more environmental-friendly option. Application of microbial geo-technology can improve the strength, texture, voids and water-resisting properties of the brick. The natural properties of soil can be altered through the process of soil stabilization. Soil stabilization is the process of modifying soil properties concerning its strength, texture, voids, and water-resisting properties, compatible with a particular application (Ivanov and Chu, 2008). Some microbes have the capacity to modify soil properties

Bacteria-induced calcite precipitation (BCP) is the term for calcite synthesis at supersaturation circumstances and temperatures, which, because of the presence of bacterial cells and their metabolic activity, induce reactions that produce a variety of calcium carbonate polymorphs (Ferral and García, 2020). Organisms can secrete one or more metabolic products that react with divalent cations in their environment, resulting in mineral precipitation. Four fundamental components have the greatest impact on BCP, calcium carbonate content, dissolved inorganic carbon concentration, pH, and the availability of nucleation sites (Yoshida et al 2010.,Anbu *et al*., 2016). The BCP technology can be employed to solve a variety of medicinal, environmental (heavy metals remediation, radionuclide remediation, and CO_2 sequestration), and engineering (biocementation and bioconsolidation) challenges (Achal *et al*., 2013), More recently, BCP has gained attention for innovative technologies in self-healing in the field of geotechnical engineering, earth science, and building materials. The stabilization of soil by boosting its strength and decreasing its compressibility is one of the few well-known BCP uses in geotechnical and geoenvironmental disciplines.Hugo Houben and Hubert Guillaud. Earth construction; A comprehensive guide. 1st ed. London UK: *International Technology Development G* Until now, many bacteria have been used to illustrate the molecular mechanisms involved in the calcite precipitation with different morphologies (Ferral and García-Galicia., 2020, Arias,et al., 2018,. Kim et al., 2016, Seifan, *et al*., 2016) Typically, the type of bacterium selected for BCP depends on the specific function and environmental factors. *Sporosarcina pasteurii* (Gollapudi, *et al*., 1995) *Bacillus* spp. (Sánchez-Navas *et al*., 2009) *Pseudomonas aeruginosa* (Li, *et al*., 2015) and *Arthrobacter* spp. (\ Rusznyak, *et al.* , 2012) are frequently used in BCP. Bacteria that produce biomolecules capable of altering the environment and promoting the precipitation of minerals are used in the biocementation process. These bacteria produce urease, which can result in the production of metabolites such as CO_2 and NH_3, which can combine with Ca^{2+} to form calcite (Dhami *et al*., 2013) Calcite precipitation by the bacteria mentioned above appears in monuments, caves, sediments, and constructions. The function and structure of biofilm geometry and exopolymeric substances (EPS) explain how these bacteria nucleate Ca^{2+} and synthesis minerals (\ Dupraz, *et al*., 2009) *roup ITDG*, 1994.

MATERIALS AND METHODS

Isolation and screening of calcite producing bacteria

The soil samples were collected from the Termites guts in Kannur, (Kerala) India, in sterile polythene bags and transported to the laboratory. The serially diluted samples were plated on nutrient agar medium and observed for morphologically different colonies at 37°C for 7 days. Five morphologically different colonies (*KRA01 – KRA05*) were cultured on nutrient Agar and screened for Calcite production using standard protocol (Mahanty *et al*, 2013). For the rapid detection and isolation of Calcite producing bacteria, Precipitation of calcium carbonate was assessed via titration with Hydrochloric acid. The pure culture of the producer strain designated KRA01. The morphology of the Calcite producer was further confirmed by Scanning Electron Microscopy. (Arya *et al*.,2016)

Measurement of dry biomass

The culture was centrifuged at 10,000 rpm for 15 minutes in order to quantify dry biomass, and the pellet was dried to constant weight at 55 °C in an oven.

Biochemical and molecular identification of Calcite producer *KRA01*

The Calcite producing strain *KRA01* was characterized by studying their colony morphology and biochemical characteristics in accordance with Bergey's Manual of Determinative Bacteriology. 6S rRNA studies were carried out to confirm the species level identification of *KRA01*. The DNA of *KRA01* was isolated using CTAB method and amplified using universal forward and reverse primers 3' AGGCCCGGAACGTATTCACC 5' and 16R 3' GTGCCAGCAGCCGCGGTAAT 5'. The PCR amplified product was sequenced using the Big Dye Terminator (BDT) v3.1 cycle sequencing kit on an ABI 3730xl Genetic Analyzer. The sequenced product was subjected to BLAST studies to identify the nearest homology and phylogenetic tree drawn using Mega 10.0 software. The evolutionary history was inferred by using the Maximum Likelihood method and Tamura-Nei model. The bootstrap consensus tree inferred from 500 replicates is taken to represent the evolutionary history of the taxa analyzed.

CALCIUM CARBONATE PRECIPITATION

Precipitation of calcium carbonate was assessed via titration with hydrochloric acid using approximately 10 grams of randomly sub sampled soil from the column. The soil taken for titration was not air-dried as described by Rajasekar *et al*., 2018. Before this procedure, the sand was washed with distilled water to remove excess or unused calcium chloride, urea, and any other by-products such as hydrochloric acid that may have been retained in the sand. The steps are as follows: Put a 1 to 10 g (0.001 g) soil sample into a 250 mL Erlenmeyer flask, add 20 mL of standardised 1N HCl using a volumetric pipette, cover the Erlenmeyer flask with a watch glass, and boil the soil-acid mixture for 5 minutes before adding 50–100 mL of deionized water using a graduated cylinder. After it has cooled down; add 2 or 3 drops of phenolphthalein indicator. Titrate the solution with 1N NaOH solution while swirling the flask and finally record the reading when a faint pink colour develops (Adharsh Rajasekar *et al*., 2021).

$$CaCO_3\text{equiv.}, \% = \left(\frac{V_{HCl}N_{HCl} - V_{NaOH}N_{NaOH}}{\text{grams of soil}}\right) \times 0.05 \times 100$$

Effect of Culture Conditions on the production of Calcite

The effect of incubation temperature, pH, carbon and nitrogen sources were determined. The growth of *KRA01* strain congruent to the production of calcite in nutrient medium under different culture conditions was also determined. The growth of the *KRA01* strain was monitored by measuring the aliquots at an absorbance of 610 nm (OD_{610}) using a UV – Visible double beam (HLS1 – 19191 India) spectrophotometer for every 24 h for 6 days. The cultures grown for calcite precipitation was evaluated Volumetric methods.

The effect of temperature and pH on the growth of the strain was determined by culturing *KRA01*at various temperatures (25, 30, 37 and 40°C) and pH adjusted to 5.0, 6.0, 7.0, 8.0 and 9.0 using 1 N HCl / 1 N NaOH in Minimal Davis broth medium and nutrient broth respectively in separate experimental setups.

Effect of Carbon, Nitrogen source and Inorganic salt on calcite production

To determine the best carbon source for calcite production, 100 mL of sterile Nutrient media with 1% w/v of different carbon sources (sucrose, lactose, starch, and sago rice powder; pH 7.0) were inoculated with 10 ml inoculum and incubated for 72 h at 37 °C after sterilization. The growth, dry weight and calcite production were estimated as described previously.

The effect of nitrogen source on calcite production was determined by supplementing the 100 ml production medium with different nitrogen sources (1% w/v) *viz*., ammonium sulphate, beef extract, malt extract and green gram powder and pH adjusted to 7.0; 10% inoculum was added to the sterilized media and incubated for 72 h at 37 °C following which growth, dry weight and calcite perception was estimated.

Preparation of bacterial earthen bricks

Nutrient broth with additional carbon, nitrogen, and inorganic sources was inoculated with KRA01. The log phase bacterial culture and clay were mixed in a 1:1 ratio, and a brick measuring 15x6.8x3.5cm was prepared and baked in a kiln at temperatures ranging from 900˚C to 1200˚C. A brick that was prepared using a conventional method served as a control. Bricks must cure in order to become strong and long-lasting. For 28 days, the bricks were sprayed with nutritional medium and wrapped with cotton fabric in order to cure. Calcite precipitation caused by microbes is enhanced by curing. (Arya *et al*.,2016)

RESULTS AND DISCUSSION

1. Isolation and screening of calcite producing bacteria

From among the five distinct strains of bacteria isolated from the soil sample, the strain *KRA01* produced calcite as revealed by calcium precipitation test. The calcite producer strain *KRA01* was confirmed as *Streptomyces sps. KRA01*based on their morphological, biochemical and molecular characteristics. The microscopic of the calcite producer strain *Streptomyces sps. KRA01* is shown in Fig. 1.

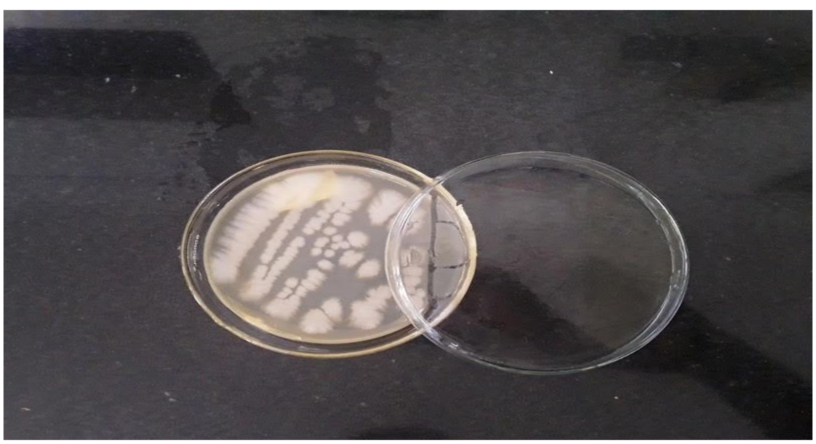

Fig. 1. Microscopic (a) and Isolation

2. Biochemical and molecular identification of Calcite producer *KRA01*

The strain was identified by its biochemical properties as a long filamentous bacterium that is Gram positive and can form chains of globose-shaped, smooth-surfaced spores that are straight to flexuous (rectiflexibile). Fig. 2 displays the phylogenetic tree created with Mega 10.0 software. GenBank received the gene sequence submission and assigned it the accession number MW186183. *Streptomyces* species—catalase-positive, indole-negative, and can utilize glucose as their carbon source. Interestingly, most of the hydrogen sulfide producers came from the dumpsite, which is known to contain alternative sources of nutrients (e.g., food waste, paper, plastic) for microbial use with hydrogen sulfide as an end product (Ko, et al.,2015., Tsuchida *et al*., 2011). In addition, Long *et al*., 2016 reported that the accumulation of hydrogen sulfide may cause an increase in soil pH, which favors the growth of *Streptomyces*. This could likewise explain the abundance of *Streptomyces* isolated from dumpsites as seen in this study. (Wilson *et al.,*2022)

3. Calcium Carbonate Precipitation

By titrating the bacteria-containing soil columns against the control soil column, the percentage of CaCO3 in each was determined (Table 1). In comparison to 15% for the control sample, the CaCO3% generated by the MICP process varied from 15% in Kerala and Tamil Nadu soil to 0% by the microbe. A larger difference in CaCO3 was anticipated given the measured differences in permeability and strength. From a scientific standpoint, this variation indicates that the organism's distribution of CaCO3 production plays a crucial role in the modification of permeability and strength. In order to confirm whether the moonmilk cultivable *Streptomyces* could indeed produce mineral deposits, we selected two phylotype representatives to be first investigated by polarized light microscopy then by ESEM in low vacuum mode for the presence of calcium carbonate precipitates. The selection of strains was based on their predispositions for $CaCO_3$ precipitation as judged by the sum of metabolic performance observed for ureolysis and peptide/amino acid ammonification—the two most significant activities observed for moonmilk *Streptomyces* (Maciejewska, 2017)

Table:1 Effect of Culture Conditions on the production of Calcite

Sno.	Sample	Soil	Temperature			pH			Calcite Precipitation	Percentage
			25	37	40	5	6	7		
1	Tamil Nadu	10g	1.25	0.94	0.72	1.46	0.84	0.76	Pink	15
2	Kerala	10g	0.91	0.63	0.32	0.67	0.54	0.44	No colour change	-
3	Control	10g	1.15	0.96	0.76	0.97	0.46	0.45	Pink	15

Figure:3 Showing the Microbially induced Calcium Carbonate Precipitation

4. Growth kinetics of *Streptomyces sps. KRA01*.

After reaching its peak growth in the nutritional medium at 25oC and pH 5 on the 72nd hour of incubation (OD610 = 1.02±0.052; calcite precipitation: 15%), the calcite-producing strain Streptomyces sps. KRA01 started to decline (Table:2). According to reports by Tian Yan Yunet *et al*., 2018), the growth of the Streptomyces strain was found to occur within a pH range of 6–8, with optimum growth at pH 7 and 28˚C. Actinobacteria, in particular, has been proposed to play a role in the formation of moonmilk deposits by acting as carbonate deposition nucleation sites (Canaveras *et al*., 2006). Classical SEM observations showed the same crystal morphologies that have also been reported in the literature, regardless of the fixation method (glutaraldehyde, ethanol, and freeze-drying) Canaveras *et al*., 1999, Bindschedler *et al*., 2010,

Table :2 Effect of Growth kinetics in *Streptomyces sps. KRA01*.

Temperature (°C)	Growth OD610 nm	pH	Growth OD610 nm	Calcite Precipitation	Percentage
25	1.02±0.052	5	1.23±0.015	Pink colour change	15
30	1.21±0.085	6	0.95±0.011		
37	0.94±0.015	7	0.48±0.38		
40	0.78±0.045	8	0.01±0.01		

Effect of temperature, on growth and production of Biomass by *Streptomyces sps. KRA01*:

Grown at various temperatures ranging from 25 to 40, the strain Streptomyces sps. KRA01 demonstrated optimal growth at pH 30, exhibiting an O.D value of 1.21±0.085 and dry mass production of 3.23±0.208 g/L (Table 3). Related findings were noted by *Streptomyces* isolates from each soil habitat examined in this study. We observed that more *Streptomyces* species were isolated from soil habitats (e.g., dumpsite and garden) at higher altitudes, with slightly acidic to alkaline pH and temperatures ranging from 29–33°C, which is consistent with the reported optimum conditions that support *Streptomyces* growth (Goodfellow,. *et al*., 2012, Barkaet, al,2016). (Jhon.*et al.,*2022)

Temperature (°C)	**Growth OD610 nm**	**Dry biomass (g/l)**
25	1.02±0.052	4.9±0.230
30	1.21±0.085	3.23±0.208
37	0.94±0.015	7.16±0.251
40	0.78±0.045	8.13±0.351

Table 3 Effect of temperature, on growth and production of Biomass by *Streptomyces sps. KRA01*,

5. Effect of pH, on growth and production of Biomass by *Streptomyces sps. KRA01*:

The strain's growth and dry mass production were found to be optimal at pH 1.23±0.015, with a dry mass yield of 8.37±0.208 at pH 7 (Table :4). Ten environmental Streptomyces spp. were grown and sporulated between pH 4.0 and 11.5, at the interval of 1.5, on starch-casein-KNO(3), tryptone-yeast extract-glucose, glycerol-arginine and tryptone-soy agars, and three their modifications.(Kontro,*et al.,* 2005).

Table 4 Effect of pH, on growth and production of Biomass by *Streptomyces sps. KRA01*

pH	Growth OD610 nm	Dry biomass (g/l)
5	0.48±0.38	2.97±0.493
6	0.95±0.011	4.47±0.251
7	1.23±0.015	8.37±0.208
8	0.01±0.01	6.2±0.3

6. Effect of carbon source on growth and production of Biomass by Streptomyces sps. KRA01:

In Streptomyces sps. KRA01, jangery as a carbon source induced a dry mass of 8.83±0.230. When different carbon source concentrations were added to the media, the amount of Jagery that grew and produced dry mass at 183±0.0017 was highest. (Table 5) This result was consistent with the research study conducted by Pridham TG and Gottlieb D 1948, as the strain's wide capacity for carbon assimilation was demonstrated by its use of a variety of carbon sources. Valine, D-galactose, α-Lactose, soluble starch, and anhydrous lactose were among the carbon sources that the strain effectively used. The taxonomic characterization of actinomycetes is significantly influenced by their utilization of carbohydrates. (Yun Tian Yan *et al*., 2018)

Table 5 Effect of carbon source on growth and production of Biomass by *Streptomyces sps. KRA01*

Carbon source	**OD VALUE**	**DRYMASS**
Jangery	183±0.0017	8.83±0.230
Sugarcane	1.33±0.040	8.93±0.251
Tody Juice	0.085±0.0040.	4.27±0.208
Tody Sugar	0.72±0.002	6.53±0.152

7. Effect of Inorganic salt on growth and production of Biomass by *Streptomyces sps. KRA01*

From a variety of inorganic and organic sources the maximum 2.73±0.21 growth was supported by sodium chloride, which was followed by yogurt and calcium carbonate. According to published research, NaCl is the ideal inorganic source for microorganism growth. (Table 6) This outcome is consistent with studies by (M. Bhavana *et al*., 2014), which found that 7.5 g/L of NaCl was the ideal concentration needed to produce antimicrobial compounds. An additional increase in NaCl concentration resulted in a sharp decline in mycelium growth and antimicrobial compound production.

Table 6 Effect of Inorganic salt on growth and production of Biomass by *Streptomyces sps. KRA01*

Inorganic salts	OD VALUE	DRYMASS
Calcium carbonate	0.87±0.06	0.79±0.08
NaCl	2.73±0.21	2.33±0.15
yogurt	3.17±0.25	5.2±0.36

8. Effect of **Nitrogen source on growth and production of Biomass by *Streptomyces spsA01***

Provides a summary of the different nitrogen sources that contributed to the obtained results. The graph showed that while Streptomyces sps. KRA01 could grow on soybean and green gramme, it could not grow on chebulic myrobalan or corn steep liquor. As a result, it was found that the medium with soybean as the sole nitrogen supplement had high growth. The inorganic sources in the medium were found to have a significant impact on Streptomyces sps. KRA01 growth. Out of all the inorganic sources used, yogurt showed the fastest rate of growth. (Table 7)

9. Preparation of bacterial earthen bricks

Bacterial earthen bricks: *Streptomyces sps. KRA01* was injected into nutrient broth containing extra carbon, nitrogen, and inorganic sources. A brick measuring 15x6.8x3.5 cm was made and baked in a kiln at 900˚C to 1200˚C using a 1:1 mixture of clay and bacterial culture at log phase. A control brick was one that was made in a traditional manner. In porous soil, the microbially induced precipitation of CaCO3 is typically achieved by the addition of the exogenous bacterium Streptomyces sps. KRA01. (Figure:4). CaCO3 deposition around the surfaces of the soil particles or in the void spaces causes the porous medium to become clogged, which lowers the porosity of the material. Recent studies have shown that waste products like fly ash, rice husk ash, and waste stone The use of microbially induced calcite precipitation to improve soil shear strength and minimize hydraulic conductivity has been demonstrated. Increased soil strength can help to increase ground bearing capacity, while decreased hydraulic conductivity can help to reduce settlement, shrink well propensity, seepage, and rainfall penetration into soils. MICP was found to be more effective in improving shear strength in residual soil than in sand in the experiments. (Krishna Kumari1 *et al*., 2021).

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