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Optimization of Effective microbes for Soil Stabilization through Bacterial Calcite Precipitation

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Abstract:
Bacterial calcium carbonate(calcite) precipitation is a naturally occurring biological process in which bacteria produce inorganic materials as part of their basic metabolic activities. This technology has been widely explored and promising with potential in various technical applications.Sporosarcinapasturii GRI-AURO, Sporosarcinapasturii, Bacillus subtilisinaquosorum and Bacillus cereus are the bacteria isolated from termite soil and termite gut and identified with accession number. The above mentioned bacteria are ureolytic in nature. These are capable of synthesis calcite crystals and take part in microbial soil stabilization. All morphological and biochemical tests emphasizes the properties of a particularly confined group of bacteria that can act as binding agents. The applications of bacterially produced carbonate biominerals for improving the durability of buildings, remediation of environment (water and soil), sequestration of atmospheric CO_2 etc. are discussed. The study also sheds light on benefits of bacterial biominerals over traditional agents and also the issues that lie in the path of successful commercialization of the technology of microbially induced calcium carbonate precipitation from lab to field scale.

Keywords: *Calcie, Ureolytic bacteria, Biominerals, Soil stabilization*

I. INTRODUCTION

Every microbes are having their functions and showing their identity like any living being in nature. All unique discoveries in Microbiology are the manipulations of microbial functions. As the primitive life in the universe micro-organisms are very related to nature. Everything we are getting from nature so we ought to give something back to nature. Environmental Microbiology is related with the microbes useful to the environment and bioremediation became an established solution for all environmental threat.

This research introducing you a group of bacteria that are capable of stabilize the soil and lead to the production of Ecobricks. Microbes especially bacteria have precipitations and secretions as aresult of metabolic activities. We can stimulate these kind of precipitations and lead to effective products. That is what happening in some soil microflora and termite microflora. Termites are nature's ecosystem engineers and well known for earthen construction. Bacteria that inhabit the termite soil, termite gut and mouth parts playing an important role by acting as the binding agent. The idea of isolating bacteria from termite mounds was that the termites are effective agents in consolidating soil and making a hard cemented soil similar to bricks. Termites mix the soil with saliva and make smalls of mud and start constructing their home. They also engulf the soil and excrete the soil.

The bacteria, got transferred from termite to the soil and start precipitating calcium carbonate($CaCO_3$). Only ureolytic bacteria can produce $CaCO_3$ with the help of urease enzyme. Calcium carbonate also known as Calcite. The presence of calcite can be seen in the pores between soil particles through Scanning Electron Microscopy (SEM) and other physico chemical parameters.

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Calcium carbonate Precipitation

$CO(NH_2)_2 + 3\ H_2O \rightarrow 2NH_4^+ + HCO^{3-} + OH^-$

(Urea) (Ammonia) (Bicarbonate)

$Ca^{2+} + HCO^{3-} + OH^- \rightarrow CaCO_3 + H_2O$

(Calcium)(Bicarbonate) (Calcium carbonate)

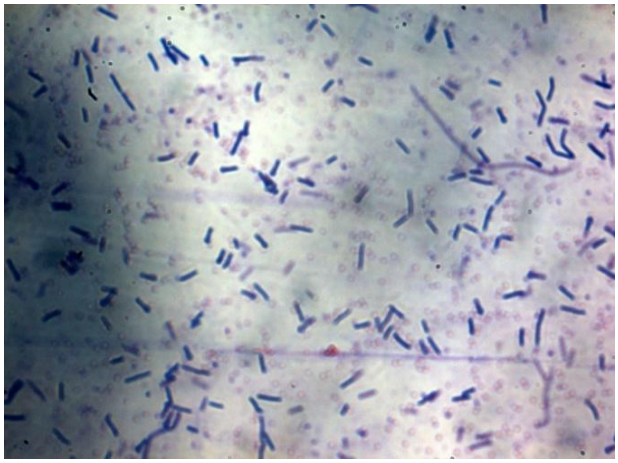
Precipitation of calcium carbonate crystals occur by heterogeneous nucleation on the bacterial cell wall. Once super saturation is achieved and these crystals precipitate inside the pore space. The bacteria not only initiate calcite precipitation, but also serve as nucleation sites for calcite crystals in association with other factors such as Ca+ ions, dissolved inorganic carbon PH and temperature in the medium. These carbonates/ calcite crystals act as cloggers in the building materials by filling the voids and thereby reducing permeability.

The objective of this paper is to evaluate the potential of bacterial carbonates/ calcite crystals as cementing/stabilizing agents to produce low energy, low CO_2 emitting, green building materials i. e. soil blocks. The effect of bio-cementation by bacterial calcite on varying densities of soil has also been checked in order to find out an optimal combination.

II. Review

In this research, urea hydrolysis was not used to induce calcium precipitation. The precipitation was done by the hydrolyzing urea, which produced ammonium and bicarbonate ions, thereby increasing the pH (Fujita et al 2008).

The bacteria can be found naturally; however, these microbial communities need to be augmented to support microbial induced calcite precipitation (MICP) in the field. If there are few ureolytic bacteria in the subsurface, then it has to be augmented with nutrient cycles. (Dejong et al 2010)



Calcium carbonate has three polymorphs: calcite, aragonite and vaterite. Calcite is the stable form under ambient conditions and aragonite is the high pressure polymorph. Vaterite is thermodynamically unstable. Although the preparation of calcium carbonate has been described many times in the chemical and geological literature, the synthesized products have not been well defined yet and their occurrence varies considerably with experimental conditions. Among these main studies, only Wary and Daniels detailed the description of experimental factors when synthesizing calcium carbonate in a direct inorganic precipitation method using soluble carbonate and calcium salts as initial materials. In this research also concluded with the microbial precipitation of aragonite and vaterite by confirming their morphology through SEM.

Aragonite is a carbonate mineral, one of the two most common, naturally occurring, crystal forms of calcium carbonate,$CaCO_3$ (the other forms being the minerals calcite and vaterite). It is formed by biological and physical processes. *Vaterite* is a mineral, a polymorph of calcium carbonate ($CaCO_3$). It was named after the German mineralogist Heinrich Vater. It is also known as mu-calcium carbonate (μ-$CaCO_3$). Vaterite belongs to the hexagonal crystal system, whereas calcite is trigonal and aragonite is orthorhombic. Vaterite, like aragonite, is a meta stable phase of calcium carbonate at ambient conditions at the surface of the earth. As it is less stable than either calcite or aragonite, vaterite has a higher solubility than either

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of these phases. Therefore, once vaterite is exposed to water, it converts to calcite (at low temperature) or aragonite (at high temperature: ~60 °C).

Bacterial calcites are filling the pores and binding the soil particles. Soil blocks production by compaction with suitable molds and machines would be a better example of eco-friendly building materials.

III. Experimental Studies

A. *Isolation of termite soil microorganisms*

The sources of microbes are termite soil and termite's body parts. Different geographical areas of Gandhigram were selected for the sample collection with the help GPS. The samples were transported aseptically to the lab by using sterile NascoSampling bags. Samples were dissected and homogenized by using sterile lab wares. Sampling was done in triplicate for accuracy. Eight isolates were specifically isolated and preserved for long term usage.

- In primary screening, eight isolates were selected and purified and preserved
- In secondary screening, four different strains were selected out of eight strains.
- Finally the strains were isolated based on the productivity of urease enzyme.

B. *Gram staining*

Gram staining was done with the isolates to determine the morphological features like form, size and Gram reaction. The colony characteristics and the gram reaction of the isolates were identified under the microscope. Four strains (TIS WS4, TIS CS3, TB1 and tb2) are gram positive.

Figure 1: Microscopical observation of Gram stained isolated strain

C. *Spore staining*

From eight isolated strains four strains (TISWS4, TISCS3,TB1 and TB2)are spore forming bacteria. During unfavorable conditions bacteria will produce spores and become inactive. Sporulation is necessary during the formation and optimization of bacterial solution

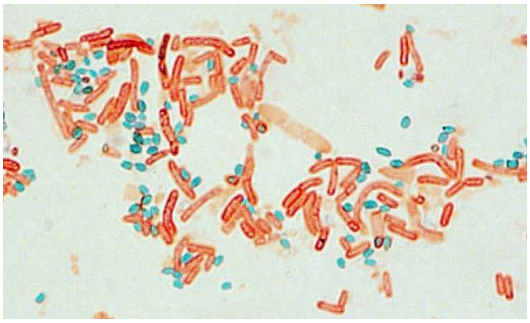

Figure 2: Microscopic observation of spore stained isolated strain

D. *Urease activity*

Urease agar with Phenol red indicator was used to test the urease activity. Among eight samples four are urease negative four are urease positive. In the case of positive tubes, original color of the medium was yellow, it change to pink in color .The color changes indicate the presence of urease. The urease enzyme inside the bacteria split the urea into ammonia and carbonate. The cell calcium combined with carbonate and form calcium carbonate (calcite).

Figure 3: Urease activity

TABLE I Urease activity of isolated bacterial strains

Colony	Urea Test
TIS WS4	Positive
TIS CS3	Positive
TB1	Positive
TB2	Positive

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E. Urease Assay

Urease the secretory enzyme inside the bacteria splits urea and liberating ammomia(NH_3) and Carbon di Oxide(CO_2). The reaction is stochiometric and enyme activity is easily determined by measuring the amount of ammonia formed and can be observed colorimetrically since NH_3 forms a brown complex in the presence of nessler's reagent (K_2HgI).

Standard Graph

A pure ammonium sulfate solution (20mg/100ml) is prepared and different aliquots of this solution are taken and the volume is made up to 30 ml with distilled water.1ml of Nessler's reagent is added. After mixing the color intensity is measured at 500 mm. A standard graph is drawn in the usual way.

Enzyme source

Horse gram seeds contain a high concentration of the enzyme. The seeds are powdered in pestle and motor and about 1 gm of finely grinned powder is suspended in 100ml of distilled water and stirred well. This suspension is filtered through coarse cloth and filtrate is used as the enzyme source.

Enzyme assay

Pepette out 1ml of substrate solution i.e 3% urea solution buffered with 1ml of 0.2M phosphate buffer PH7 add 1ml of enzyme extract and incubate 55^0C for 15 minutes. At the end of incubation time quickly place the tubes on the ice. Add 1ml of H_2SO_4 to stop the reaction and 1ml of 1M sodium tungstate solution to precipitate the protein. Filter (or) centrifuge to assay for NH_3 and the enzyme activity is calculated.

TABLE II Standard table of urease assay

	De-ionized water	Ammonium standard (nmol/mg)	NCR	O.D
Std blank	1.00	0.00	5. 00	0.00
Std 1	0.80	2.00	5. 00	0.19
Std 2	0.60	4.00	5. 00	0.42
Std3	0.40	6.00	5. 00	0.62
Std4	0.20	8.00	5. 00	0.85
Std5	0.00	10.00	5. 00	1.00

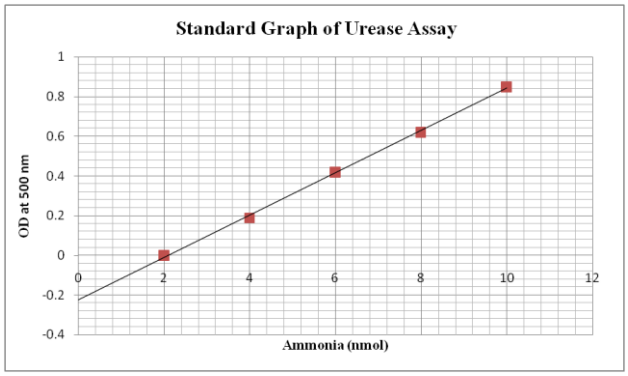

Figure 4: Standard graph of urease assay

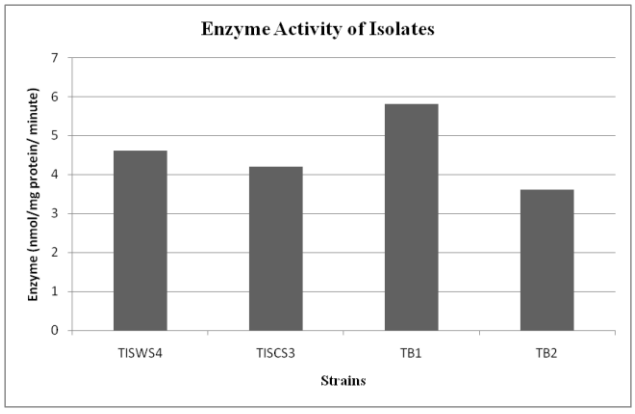

Figure 5: Enzyme activity of isolates

From the graph we can analyze that the isolated strains are producing urease enzyme. The presence of ammonia indicated the lysis of urea into ammonia

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and carbonate. The quantity of ammonia [nmol] depends on the quantity of urease secreted by the bacterial strain. TB1 bacterial strain showed the highest activity of urease enzyme. The bacteria will precipitate Calcite by stimulating the production of urease.

F. Haemocytometer

Optimized bacterial consortium is the final product. So, it is very important to find out how much is the cell count per 1 ml of bacterial solution. Haemocytometer is used to estimate the total cell count. It is also known as Neubauer chamber.

Calculation

Number of cells /ml =

$$\frac{\text{Number of cells counted X Dilution factor}}{\text{Squares counted}}$$

$$= \frac{65X10^3}{5}$$

$$= 13 \text{ X } 10^3 \text{ cells/ml}$$

The given bacterial culture [TSS1] contains = 13 X 10^3 cells/ml

TABLE III

Cell count of isolated bacterial strains

Colony	Cells/ml
TB1	13x10^3 cells/ml
TB2	14x10^3 cells/ml
TIS WS4	15x10^3 cells/ml
TIS CS3	12x10^3 cells/ml

G. Optimization of media

It is indeed interesting to observe that every organic material can be a good media for microbial growth. So the microbe especially bacteria would grow in the organic material and stabilize the soil. Hence it is called bacterial soil stabilization.

Media and different cultural conditions are optimized under standard condition with microbial consortium. The media showed maximum biomass production and growth under specific carbon and nitrogen requirements. The medium with carbon source i.e.; Jaggery and nitrogen source Egg white showed maximum activity and biomass productivity. *Terminaliachebula*is used as a stabilizing agent. Green gram is also used as the nutrient source. All other carbon and nitrogen sources were showed diverse biomass production. Apart from all Optimization of media lead to maintain the cost benefit ratio.

TABLE IV

Enumeration bacteria in different organic materials

Sl No	Microbial Consortium in Different Organic materials	CFU/ml
1	Jaggery	10^3
2	Green Gram Flour	10^6
3	Egg White	10^6
4	*Terminaliachebula*	10^5
5	Jaggery+ Green gram+ egg white+ *Terminaliachebula*	10^9

H. Centrifugation of bacteria

Bacterial centrifugation method is used to extract the calcium carbonate from the bacteria. This method is used to test the ability of bacteria produce calcium carbonate.Secondary metabolites produced by bacteria are collecting through centrifugation with TCA (Trichloro Acetic Acid) and cold ethanol. The

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white semisolid component contains calcium carbonate. 7 grams of calcite collected from 50 ml of bacterial solution.

Figure 6: Secondary metabolite

Figure 7: precipitation settlement

Secreted by bacteria in the bottom of conical flask

Figure 8: Bacterial solution

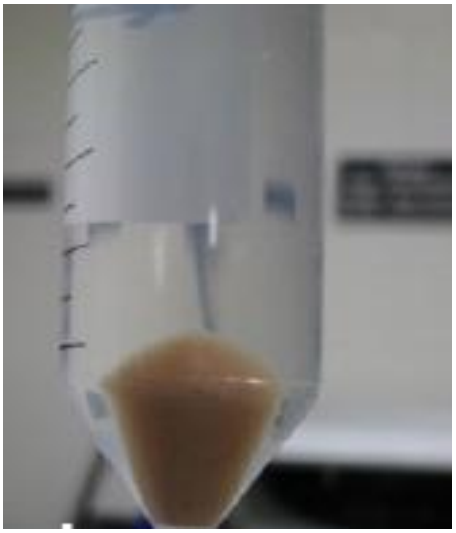

Figure 9: Pellet after centrifugation

Physico- Chemical Analysis

I. Scanning Electron Microscopy [SEM] and Enegy Dispersive X-Ray analysis [EDAX]

Four urease positive strains [TIS WS4, TIS CS3, TB1, TB2]among the eight isolated strains were observed for morphological analysis in Scanning Electron microscopy.

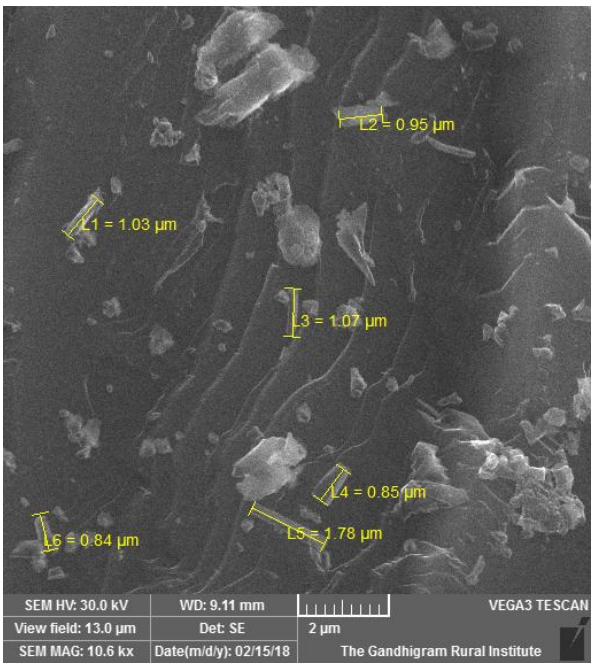

Figure 10: SEM image of TIS WS4

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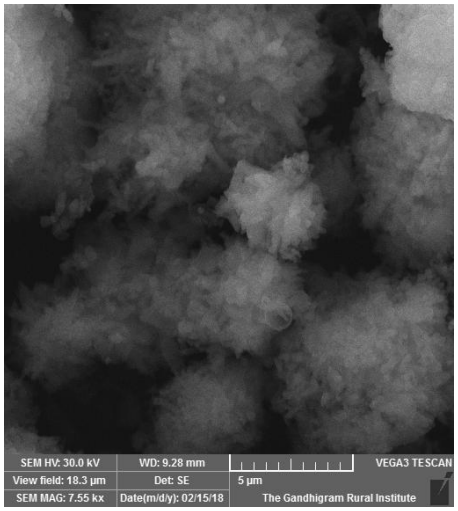

Figure 11: SEM image of TIS CS3

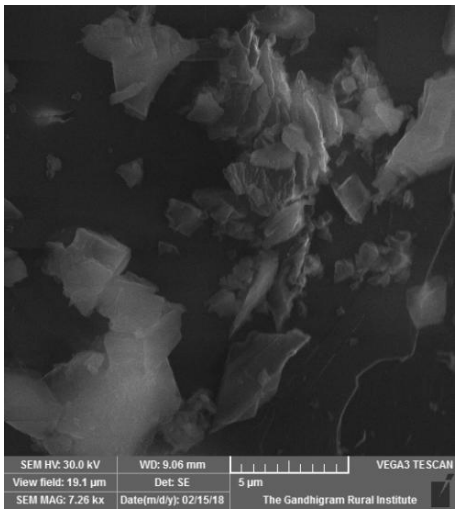

Figure 12: SEM image ofTB1

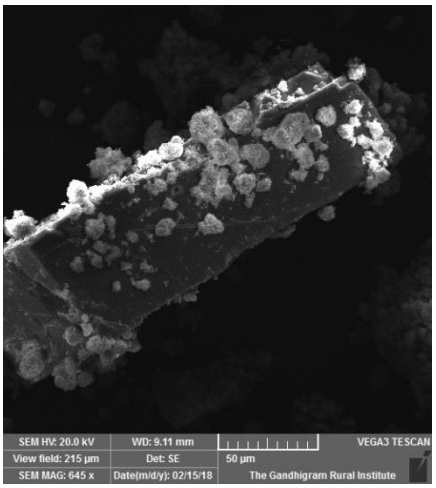

Figure 13: SEM image of TB2

TS3 and TB2 are a particular strains of bacteria that can precipitate polymorphs of calcite[calcium carbonate crystals]. TS3 precipitated aragonite and TB2 precipitated vaterite.

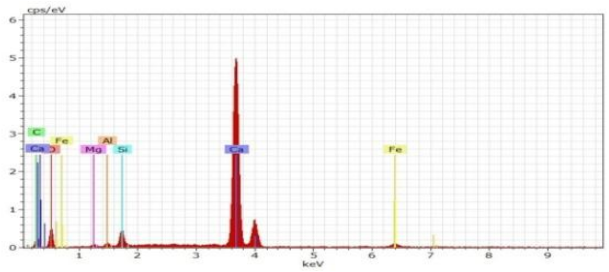

Figure 14: EDAX of TIS WS4

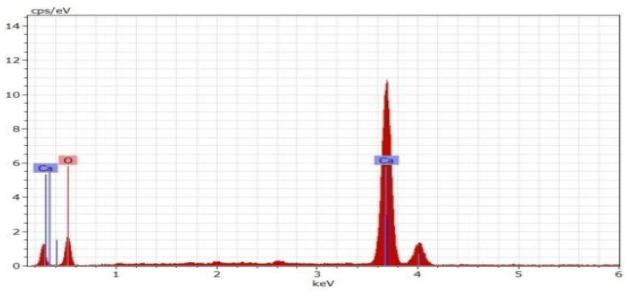

Figure 15: EDAX of TIS CS3

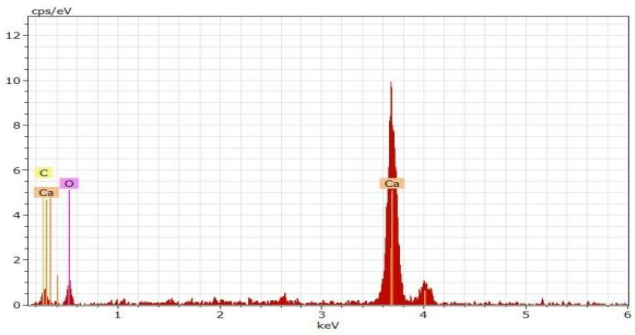

Figure 16: EDAX of TB1

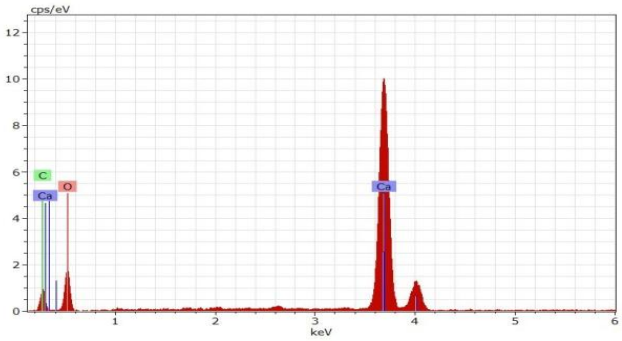

Figure 17: EDAX of TB2

Caaccumulations by microbial action lead the formation of mineral deposits such as calcium carbonate or calcium silicate. The involvement of bacteria in calcite bio mineralization was very evident as bacterial cells in close contact with calcite crystals were visible. Rod shaped impressions of bacterial cells within calcite crystals proved that they had been occupied by bacteria at some stage of

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crystallization or the cell had completely colonized by the crystals. The presence of calcite associated with bacteria proves that bacteria served as nucleation sites during the mineralization process. Point EDAX scanning was analyzed and confirmed the presence of $CaCO_3$, calcium silicate and other minerals.

J. Fourier Transform Infra Red Spectroscopy

7 grams of precipitate(dried) got from 50 ml of bacterial consortium. The C=O bond of carbonate indicates the presence of calcium carbonate (Presence of calcium already confirmed by EDX). The C=O bonds (of carbonate group) would exhibit in-plane bending and out-of-plane bending about 1415,1487,1434 and 1460 cm-¹. The FT-IR spectroscopy is a simple and accurate technique to identify and quantify $CaCO_3$ polymorphous. Due of their crystalline structure, they can be discriminated by FT-IR; a different spectrum is observed for each of the structural forms.

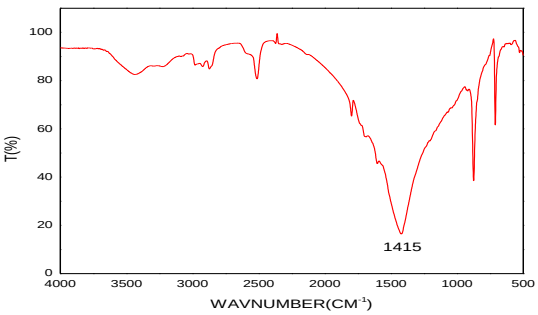

Figure 18: FT-IR of TIS WS4

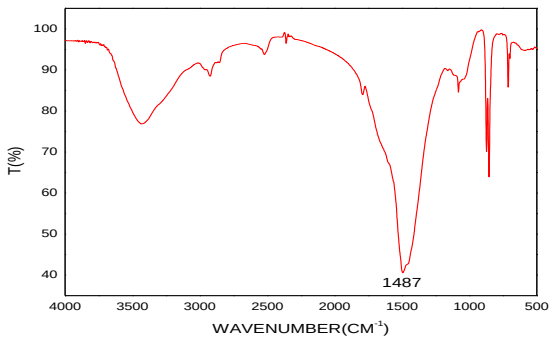

Figure 19: FT-IR of TIS CS3

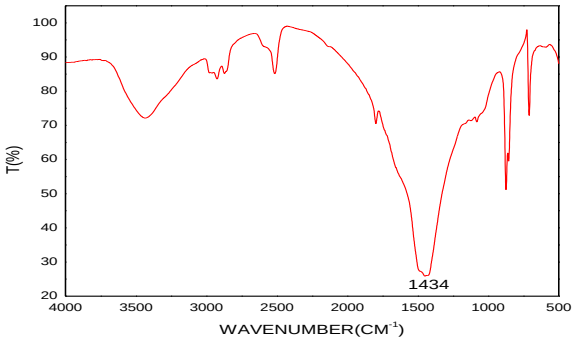

Figure20 :FT-IR of TB1

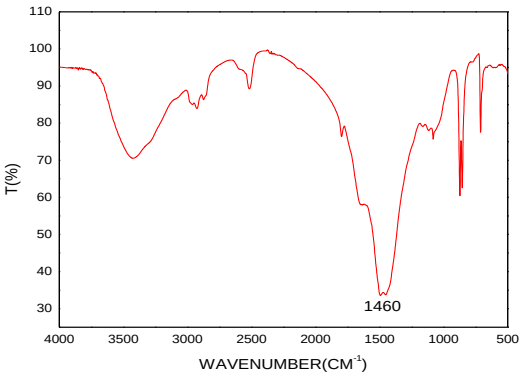

Figure21:FT-IR of TB2

The FT-IR spectra obtained from the samples of pure commercial calcite indicated the in-plane bending vibrations of the carbonate group with wave numbers 1415, 1487, 1434 and 1460 cm-¹ respectively. Hence it confirmed the precipitation of $CaCO_3$.

K. DNA sequencing

The selected organisms were dispatched to the YAAZH XENOMICS Pvt. Ltd. for the sequencing of its DNA. The Quality and purity of the culture were analyzed through the conventional microbial methods.

The amplified DNA sample was sequenced by Applied Bio System Sequencer. The sequenced DNA was further treated with Applied Bio System Sequencer Scanner V1.0 software and the organism was identified by using BLAST at NCBI. The representative 16S rRNA sequences were submitted to the NCBI review panel for getting GenBank accession number by using Bank It tool.

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There are four strains of bacteria that are accepted by The National Centre for Bioinformatics USA, (NCBI) and got accession numbers. All the microbiological and physico-chemical parameters have confirmed the potential of these four bacteria to precipitate Calcite crystals.

This bacterial calcite can be further undergoing through engineering parameters to alter the mechanical properties of soil. This will lead to a new strategy to restore earthen constructions.

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TABLE V

Identification of bacteria (16s rRNA Sequencing)

Sl. No	Accession No	Organism	Title	Authors & Year
1	KP938774	*Sporosarcinapasteurii* strain GRI-Auro	A study on the mechanical properties of microbial stabilized Compressed earth blocks (MSCEB)	Haris,P., Arya,F.C., Joseph,A., Fazil,S., Satprem,M., Lara,D. andAbraham,RD.,2015
2	KSACF2	*Sporosarcinapasturii*	Optimization of Organic Media for microbial Soil Stabilization	K.Suvetha, Arya C F and David A R, 2017
3	KSACF1	*Bacillus subtilisinaquosorum (Sub Sps)*	Optimization of Organic Media for microbial Soil Stabilization	K.Suvetha, Arya C F and David A R,2017
4	KX926491	*Bacillus cereus*	Application of *Bacillus subtilis* to geotechnical engineering for biomineralizatin and stabilization of soil	Arya,C.F.,Vignesh,N.P,Mahendra n,K. and David,A.R, 2016

This bacterial calcite can be further undergoing through engineering parameters to alter the mechanical properties of soil. This will lead to a new strategy to restore earthen constructions.

IV. Conclusion

Sporosarcinapasturii GRI-AURO, Sporosarcinapasturii, Bacillus subtilisinaquosorum and Bacilluscereus are the bacteria isolated from termite soil and termite gut and identified with accession number. The above mentioned bacteria are ureolytic in nature. These are capable of synthesis calcite crystals and take part in microbial soil stabilization. All morphological and biochemical tests emphasizes the properties of a particularly confined group of bacteria that can act as binding agents. It is hoped that the study would contribute some highlights towards an eco-friendly, energy efficient, durable and low cost building material.

This paper provides the way for manufacturing bricks using bacteria. Even though the bacteria can be used for many other purposes, now it is proved that it can be used in the field of civil engineering through the manufacture of bricks. The microbial

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perceptive of earthen construction is nothing but the bacteria producing a bio cement to bind soil particles and fill in between the grains of sand. This process reduces carbon dioxide emission compared to the port land cement production. If you manufacture one ton of cement then you inject one ton of carbon dioxide to the atmosphere. Anyway microbial soil stabilization suggests a comfortable zone of habitat related to nature. For that we should make our soil strong and efficient through potential effective micro-organisms.

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