



## Studies on isolation and characterization of calcium carbonate bio-precipitating bacteria

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### Abstract

A variety of bacteria involved in precipitation of calcium carbonate in nature and *in vitro* methods. The present work involves isolation of bacterial strains, morphological studies, characterization, calcium carbonate precipitation and studies on shrinkage properties. The soils collected from construction site and bacterial strains were isolated by soil dilution method using calcium carbonate precipitating medium (CCPM). The selected colonies were identified by morphological and biochemical tests. The urease activity of selected strains along with metal ion concentration also determined. The precipitated calcium carbonates were quantified, characterized by SEM and FTIR. The specific gravity of soil solids determined by pycnometer. The shrinkage factors of soil like shrinkage ratio, shrinkage limit and volumetric shrinkage compaction properties were studied by standard methods. Of the total nine bacterial strains four strains (marked as isolate 1, 5, 6, 9) were selected for characterization and further studies. Among this two are Gram positive and other two are Gram negative. On morphology 3 are rods and one is coccus. They were also showed varied level of tolerance to Ni and Co. Among them isolate 5 showed highest urease activity followed by isolate 1 and so on. Isolate 5 also showed 2.34g/100 ml bio precipitation of calcium carbonate followed by 1.94g by isolate 9, 1.85g by isolate 6 and 1.42 by isolate 1. The crystal size varied from 1.08-4.61  $\mu\text{m}$ . FTIR analysis showed the highest peak at 1421-1424 for all isolates including control. The peaks located at 710 $\text{cm}^{-1}$  and 876 $\text{cm}^{-1}$  correspond to the in-plane bending and out-of-plane bending modes of  $\text{CO}_3^{2-}$ ,  $^{2-}$  and they are characteristic of calcite. The peak at 1421  $\text{cm}^{-1}$  is assigned to the asymmetric C-O stretching vibration. A weak peak at  $\sim 2514 \text{ cm}^{-1}$  is also characteristic of calcite. Isolate 6 showed higher specific gravity and isolate 9 soil shrinkage properties. © 2019 ijrei.com. All rights reserved

**Keywords:** Bacterial isolates, Bio precipitation, Calcium carbonate precipitation, FTIR, SEM, Shrinkage properties

### 1. Introduction

Calcium carbonate is a common substance found in rocks as the minerals calcite and aragonite and is the main component of pearls and shells of marine organisms, snails, and eggs. Calcium carbonate is the active ingredient in agricultural lime and is created when calcium ions in hard water react with carbonate ions to create lime scale. It is medicinally used as calcium supplement or as an antacid, but excessive consumption can be hazardous.

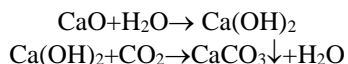
As some of the most abundant minerals on earth, carbonates are ubiquitous and highly reactive component of natural environments. Carbonate mineral plays important roles in global carbon cycling, alkalinity generation, cycling of major and trace elements, and transfer of matter among the oceans, the continents, and the atmosphere.

Calcium carbonate exists in a variety of polymorphs, including

calcite, aragonite and vaterite which differ in their crystal structure. Calcite, the thermodynamically stable form, exhibits hexagonal rhombohedral crystal structure; aragonite, a metastable crystalline form displays orthorhombic crystal structure, often appearing as needles; and vaterite, another metastable crystalline form, displays hexagonal structure, commonly exhibiting spherulitic or disc like precipitates (Chakraborty et al., 1994). Most research to date has focused on microbial precipitation of aragonite and calcite (Krumbein and Giele, 1979; Buczynski and Chafetz, 1991). Precipitation of vaterite to aragonite and calcite, has also been reported (de Leeuw and Parker, 1998). The different polymorphs are well known to have different stabilities (Chakraborty et al., 1994; de Leeuw and Parker, 1998).

Chemically it is produced by reacting quick lime with water to give calcium hydroxide, which is in turn treated with carbon

dioxide to precipitate the calcium carbonate salts.



Bacteria are ubiquitous in every habitat on earth, like growing in soil, acidic hot springs, radioactive waste water. Hence these are widely used for the production of any important materials and also for the bioconversion. There are three main groups of microorganism that can induce the carbon precipitation. They are 1) Photosynthetic microorganism such as cyanobacteria and microalgae, 2) sulphate reducing bacteria and 3) microorganism involved in nitrogen cycle.

Microbial degradation of urea is a potential geochemical catalyst for calcium carbonate precipitation. Hence the importance of bacteria in mineral precipitation is becoming increasingly appreciated in the geosciences. Microbially mediated precipitation of a variety of important geochemical mineral phases has been reported in the literatures of the environment (Sunda and Huntsman, 1987; Beveridge, 1992; Mandernak et al., 1995; McLean et al., 1996; Mojzsis et al., 1996; Beard and Johnson, 1999).

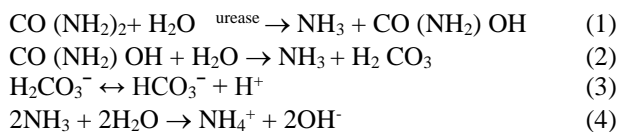
### 1.1 Bio-precipitation mechanism

Bio-precipitation occurs as a by-product of the microbial metabolism.

- Extracellular molecules are involved in the carbonate mineralization process.
- A nucleation process of carbonates occurs in the cell wall of microorganisms.

The bacterial surface plays an important role in the precipitation of carbonates due to the presence of various negatively charged groups at neutral pH, positive ions can bind to the bacterial surface favoring nucleation (Dhami et al., 2013).

This method basically consist of the microorganism's ability to alkalyze the surrounding environment according to the physiological activities they perform. These bacteria are widely distributed in the nature and their role is to catalyze the hydrolysis of urea to produce carbonic acid and ammonium (Philips et al., 2013). These products, in solution, have as final result to induce a change of pH in the medium (Al-Thawadi, 2014).



The increase in pH leads to an adjustment of the bicarbonates equilibrium to form carbonates ions, further favouring the formation of  $\text{CO}_3^{2-}$  from  $\text{HCO}_3^-$  (Knoll, 2003). A high carbonate concentration induces  $\text{CaCO}_3$  precipitation around the cells and the presence of calcium ions in the surrounding environment. Equations 5-7 (Kumar et al., 2016).



Under natural conditions, the precipitation of carbonates occurs very slowly. Microorganisms would act as a catalyst in the carbonate formation process.

The urease enzyme is present in a great diversity of microorganisms (Anbu et al., 2016). Enabling the cell to use urea as a source of nitrogen (Burbank et al., 2012). The ureolytic bacteria are mostly used as an example of microbial induced calcium carbonate precipitation (MICP) and present an active intracellular urease (Dejong et al., 2011). The process of urease aided calcium carbonate precipitation is triggered by the catalytic action of urease in the hydrolysis of urea. The products of the reaction are carbonic acid and ammonia. The released ammonia can be used as a parameter to check the amount of urease activity exhibited by microorganism.

The performance of coupled biogeochemical systems can be severely affected by the presence of heavy metals. It is thus important to study the interactions among heavy metals and native organisms. Heavy metal toxicity to microorganisms has been assessed in a number of studies (Gikas et al., 2008). In general, it has been shown that the presence of heavy metals can inhibit or cease microbial growth (Madoni et al., 1996; Sani et al., 2003). However, small amounts of selected heavy metals may act as growth stimulants (Burgess et al., 1999; Gikas, 2006, 2009). This is because some heavy metals, at low concentration, are utilised by microorganism in various biochemical pathways as enzymes cofactors, usually in the form of metalloproteinase (Gadd, 1993; Ji and Silver, 1995). The effects of heavy metals on microorganism depend on the type (Mowat, 1976), speciation (Sandrin and Maier, 2003; Gikas et al., 2006) and concentration (Vankova et al., 1999) of the metal and type of microorganisms (Babich and Stotzky, 1982).

They may be coupled on the microbial membrane, causing irreversible damage such as loss of membrane integrity (Cervantes and Gutierrez-Corana, 1994; Stohs and Bagchi, 1995). Absorbed in the cytoplasm oxidising vital enzymes or inactivating microbial organelles (Peitzsch et al., 1998) or affect the genetic material of the microbial cell by reacting directly with DNA (Morby et al., 1993).

### 1.2 Scope of work

The evidence of microorganism involvement in carbonate precipitation, has lead the development of bioprocess technology in the field of construction material (De Muyneck et al., 2010). The amount of organic matter in the soil significantly affects its geotechnical properties, including specific gravity, water content, liquid limit, plastic limit, bulk density and shrinkage limit. In the past few years, the use of bacterial calcium carbonate precipitation (BCCP) has become popular as a ground improvement technique. It is presented as a new and environmentally friendly method (Dejong et al., 2006). This new method has advantage over conventional chemical treatments, which can be toxic and environmentally harmful and have a

limited injection distance (Karol, 2003). The method is also cost effective in comparison to chemical treatments (Ivanov and Chu, 2008).

Gollapudi et al. (1995) used microbial mineral plugging to reduce the porosity of rock fractures. Dejong et al. (2006) demonstrated that microbially cemented specimens exhibited increase in the strength of sandy soil. Whiffin et al. (2007) examined the effect of microbial carbonate precipitation on the permeability and shear strength of sandy soil. In all the previous studies BCCP technique was an attempt to improve the geotechnical properties of granular soil and successful results were obtained.

Objectives of the study include isolation and characterization of calcium carbonate precipitating microorganism, evaluation of metal ion concentration on selected bacterial isolates and bioprecipitation of calcium carbonate for texturing soil character enhancing property by isolates.

## 2. Materials and methods

### 2.1 Collection of samples

The non-rhizospheric soil samples were collected from construction site near Alva's College campus, Vidyagiri, Moodbidri.

### 2.2 Media for isolation of microorganism

The calcium carbonate precipitating organisms were isolated from dry non-rhizospheric soil samples by using CPM media (Sensoy et al., 2016). The pH of medium was maintained at 7.5 (Table 1).

Table 1: Composition of CPM agar Medium

Ingredients	g/litre
Urea	20.0
Sodium bicarbonate	2.12
Ammonium chloride	10.0
Nutrient broth	3.0
Calcium chloride	25.0
pH	7.5 – 8.0

One gram of soil sample was dissolved in 100 ml of sterile distilled water. Followed by one ml of soil suspension from above sample was serially diluted from  $10^{-1}$  to  $10^{-7}$  using sterile distilled water. Then 0.1ml of each dilution sample was taken and inoculated by pour plate technique on calcium carbonate precipitating medium pH was maintained at 7.5 to 8.00. The plates were incubated at room temperature. Bacterial colonies were observed every 5 days with stereo microscope at regular intervals until the crystal formation around the colonies. The crystalline colonies were further sub cultured on CP medium.

### 2.3 Identification of the microorganism

Microbial isolates were identified using cell morphology by Gram's staining and characterized by biochemical tests like Methyl-Red (MR) test, Urease test, Citrate utilization test, Starch

solubilizing test, Indole test and Lipase test. Evaluation of metal ion concentration on bacterial biomass culture by the procedure of Chaurasia et al. (2014), urease activity of bacterial strains by standard protocol.

### 2.4 Bio-precipitation of calcium carbonate by bacterial strains

The bio-precipitation of calcium carbonate was done by calcium carbonate precipitating broth (CCP broth) which consists mainly of calcium chloride, sodium bicarbonate, ammonium chloride, nutrient broth powder, urea and pH maintained at 7.5-8.0. Further these broths were inoculated with respective isolates and incubated for 7 days at room temperature on rotary shaker. A control was maintained. After 7 days the broth was washed with ethanol and filtered using Whatman No. 1 filter paper and weighed.

### 2.5 Characterization of precipitated calcium carbonate

The calcium carbonates precipitated were further characterized by scanning electron and Fourier transition infrared microscopy.

#### 2.5.1 Tests for checking the property of soil after treating it with bacteria

The dry soil samples were collected from Moodbidri near Alva's College. The soil samples were inoculated with the different isolates and incubated for seven days, to conduct the soil property tests after treating it with bacteria.

Specific gravity of soil of solids was determined using Pycnometer for coarse-grained soil or specific gravity bottle for fine-grained soil. The value of specific gravity helps in identification and classifying the soil type and also gives an idea about the suitability of the soil as a construction material. Higher value of gravity indicates more compactness and gives more strength for roads and foundations. It is also used in the critical hydraulic gradient in soil when a sand boiling condition is being studied and a zero air-void calculations in the compaction theory of soils. Shrinkage factors of soil like shrinkage limit, shrinkage ratio and volumetric shrinkage determined by shrinkage dish method and compaction properties of soil using Standard Proctor test.

## 3. Results

### 3.1 Isolation of bacteria

The bacteria were precipitated from dilution of  $10^3$  and  $10^4$ . Further the purified colonies were also found to be precipitated on calcium carbonate medium. Altogether nine bacterial colonies were isolated from the soil sample, out of nine; four strains were selected based on their culture/growth on the media. These four strains viz., 1, 5, 6 and 9 were later used for characterization and for further studies (Plate 1, 2).

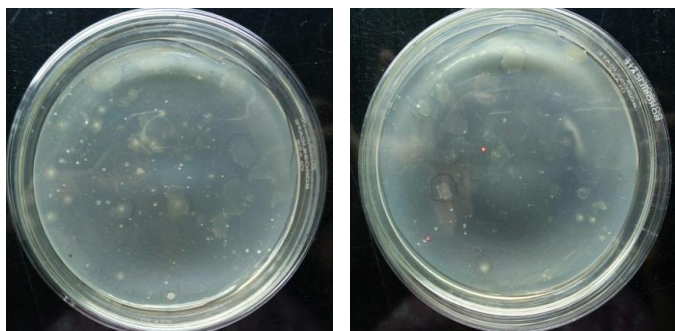


Plate 1: Bacterial CaCO<sub>3</sub> colonies precipitated on master plate



Plate 2: Purified precipitated CaCO<sub>3</sub> by bacterial isolates

### 3.2 Identification bacteria

The bacterial isolates were identified by observing their cell morphology and biochemical analysis. The cell morphology of isolate 1 was +ve rod, isolate 5 was +ve cocci, isolate 6 were -ve rod and isolate 9 was -ve short rod. The Gram’s reaction for isolates showed positive results. The results of biochemical tests also presented in table 2.

Table 2: Morphology and biochemical characters of different bacterial isolates

Morphology/biochemical	Isolate 1	Isolate 5	Isolate 6	Isolate 9
Gram’s reaction	+ve	+ve	-ve	-ve
Shape	Rod	Cocci	Rod	Short rod
Urease hydrolysis test	+ve	+ve	+ves	+ve
Starch hydrolysis test	+ve	+ve	+ve	-ve
Citrate utilisation test	+ve	+ve	+ve	+ve
Indole test	+ve	+ve	+ve	+ve
Methyl red test	+ve	-ve	-ve	-ve
Lipase test	-ve	-ve	-ve	-ve

### 3.3 Evaluation of metal ion concentration on bacterial biomass

Nickel ammonium sulphate and cobalt chloride of varying concentrations from 1000µl, 500µl, 100µl, and 10µl were added to urea broth for evaluating the metal ion effect on bacterial biomass for different isolates. The lowest concentration of nickel that is 10 µl was found to be most favorable for the biomass production of the bacterial isolate (isolates- 1, 5, 6, 9). Whereas in case of cobalt there was varying results for different bacterial strains. The concentration of 10µl was found to be more favorable for isolates 1 and 5. Concentration of 500µl for isolate 6 and 100µl for isolate 9 (Fig. 1, 2).

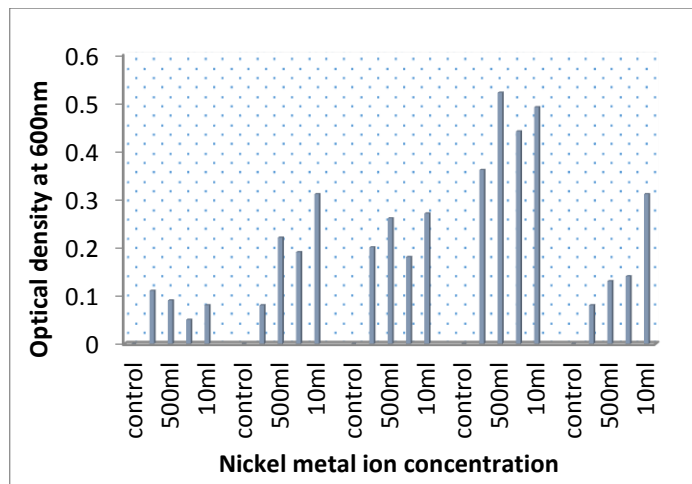


Figure 1: Effect of nickel metal ion on different bacterial biomass

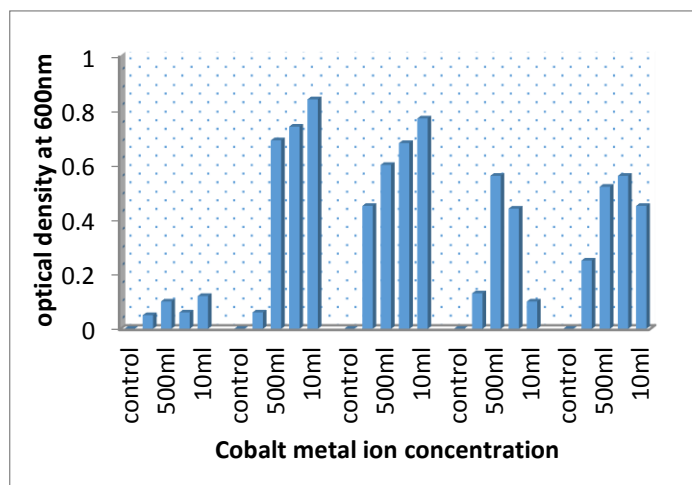


Figure 2: Effect of cobalt metal ion on different bacterial biomass

### 3.4 Estimation of urease enzyme activity of bacterial isolates

The ability of bacterial isolates to produce urease was evaluated. Urease activity of bacterial isolates was found to be more as compared to control. Highest activity was exhibited by isolate 5 followed by isolate 1, 6 and 9 respectively (Fig. 3).

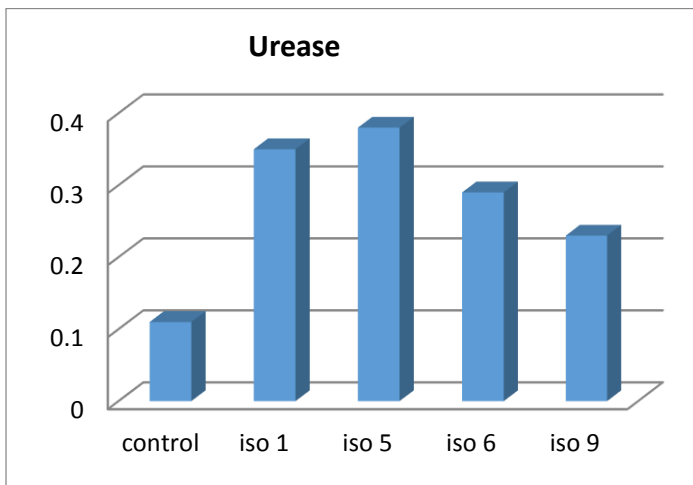


Figure 3: Urease enzyme activity of bacterial isolates

### 3.5 Bio-precipitation of calcium carbonate

The different isolates were inoculated in to the urea broth to check their efficiency for precipitation of calcium carbonate. Isolate 5 precipitated 2.34 g of calcium carbonate followed by isolate 9 1.94gms/100ml, isolate 6 1.85gms, isolate 1 1.42gm which were higher than the control (Table 3, Plate 3).

Table 3: Calcium carbonate precipitated by different isolates in urea broth

Isolates	Weight (g)
Control	0.86
Isolate 1	1.42
Isolate 5	2.34
Isolate 6	1.85
Isolate 9	1.94



Plate 3: Broth with isolates of bacterial culture and respective precipitated carbonates

### 3.6 Characterization of precipitated calcium carbonate

Calcium carbonate crystals were precipitated by all the bacterial isolates. The crystal of isolates were found to be larger compared to control. The crystal of isolate 1 was 2.12µm-2.34µm, isolate 5 1.47µm - 2.94µm, isolate 6 1.89µm - 4.61µm, isolate 9 1.08-24.9µm. and that of control 1.64µm – 2.04µm (Plate 4-8).

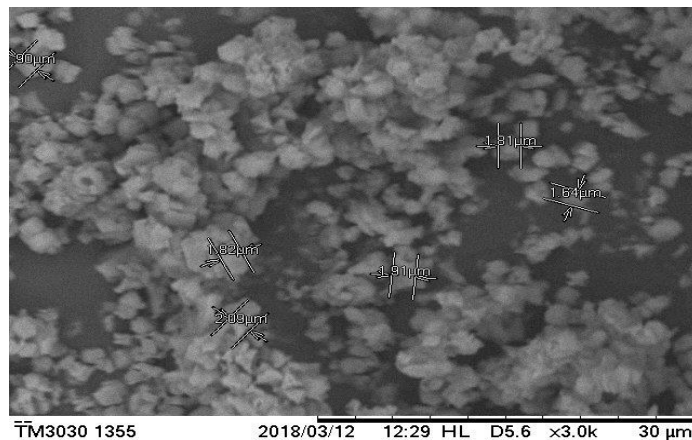


Plate 4: SEM image of Control crystal of precipitated CaCO<sub>3</sub>

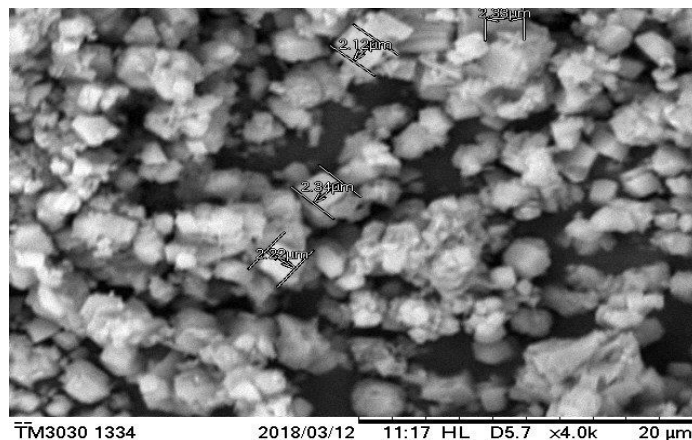


Plate 5: SEM image of crystal precipitated by isolate 1

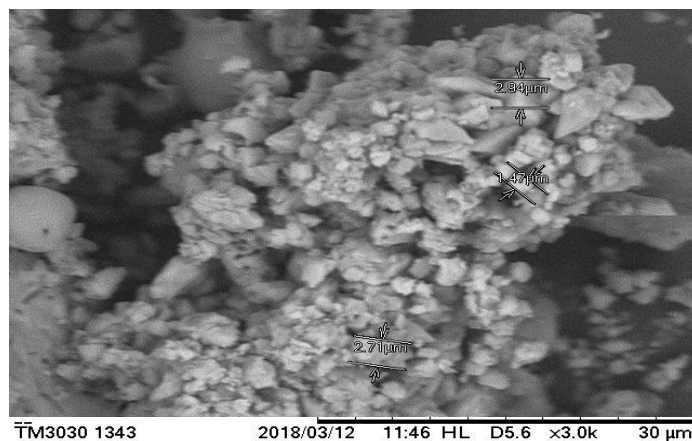


Plate 6: SEM image of crystal precipitated CaCO<sub>3</sub> by isolate 5

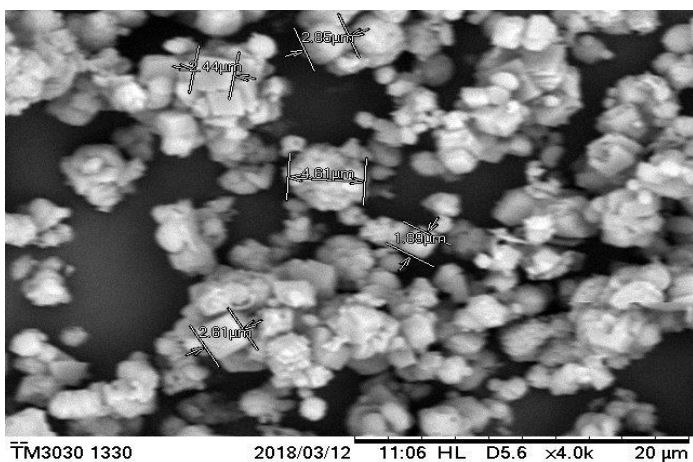


Plate 7: SEM image of crystal precipitated  $\text{CaCO}_3$  by isolate 6

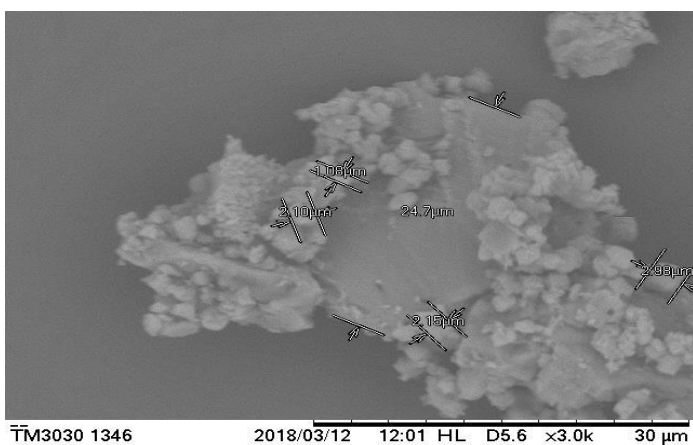


Plate 8: SEM image of precipitated  $\text{CaCO}_3$  crystal of isolate 9

### 3.7 Fourier transform infrared spectroscopy

The FTIR peaks were observed for precipitated calcium carbonate of all isolates (1, 5, 6 and 9) including control. The FTIR of precipitated  $\text{CaCO}_3$ , of the control showed various peaks, at 3431.94 – strong broad O-H stretching alcohol group with intermolecular bonding, 2348.65 – strong O=C=O stretching carbon dioxide. 1796.94 – strong C=O stretching acid halide, 1424.90- medium C-H stretching bending alkane methyl group. 1024.04- strong broad CO-O-CO stretching anhydride. 874.32- strong bending 1, 3-disubstituted (Fig. 4)

The FTIR of precipitated  $\text{CaCO}_3$  of isolate 1 showed various peaks at 2512.67- strong broad O-H stretching carboxylic acid. 1797.41- strong C=O stretching conjugated acid halide, 1421.05- medium O-H bending carboxylic acid, 874.05- strong C-H bending 1,2,4- trisubstituted. 721.70- strong C-H bending monosubstituted bending derivative (Fig. 5). The FTIR of precipitated  $\text{CaCO}_3$  of isolate 5 showed various peaks at 3406.06- medium N-H stretching primary amine. 2513.14 - strong broad O-H stretching carboxylic acid usually centered on  $3000\text{ cm}^{-1}$ , 1421.98 – medium C-H bending alkane, 712.14 – strong C=C bending (Fig. 6). The FTIR of precipitate  $\text{CaCO}_3$  of isolate 6

shows various peaks at 3428.02 – medium N-H stretching primary amine. 2934.42 – strong broad N-H stretching amine salt, 2513.16- weak S-H stretching thiol, 1423.94 – medium O-H bending carboxylic acid, 712.35 - strong C=C bending alkene disubstituted (cis) (Fig. 7). The FTIR of precipitated  $\text{CaCO}_3$  of isolate 9 shows various peaks at 3430.17 – medium N-H stretching primary amine, 2925.01 – strong broad O-H stretching carboxylic acid, 1797.40 – strong C=O stretching anhydride. 1424.07 medium O-H bending carboxylic acid, 874.48 – strong C=C bending alkene (Fig. 8).

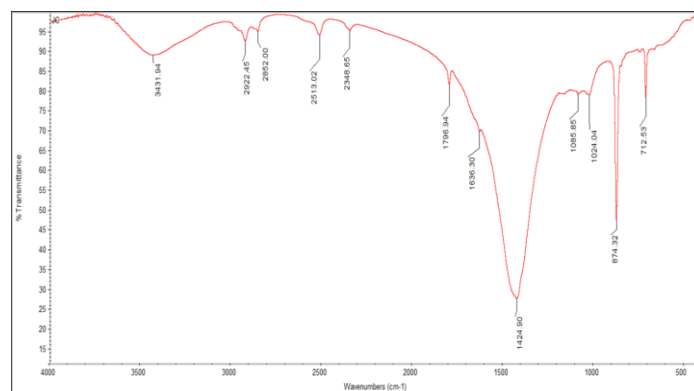


Figure 4: FTIR peaks of precipitated  $\text{CaCO}_3$  of control

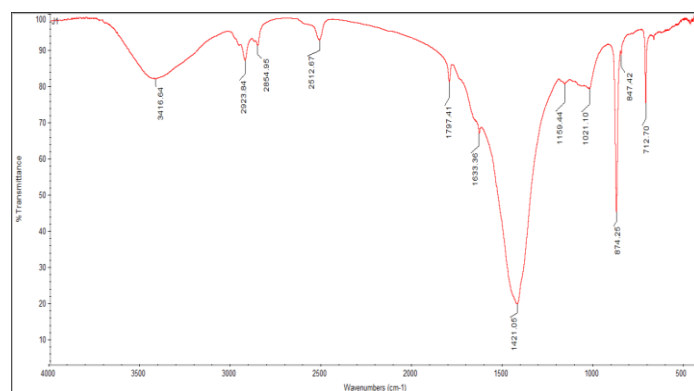


Figure 5: FTIR peaks of precipitated  $\text{CaCO}_3$  of isolate 1

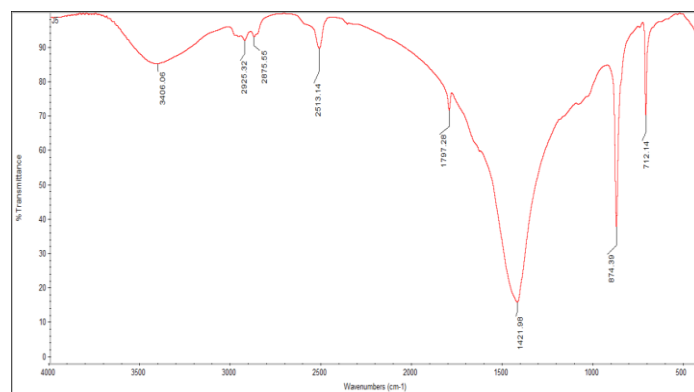


Figure 6: FTIR peaks of precipitated peak  $\text{CaCO}_3$  isolate 5

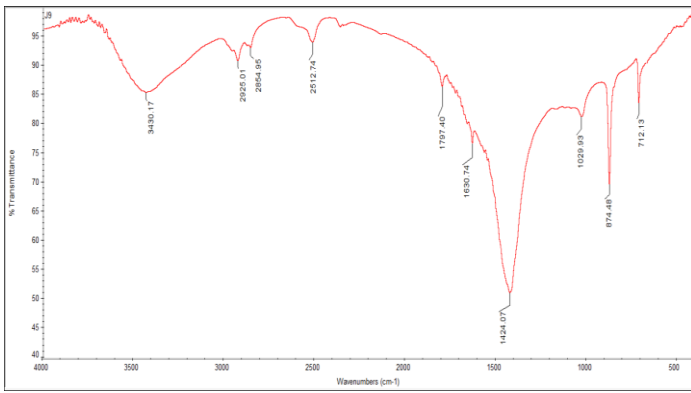


Figure 7: FTIR peaks of precipitated peak  $CaCO_3$  of isolate 6

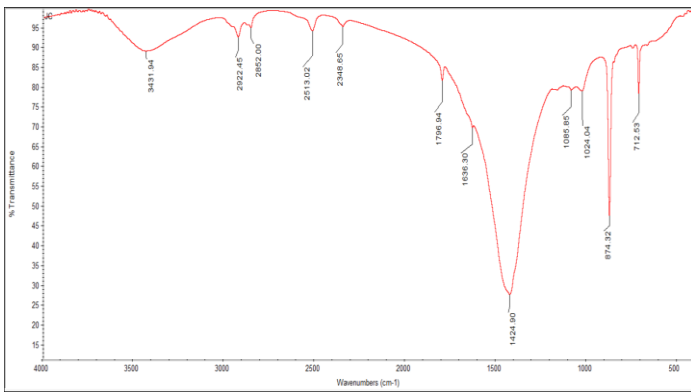


Figure 8: FTIR peaks of precipitated peak  $CaCO_3$  of isolate 9

The specific gravity of the control soil sample is 2.66, the soil sample treated with bacterial isolates are 1.99 for isolate 1, 1.42 for isolate 5, 3 for isolate 6 and 2.94 for isolate 9 (Fig. 9)

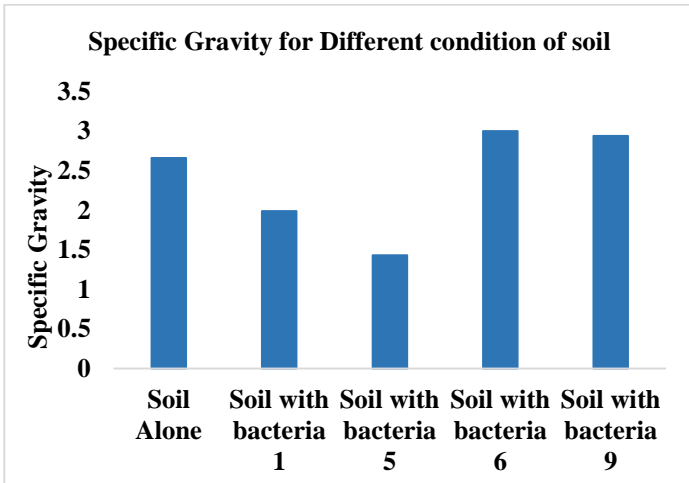


Figure 9: different range of values for specific gravity of soil sample treated with bacterial isolates

The shrinkage limit of bacterial soil samples were 39.70% for isolate 1, 35.85% for isolate 5, 36.6% for isolate 6, 40.13% for isolate 9 and 12.04% for the control soil sample (Fig. 10).

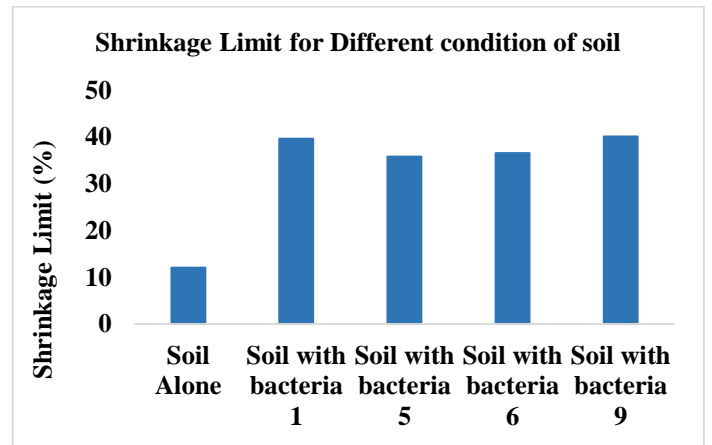


Figure 10: Different range of values for Shrinkage Limit

Bulk density is the weight of soil in a given volume. It also increases with the compaction of soil. The control soil sample showed bulk density of 1.121 gm/cc. The soil sample treated with bacterial isolates showed bulk density of 1.39 gm/cc of isolate 1, 1.46 gm/cc of isolate 5, 1.58 gm/cc for isolate 6 and 1.56 gm/cc for isolate 9 with optimum moisture content of 12.20 % for control, 14.00% for isolate 1, 13.10% for isolate 5, 13.00% for isolate 6 and 12.90% for isolate 9 respectively (Fig. 11-17).

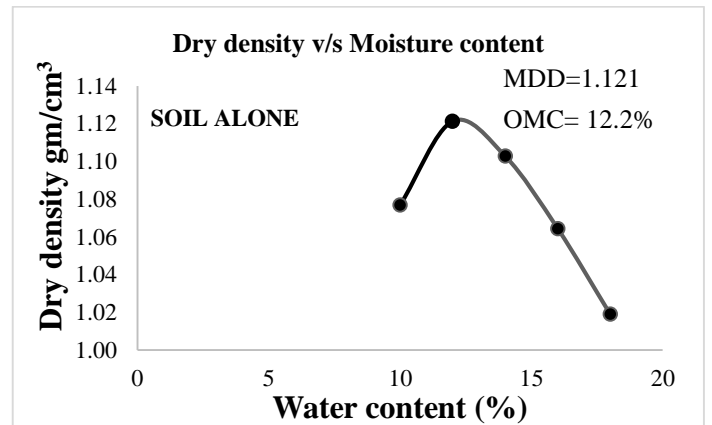


Figure 11: Dry density v/s moisture content for only soil sample (control)

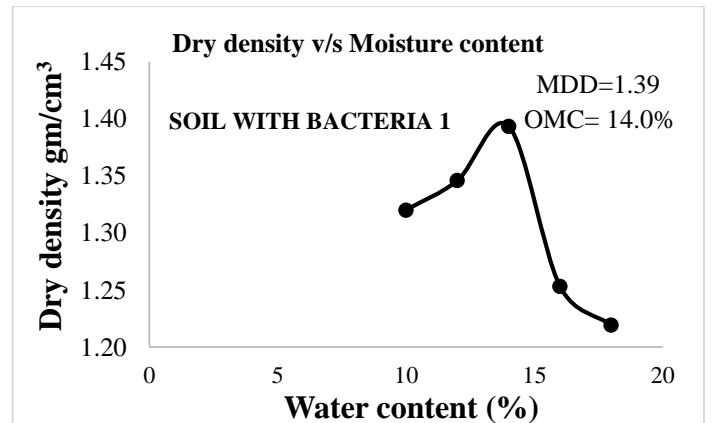


Figure 12: Dry density v/s Moisture content for soil with bacteria 1

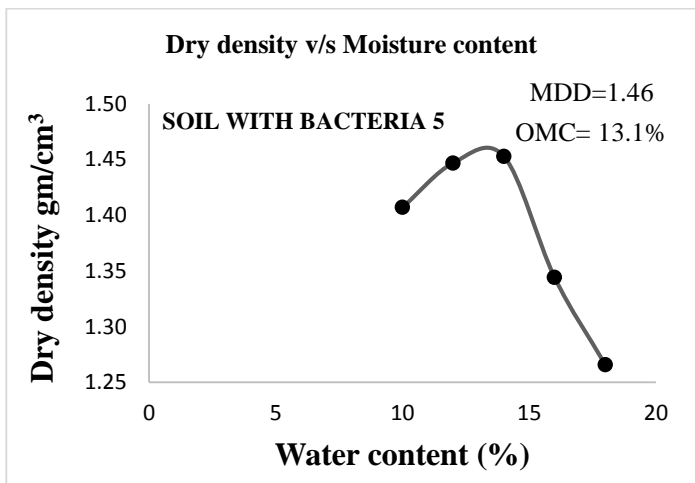


Figure 13: Dry density v/s Moisture content for soil with bacteria 5

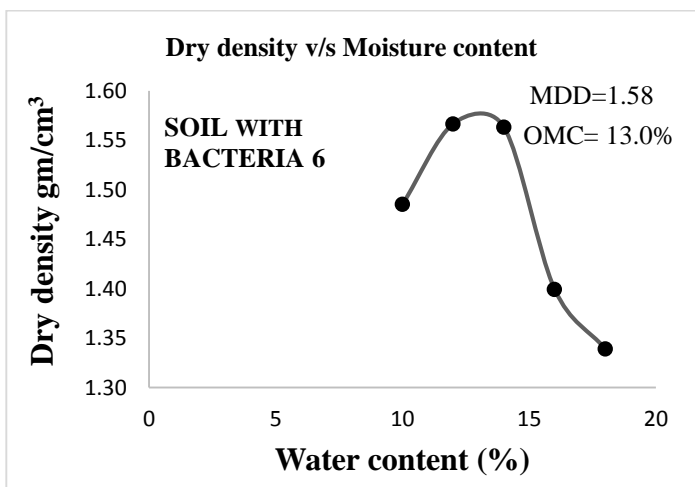


Figure 14: Dry density v/s Moisture content for soil with bacteria 6

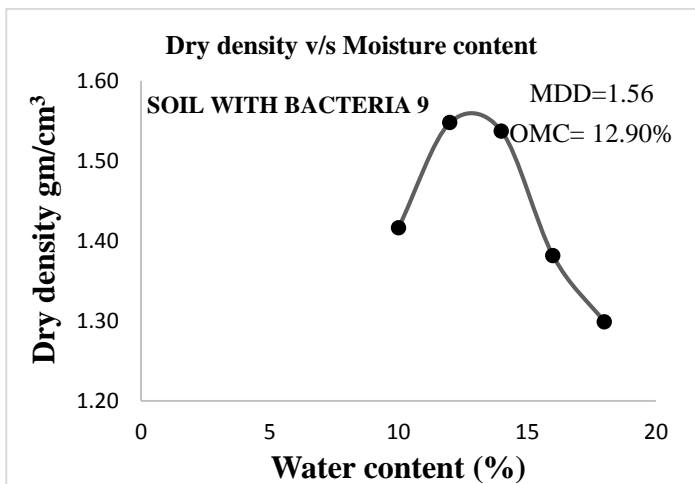


Figure 15: Dry density v/s Moisture content for soil with bacteria 9

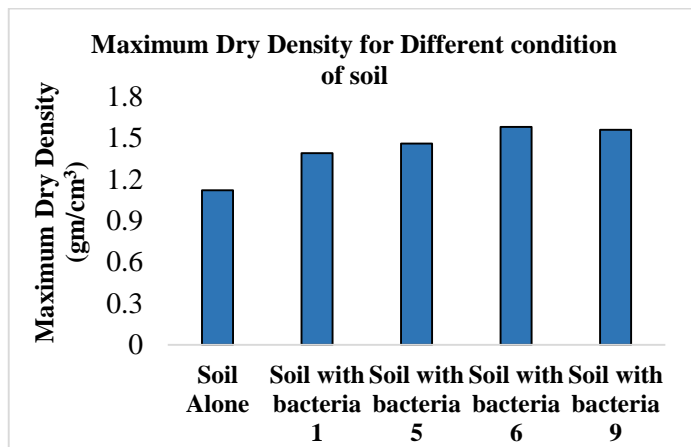


Figure 16: Different range of values for maximum dry density

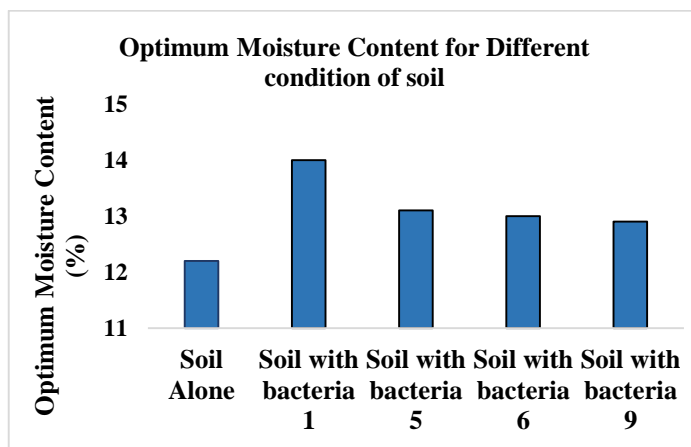


Figure 17: Different range of values for Optimum Moisture Content

#### 4. Discussion

Abundant diverse bacterial species participate in the precipitation of mineral carbonates in various natural environments including soils, geological formation fresh water Biofilms Ocean and saline lakes (Peckman et al., 1999; Rivadeneyra et al., 2000).

The bio-precipitation of calcium carbonate was carried out by different bacterial isolates. In the present study bacteria were isolated from the dry soil sample by serial dilution and pour plate method. The precipitated colonies were formed which were compared with study of (Chahal et al., 2011). Based on the precipitation of calcium carbonates on agar medium four bacterial strains were selected. In the similar study conducted by Dhama et al. (2012) isolated the bacteria from rhizospheric soil sample. Five strains were selected based on their high urease, carbonic anhydrase, and carbonate precipitation.

Morphology of bacterial isolates were found to be Gram positive and Gram negative rod in case of isolate 1 and 6, Gram positive cocci for isolate 5 and Gram negative short rod for isolate 9. Whereas in a study carried out by Kumar et al. (2013) isolate 1,3,5,6 were found to be Gram positive bacilli, isolate 2 and 4 were Gram positive cocci.

The urease producing bacteria utilizes urea present in the media and then degrades phenol red giving pink colour (Andrew et al.,



1995). In the present study four selected bacterial isolates showed positive result for qualitative urea hydrolysis test. As the Bacteria exhibits wide range of urease activity the ability to precipitate Calcium carbonate is directly related to the amount of urease produced, hence it is regarded as cementing enzyme (Kumar et al., 2003). The ability of bacteria to produce urease has been studied quantitatively using the Nessler's reagent in the present study, all isolates showed higher urease activity as compared to the control. Among the isolates 1,5,6 and 9, isolate 5 (0.38µg/ml/hour) showed the maximum the maximum urease activity followed by isolate 1- 0.35µg/ml/hour, isolate 6- 0.29µg/ml/hour, isolate 9-0.23 µg/ml/hour. This indicates the bacteria secretes the extracellular enzyme to cleave the urea to ammonia and carbonate salts. Urease induced calcite precipitation is accelerated by the subsequent increase of pH in the medium due to presence of ammonia ions and the release of CO<sub>2</sub> from the enzymatic urea hydrolysis (Krulwich et al., 1989). Achal et al. (2010) reported the urease activity for two bacterial culture by phenol-hypochlorite assay, the two isolates CT2 and CT5 produced 575.87 U/ml and 670.71 U/ml respectively.

#### 4.1 Metal ion concentration on bacterial biomass

Addition of trace amounts of heavy metals to the environment of microbial cells often stimulates microbial growth (Gikas, 2007, 2008). Higher concentrations may result in severe reduction of microbial activity. Addition of trace amount of metal ions may increase the biomass of bacteria, in the present study nickel ammonium sulphate and cobalt chloride were used as metal ions of varying concentration (1000µl,500µl,100µl and 10µl), were added to the culture broth. The optical reading of all the concentration showed increased bacterial growth means increased pellet and thus increased dry biomass. The lowest concentration of Nickel that is 10 µl was found to be most favourable for the biomass production of the bacterial isolates 1, 5, 6 and 9. Where as in case of cobalt there was varying results for different bacterial strains. The concentration of 10µl was found to be more favourable for isolates 1 and 5. Concentration of 500µl for isolate 6 and 100µl for isolate 9 compared to control. The results obtained for nickel metal ion were the same as in the study conducted by (Chaurasia et al., 2014). As certain metal have recognised to have important biological functions as these makes transport and homeostatic control of bacteria and is also required as metalloproteins and serves as cofactors or structural element for enzyme.

#### 4.2 Bio-precipitation of Calcium Carbonate

The calcium carbonates were precipitated by all isolates in CCP broth which contain calcium chloride, urea, sodium bi carbonate, Ammonium chloride maintained at pH of 7.5-8.00. Calcium carbonate precipitation is dependent on the concentration Ca<sup>+</sup> and CO<sub>3</sub><sup>2-</sup> ions (Qian et al., 2010) in the solution. An increase in the CO<sub>3</sub><sup>2-</sup> concentration occurs in alkaline condition (Lian et al., 2006). The calcium chloride from calcium sources increases the urease activity and produces more calcium carbonate (Achal and

Pan, 2014). In the present study isolate 5 (2.34g/100ml) precipitated more carbonate, then followed by isolates 9 (1.94 g/100ml), 6 (1.85 g/100ml) and 1 (1.42g/100ml). In a study conducted by Dhama et al. (2012) reported that the precipitated CaCO<sub>3</sub> by *B. megaterium* was 187mg/100ml, followed by *B.subtilis* 178mg/100ml, *B. thuringiensis* 167mg/100ml, *B.cereus* 156mg/100ml and *L.fusiform* 152mg/100ml.

#### 4.3 Characterization of precipitated CaCO<sub>3</sub>

The characterization of precipitated CaCO<sub>3</sub> was done by scanning electron microscope. The sizes of CaCO<sub>3</sub> crystals precipitated by bacteria were large as compared to control. Highest crystals size was of isolate 9 (1.08-24.9µm), followed by 6- (1.89-4.61µm), 5 (1.47-2.94µm) and 5 (1.47-2.94µm) control (1.64-2.09µm.). On the other hand, the size of the crystal in a study by Dhama et al. (2013) were found to be *B. megaterium* 30-50µm in diameter followed by 15-40 µm in *B.cereus*, 10-50µm in *B.subtilis*, 2-15µm in *B.thuringiensis* and 2-15µm in *L.fusiform*.

#### 4.4 Fourier transform infrared spectroscopy

FTIR spectrum of CaCO<sub>3</sub> obtained from isolates showed the highest peak at 1421-1424 for all isolates including control. The peaks located at 710 and 876cm<sup>-1</sup> correspond to the in-plane bending and out-of-plane bending modes of CO<sub>3</sub><sup>2-</sup>, and they are characteristic of calcite. The peak at 1421 cm<sup>-1</sup> is assigned to the asymmetric C-O stretching vibration. A weak peak at ~2514 cm<sup>-1</sup> is also characteristic of calcite. FTIR result suggests the formation of most stable calcite. Similar results were reported in earlier study also (Chen et al., 2012).

#### 4.5 Soil tests

Tests on soils were conducted by inoculating the isolated bacteria. This was done mainly to check the capacity of isolates in improving soil properties. The tests like specific gravity, shrinkage limit and bulk density were done. Specific gravity is the unit weight of soils solids only to unit weight of water. It is mainly needed in calculation of soil properties like void ratio and degree of saturation. There was the variation among the soil samples with control soil sample and soil treated with bacteria. The specific gravity of control soil sample and soil sample of isolate 9 was found to be 2.66 and 2.94. Soil sample of treated with isolate 1 and 5 was 1.99 and 1.42 which specifies that soil contains organic matter. Soil sample treated with isolate 9 alone showed the specific gravity of 3.0 indicating the soil contains heavy substances.

Shrinkage limit is the water content where further loss of moisture will not result in any more volume reduction. The consistency and behavior of a soil are different and consequently so are its engineering properties. Soil alone sample undergone shrinkage when compared to soil inoculated with bacterial isolates. Soil inoculated shows less shrinkage, indicating that increase in the shrinkage limit which holds good for the construction of foundation on such soil.

Bulk density is an indicator of soil compaction, which is necessary in determining the amount of compaction required by soil and optimum water content at for compaction, the compaction tests are conducted on the soil. It is calculated as the dry weight of soil divided by its volume. This volume includes the volume of soil particles and the volume of pores among soil particles. Bulk density is typically expressed in  $\text{g}/\text{cm}^3$ . High bulk density is an indicator of low soil porosity and soil compaction. The result obtained in the present study for bulk density on soil treated with different bacterial isolates are follows untreated soil sample showed 1.121  $\text{gm}/\text{cc}$  with water holding capacity of 12.2%. among the soil samples treated with four different bacteria isolate 6 was found to show the maximum bulk density of 1.58  $\text{gm}/\text{cc}$  followed by 1.56 (isolate 9), 1.46 (isolate 5) and 1.39 (isolate 1) respectively.

## 5. Conclusion

The finding of this study suggests the bacteria are novel method for precipitating of  $\text{CaCO}_3$ . This is carried out in alkaline condition at high pH. The enzyme urease plays vital role in precipitation of calcium carbonate as it cleaves the urea into ammonium and carbonates. Hence it is the natural biological process occurring in the nature. The addition of trace metals like nickel and cobalt enhances the growth of microbes in medium. Bacteria produce extracellular enzyme urease. The activity of urease enzyme varies among the bacteria.

Calcium carbonate precipitated by the bacteria is confirmed by the SEM and FTIR analysis. Carbonate precipitation widely helps in enhancing the strength of soils, which lay the basic foundation for the construction purposes.

## References

Achal, V., Mukherjee, A. and Reddy, M.S. 2010. Biocalcification by *Sporosarcina pasteurii* using corn steep liquor as nutrient source, Industrial Biotechnol 6: 170-174.

Achal V, Mukherjee A, Basu PC and Reddy MS. 2009. Lactose mother liquor as an alternative nutrient source for microbial concrete production by *Sporosarcina pasteurii*. J Industrial Microbiol Biotechnol, 36(3): 433-438.

Achal V and Pan X. 2014. Influence of calcium Sources on microbially induced calcium carbonate precipitation by *Bacillus* sp. CR2. Appl Biochem Biotechnol. 173: 307-317.

Anbu P., Kang CH., Shin YJ. and So SS. 2016. Formation of calcium carbonate minerals by bacteria and its multiple applications. Springer Plus 32: 5-250.

Al-Thawadi SM. 2011. Ureolytic bacteria and calcium carbonate formation as a mechanism of strength enhancement of sand. J. Adv. Sci. Eng. Res, 1(1): 98-114.

Beard BL. and Johnson CM. 1999. High precision iron isotope measurements of terrestrial and lunar materials. Geochimica Cosmochimica Acta 63:1653-1660.

Beveridge TJ. 1989. Calcium carbonate concentration formed by the decomposition of organic matter. Science 159: 195-197.

Buczynski C. and Chafetz HS. 1991. Habit of bacterially induced precipitates of calcium carbonate and the influence of medium viscosity on mineralogy. J Sedimentary Petrol. 61: 226-233.

Burbank M. B., Weaver T. J., Williams B. C. and Crawford R. L. 2012. Urease activity of ureolytic bacteria isolated from six soils in which calcite was precipitated by indigenous bacteria. Geomicrobiol. J. 29 389-395.

Burgess JE, Quarmby J and Stephenson T. 1999. Role of micronutrients in

activated sludge-based industrial effluents. Biotechnol. Adv. 17: 49-71.

Cervantes C and Gutierrez-Corana F. 1994. Copper resistance mechanisms in bacteria and fungi. FEMS Microbiol Rev. 14:121-137.

Chahal N., Rajor A. and Siddique R. 2011. Calcium carbonate precipitation by different bacterial strains. Afr J Biotechnol. 10(42):8359-8372.

Chakraborty D. and Bhatia SK. 1996. Formation and aggregation of polymorphs in continuous precipitation 2. Kinetics of  $\text{CaCO}_3$  precipitation of calcium carbonate. Industrial and Engineering Chem Res. 35:1995-2006.

Chaurasia L., Verma RK. and Bisht V. 2014. Microbial carbonate precipitation by urease producing bacteria in cementitious materials. Int J Adv Biotechnol Res. (IJBR) 5: 671-679.

Chen L., Zhang H., Qi Z., Yao W., Wan J., Shao M. and Zhang C. 2012. Synthesis of Calcium carbonate Crystals by using Bacteria. Synthesis and reactivity in Inorganic, Metal- Organic , and Nano-Metal Chemistry 42: 972-975.

Dejong J., Fritzes M. and Nusslein K. 2006. Microbially induced cementation to control sand response to undrained shear. ASCE J Geotechnical and Geoenvironmental Engineering 132(11): 1381-1392.

De Leeuw NH and Parker SC. 1998. Surface structure and morphology of calcium carbonate polymorphs calcite, aragonite and vaterite: an atomistic approach. J Physical Chem 102: 2914-2922.

De Muynck W, De Belie N, and Verstraete W. 2010. Microbial carbonate precipitation in construction materials: A review. Ecol Eng 36:118-136. <https://doi.org/10.1016/j.ecoleng.2009.02.006>

De Muynck, W., Verbeken, K., De Belie, N., and Verstraete, W. 2010b. Influence of urea and calcium dosage on the effectiveness of bacterially induced carbonate precipitation on limestone. Ecol Eng, 36(2), 99-111. <https://doi.org/10.1016/j.ecoleng.2009.03.025>.

Dhami NK, Reddy SM and Mukherjee A. 2012. Biofilm and microbial applications in biomineralization concrete. Adv Topics in Biomineralization. Intech Open

Dhami NK., Reddy MS. and Mukherji A. 2013. Biomineralization of calcium carbonate polymorphs by the bacterial strains isolated from calcareous sites. J Microbiol Biotechnol. 23(5):707-714.

Gadd GM (2000) Bioremediation potential of microbial mechanisms of metal mobilization and immobilization. Curr Op in Biotechnol 11:271-279.

Gavimath C., Mali BM., Hooli VR., Mallpur JD., Patil AB., Gaddi DP. and Ternikar CR. 2012. Potential application of bacteria to improve the strength of cement concrete. Int J Adv Biotechnol Res. 3: 541-544.

Gikas P. 2008. Single and combined effects of nickel (Ni(II)) and cobalt (Co(II)) ions on activated sludge and on other aerobic microorganisms : A review, J Hazard Mater. 159, 187-203.

Gikas P. and Romanos P. 2006. Effects of tri-valent (Cr(III)) and hexa-valent (Cr(VI)) chromium on the growth rate of activated sludge, J Hazard Mater B 133, 212-217.

Gikas P., Şengör S.S., Ginn T.R., Moberly J. and Peyton B.M. 2009. The effects of heavy metals and temperature on microbial growth and lag, Global Nest J. 1(3), 325-332.

Gollapudi UK., Knutson CL., Bang SS. and Islam MR. 1995. A new method for controlling leaching through permeable channels. Chemosphere 30: 695-705.

Ivanov V. and Chu J. 2008. Applications of microorganisms to geotechnical engineering for bioclogging and biocementation of soil in situ. Review Environmental Sci Biotechnol. 7: 53- 139.

Ji, G and Silver, S. 1995. Bacterial resistance mechanisms for heavy metals of environmental concern. J Industrial Microbiol. 14: 61-75.

Karol RH (2003) Chemical grouting and soil stabilization. Marcel Dekker, New York (NY), p 558.

Knoll, A.H. (2003) Biomineralization and evolutionary history. Rev Mineral Geochem. 54: 329-356.

Krumbein WE. and Giele C. 1979. Calcification in coccoid cyanobacterium associated with the formation of desert stromatolites. Sediment l. 26: 593-604.

Kumar JBG., Prabhakara R. and Pushpa H. 2013. Biomineralisation of calcium carbonate by different bacterial strains and their application in concrete crack remediation, Int J Engg and Adv Technol. 2(3):486-491.

Kumar D., Qian XY., Pan X., Achal V., Li Q. and Gadd GM. 2016. Microbially -induced carbonate precipitation for immobilization of toxic metals. Adv Appl Microbiol 94: 79-108.

Lian, B., Hu, Q., Chen, J., Ji, J. and Teng, H.H. 2006. Carbonate biomineralization

- induced by soil bacterium *Bacillus megaterium*. Geochim. Cosmochim. Acta, 70, 5522–5535.
- Madoni P, Davoli D, Gorbi G and Vescovi L. 1996. Toxic effects of heavy metals on the activated sludge protozoan community. Water Res. 30: 135-141.
- McLean RJC., Fortin D. and Brown DA.1996. Microbial metal-binding mechanics and their relation to nuclear waste disposal. Can J Microbiol. 42: 392-400.
- Mojzsis SJ., Arrhenius G., McKeegan K., Harrison TM., Nutman AP. and Friend CRL.1996. Evidence for life on earth before 3,800 million years ago. Nature 384: 55-59.
- Morby AP, Turner JS, Huckle JW and Robinson NJ. 1993. Smt B is a metal dependent repressor of the cyanobacterial metallothionein gene smt A: Identification of a Zn inhibited DNA-protein complex. Nucleic Acids Res. 21: 921-925.
- Peitzsch N, Eberz G, Nies BH. 1998. Alcaligenes eutrophs as bacterial chromate sensor. Appl Environ Microbiol. 321-324.
- Qian C, Yu X, & Wang X. 2018. Potential uses and cementing mechanism of bio-carbonate cement and bio-phosphate cement. AIP Advances 8(9): 095224.
- Rivadeneyra MA, Ramos-Cormenzana A, Delgado G, Delgado R. 1996. Process of carbonate precipitation by *Deleya halophila*. Curr Microbiol. 32:308–313.
- Sani RK, Peyton BM and Jhandyala M. 2003. Toxicity of lead to *Desulfovibrio desulfuricans* G20. Environ Toxicol Chem. 22: 252-260.
- Sensoy T, Bozbeyoglu N, Dogan N, Bozkaya O, Akyol E. 2017. Characterization of Calcium Carbonate Produced by ureolytic bacteria (*Sporocarcina pasteurii* ATCC 6453 and *Bacillus aerius* U2) and effect of environmental conditions on production of calcium carbonate.15th International Conference on Environmental Science and Technology.
- Stohs GJ Bagchi D. 1995. Oxidative mechanisms in the toxic metal ions. Free Rad Biol. Medi 18: 321-336.
- Sunda W G and Huntsman SA. 1987. Microbial oxidation of manganese in a North Carolina estuary . Limnol Oceanography 32:552-564.
- Whiffin VS., Van Paassen L. and Harkes MP. 2007. Microbial carbonate precipitation as a soil improvement technique. Geomicrobiol. 24: 417-423.

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