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Calcium carbonate precipitation by *Paenibacillus fonticola* and *Sporosarcina luteola* for its potential application in bio concrete

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ABSTRACT

Concrete is one of the most widely used construction materials in various infrastructures. However, despite of several advantages, it has a high tendency to form cracks due to climatic effects and parameters like temperature fluctuations, exposure to corrosive and toxic chemicals, harmful gases, natural disasters, etc. These factors reduce the longevity of concrete which adds up to the cost of the maintenance and repair. Existing treatments have various limitations like weak resistance to weather, moisture sensitivity, heat and low sustainability. Hence, there is a need for alternative and sustainable treatment technologies. One such technology is bio concrete formation by the process of Microbially Induced Calcium Carbonate Precipitation (MICP). In nature, MICP occurs through different processes like urea hydrolysis, photosynthesis, denitrification, etc. Bio concrete is a combination of concrete and bacteria possessing the ability to precipitate calcium carbonate ($CaCO_3$) that aids in sealing the cracks that appear in it. This self-healing property of bio concrete can be a potentially sustainable and economic technology. This study was aimed to enrich, isolate and screen bacteria capable of precipitating $CaCO_3$ and their use in production of bio concrete. Total 11 samples predominantly containing speleothems and related materials were collected from different locations in Satara and Nashik districts of Maharashtra, India. The samples were further processed independently in B4 and Calcium Carbonate Precipitation (CCP) media for enrichment. A total of 94 isolates were obtained from the enriched samples. Subsequent screening and characterization of these isolates revealed that *Paenibacillus fonticola* and *Sporosarcina luteola* can substantially precipitate calcium carbonate.The Fourier Transform Infrared (FTIR) spectra, Scanning Electron Microscopy (SEM) and Energy Dispersive X-ray (EDX) microanalysis of the $CaCO_3$ precipitates formed by these bacteria renders both *Paenibacillus fonticola* and *Sporosarcina luteola* the potential candidature for bioconcrete formation.

Key words : *Paenibacillus fonticola, Sporosarcina luteola, Bioconcrete, Speleothems, Urease, Calcium carbonate precipitation*

Introduction

The ever-increasing urbanization and industrialization has led to an unprecedented use and demand of concrete. Concrete is one of the most extensively used materials in construction (Stajanca and Estokova, 2012). It is used in building several infrastructures like roads, dams, bridges, tunnels, buildings, etc. Concrete is a mixture of cement, water, aggregates and admixtures (Seifan *et al.*, 2016; Jawaid *et al*., 2018; Miller and Moore, 2020). Concrete offers a range of advantages which makes it a desirable material of choice. Low price, abundant availability, durability, high compressive strength,

adaptability with steel bars and quick mixing are the factors that make it an ideal choice (Jawaid *et al*., 2018; Castro *et al*., 2016). Every year more than ten billion tons of concrete is used worldwide and according to experts, this requirement is likely to exceed to 15 billion tons by the year 2050 (Alonso *et al*., 2019).

The production of cement is a highly complex and energy consuming process that leads to release of toxic gases and hazardous solid wastes. Raw materials like lime and clay are collected by blasting rock quarries and then finely crushed into a raw meal (Dunuweera and Rajapakse, 2018). This meal is then blended and heated in a pre-heating system which separates carbonates to form calcium and CO_2. This meal is then heated at a temperature of 1500 °C in rotary kiln. This resulting mixture is known as clinker (Babor *et al*., 2009; Habert, 2013). Clinker is further processed, ground and mixed with lime and gypsum to produce fine cement (Dunweera and Rajapakse, 2018; Mohamad *et al*., 2021). This production process leaves a detrimental impact on the environment, energy reserves and economy. Cement production accounts for 8-10 % of global CO_2 emissions resulting from man-made activities and 3-4 % of total global CO_2 emissions (Irfan *et al*., 2019; Achal *et al*., 2016 and Suhendro, 2014). Apart from this, 7.8 % nitrogen oxide, 4.8 % sulphur oxide and 6.4 % of particulate emissions have also been reported as a result of cement production (Miller and Moore, 2020). And hence, any measures to reduce usage of cement wherever possible is always warranted.

One of the reasons of increased consumption of concrete is its inefficiency to withstand biological, physical and chemical stresses. Factors like climatic variations, natural disasters, exposure to corrosive chemicals and harmful gases, microbial growth, etc. lead to formation of micro cracks and macro cracks in concrete structures (Anbu *et al*., 2016; Jroundi *et al*., 2014). These cracks often lead to leakage and entry of oxygen, CO_2 and moisture which in turn cause metal corrosion and ultimately adversely affect the concrete structure. Thus reduced durability, strength and overall longevity of the concrete increases the cost of repair and maintenance (Ponraj *et al*., 2015; Sashank *et al*., 2018; Narwaria and Tiwari, 2016; Doshi *et al*., 2020). Conventional repair mechanisms involve the use of surface treatments and penetrating sealants that include acrylic resins, epoxy resins, siloxanes, polyurethanes and chlorinated rubbers. However, these treatments offer only a temporary solution as they often show less resistance towards heat, exhibit moisture sensitivity, different thermal expansion coefficients and are often unsustainable (Seifan *et al*., 2016; De Muynck *et al*., 2008; Tittleboom *et al*., 2010; Andalib *et al*., 2014). Repeated use of these chemicals to seal cracks is not economically feasible as it increases the cost of repair and maintenance. Considering the various drawbacks of existing treatments, there is a need of alternative and sustainable treatment options that can tackle these issues.

One such alternative is the use of 'self-healing' concrete or 'bioconcrete' that is produced as a result of biomineralization. Biomineralization is a phenomenon in which microorganisms produce minerals through various metabolic pathways (Shukla and Cameotra, 2016). The first mechanism is the Biologically Controlled Mineralization (BCM), in which the organism's metabolism controls the growth and formation of the minerals. Second mechanism is the Biologically Induced Mineralization (BIM) in which the minerals are formed by the virtue of reactions between various ions present in the environment and metabolic byproducts of the organism. The third mechanism is the Biologically Mediated Mineralization (BMM) in which minerals are formed as a result of organic matrix and inorganic compounds irrespective of biological activity (Achal and Mukherjee, 2015; Ronholm *et al*., 2014; Nonakaran *et al*., 2015; Dikshit *et al*., 2020). Among these, the precipitation of $CaCO_3$ is the most studied mechanisms mainly due to two reasons: firstly, $CaCO_3$ is the most plentiful mineral found on earth. Secondly, its precipitation occurs naturally in various environments by microorganisms, a process known as Microbially Induced Calcium carbonate Precipitation (MICP) (Castro *et al*., 2016). The major metabolic pathways responsible for MICP include urea hydrolysis (Acuna *et al*., 2018; Gebru *et al*., 2021; Mountassir *et al*., 2018), denitrification, sulfate reduction (Rajaskar *et al*., 2017), ammonification of amino acids (Zhu and Dittrich, 2016), photosynthesis (Zhu and Dittrich, 2016; Rajaskar *et al*., 2017), oxidation of organic acids and methane oxidation (Seifan and Berejian, 2019; Tsesarsky *et al*., 2019; Liu *et al*., 2021). Till date bacteria like *Sporosarcina pasteurii*, *Bacillus sphaericus*, *Bacillus megaterium* and few other species of genus *Bacillus* are been used in MICP based applications. These bacteria are known to precipitate $CaCO_3$ in the environment. Their characteristic features such

as spore formation, urease production, capability to hydrolyze urea and precipitate $CaCO_3$, often makes them suitable candidates for bioconcrete related applications (Chuo *et al*., 2020).

Bioconcrete is the concrete in which bacteria capable of precipitating $CaCO_3$ are added. Once cracks are formed in the concrete, organisms precipitate $CaCO_3$ that seals these cracks, a property known as the self – healing property (Alonso *et al*., 2019). Studies have shown the importance of bioconcrete in increasing the durability, compressive strength and reducing water permeability of the concrete. These findings describe the significance of this technology and its role in development of sustainable alternatives. However, the successful implementation and commercialization of this technique has not been possible yet due to several reasons. The number of bacteria having the $CaCO_3$ precipitation potential is quite less. The spore viability and survival of microorganisms under harsh conditions of high pH, salts and low nutrients, limits the application of this technique. Also, most of the studies till now have focused more on the engineering outlook and less on the biological mechanisms of $CaCO_3$ precipitation. Therefore, there is a need to look for more applicable bacterial isolates. For successful commercialization of this technique, economically feasible alternatives for nutritional requirements, encapsulation and delivery of organisms need to be explored. The present study deals with isolation of bacteria from different environments and assessing their $CaCO_3$ precipitation potential for their possible use in bioconcrete.

Materials and Methods

Sample location and collection

A total of 11 individual samples were collected from the mountain regions, caves, prehistoric locations and agricultural field of Satara and Nashik districts of Maharashtra, India. The samples principally included speleothems, coarse and fine aggregates and agricultural soils. The samples were collected aseptically using sterile scalpel and plastic containers and stored at 4 °C for further analysis.

Enrichment and isolation of bacteria

All samples were subjected to enrichment using B4 medium (2.5 g l^{-1} calcium acetate, 10 g l^{-1} glucose and 4 g l^{-1} yeast extract, pH 7) and Calcium Carbonate Precipitation (CCP) medium (20 g l^{-1} urea, 2.12 g l^{-1} $NaHCO_3$, 10 g l^{-1} NH_4Cl, 3 g l^{-1} Nutrient broth, 30 mM l^{-1} $CaCl_2$, pH 6.5) (Ojha *et al*., 2021; Baskar *et al*., 2018). Speleothems, coarse and fine aggregates were finely crushed using sterile mortar and pestle. 0.1 g of crushed sample was independently inoculated in both B4 and CCP media and incubated at 32°C for 10 days. Appropriate media controls were maintained throughout. Enriched samples were serially diluted and dilutions ranging from 10^{-1} to 10^{-6} were spread on B4 and CCP agar respectively. Plates were incubated at 32 °C for 4 days.

Screening of isolates for $CaCO_3$ precipitation

All the obtained isolates were screened for their ability to precipitate $CaCO_3$ based on formation of $CaCO_3$ crystals. Isolates were grown on B4 agar, CCP agar and Nutrient agar at 32 °C for 7 days. Presence of crystals on isolated bacterial colonies was confirmed through stereo microscopy (Wei *et al*, 2015) and light microscopy on B4 agar and CCP agar. Herein nutrient agar acted as a negative control for these screening studies.

Urease activity of screened isolates

Screened isolates capable of $CaCO_3$ crystal formation were streak inoculated on Christensen's urea agar (1 g l^{-1} peptone, 1 g l^{-1} dextrose, 5 g l^{-1} NaCl, 1.2 g l^{-1} Na_2HPO_4, 0.8 g l^{-1} KH_2PO_4, 0.012 g l^{-1} phenol red, 15 g l^{-1} agar, pH 6.8 ± 0.2) and incubated at room temperature for 2 days (Zhulaika *et al*., 2021). Post incubation, medium was observed for change in colour.

Characterization of $CaCO_3$ precipitate

Further quantitative $CaCO_3$ precipitation potential of all positive isolates was assessed gravimetrically (Wei *et al*., 2015). Overnight grown culture in Nutrient broth was used as standardized inoculum (0.5 McFarland) for these studies. 30 µl of standardized inoculum was inoculated in triplicates in conical bottom tubes of 15 mL capacity containing 8 ml CCP broth. All tubes were incubated at 32 °C for 4 days. Uninoculated liquid media were kept as controls. Post incubation culture broths were centrifuged at 4000 rpm for 20 minutes. The resulting pellets containing bacteria and $CaCO_3$ crystals were resuspended in 4 ml TE buffer (10 mM Tris base, 1 mM EDTA, pH 8.5, lysozyme 1 mg ml^{-1}). The entire mixture was incubated at 37 °C for 1 hour. Tubes were centrifuged at 4000 rpm for 20 minutes to remove cell debris. The pellet was further washed with ster-

ile distilled water. The tubes were placed under desiccating condition at 37 °C for 24 hours for drying. The dried precipitates thus obtained, were subjected to various tests and analysis as follows.

Acid fizz test

$CaCO_3$ precipitates formed by each isolate were independently subjected to acid fizz test by overlaying the precipitate with 10% (v/v) HCl in each tube (Mahmoud *et al*., 2021; Abudoleh *et al*., 2018). Generation of effervescence after adding HCl is to be treated as a positive test.

Quantification of $CaCO_3$ precipitates

Gravimetrically the weight of $CaCO_3$ precipitates was determined in each tube and the percentile precipitate formed by each isolate was determined independently.

Physico chemical characterization of $CaCO_3$ precipitates

$CaCO_3$ precipitates of three selected isolates were subjected to SEM (Scanning Electron Microscopy), FTIR (Fourier Transform Infrared Spectroscopy) and EDX (Energy Dispersive X Ray) microanalysis (Sun *et al*., 2020; Bibi *et al*., 2018; Cacchio *et al*., 2019). Morphology of the crystals was observed through SEM (JSM-IT200). Dried precipitates were coated with gold and placed on carbon tape. The resolution was maintained at 100 μm with an accelerating voltage of 5.0 kV (Wen *et al*., 2021). Elemental analysis of the precipitates was done using EDX by ZAF method (Carillo *et al*., 2012). FTIR spectroscopy was done using Lambda 76000 FTIR by KBr pellet method (Vagenas *et al*., 2003). Spectra were recorded in the region 4000 – 400 cm^{-1}.

Identification of bacterial isolates

The primary characterization of bacterial isolates with $CaCO_3$ precipitating ability was done by Gram staining (Claus, 1992) and spore staining methods (Schaeffer and Fulton, 1993). Three isolates viz., 15C S9, 19C S5 and 4C S6, were selected for subsequent identification based on their $CaCO_3$ precipitation potential and urease activity. The isolates were identified by 16S rRNA gene sequencing. The 16S rRNA gene for all three isolates was amplified using gene specific primers 27F and 1488R (Sambrook *et al*., 1989). Purified PCR amplicons were sequenced on ABI 3730 xl DNA analyser with Big Dye terminator kit from Applied Biosystems. The gene sequences were analysed using the basic local alignment search tool (BLAST) on the NCBI database.

Results and Discussion

Sample location and collection

The environmental samples obtained from mountain regions, caves (Baskar *et al*., 2018; Rautela and Rawat, 2020), and agricultural soils (Dikshit *et al*., 2010) are often reported to contain aragonite, vaterite and calcite, the polymorphs of $CaCO_3$ precipitate. Formation of these polymorphs in not necessarily always attributed to geological activities, but microbial activities as well. Hence, it was thought worth while to try and collect samples from such locations. The choice of locations was combinations of prehistoric mountain regions and caves to maximize the possibility of isolating bacteria actively involved in $CaCO_3$ precipitation. Hence, total of 11 samples encompassing the desired sampling locations were collected. Out of these five were from Satara district and six from Nashik district. The sampling location in Satara was Buddhist caves popularly known as Agashiv caves located at the coordinates Lat. 17.255224^0N Long. 74.16304^0E. The five samples collected at Agashiv caves comprised of speleothems, coarse and fine aggregates. The two sampling locations in Nashik were Anjaneri hills located at the coordinates Lat. 19.947129^0N Long. 73.591031^0E and Makhmalabad village located at the coordinates Lat. 20.053934^0N Long. 73.762596^0E. Samples collected at Anjaneri comprised of speleothems, coarse and fine aggregates. However sample collected at Makhmalabad was a composite agricultural soil enriched with urea. The speleothems that have previously been broken by unintended human interaction or natural causes such as erosion, seismic activity or collapse were collected without impacting the sampling sites. The speleothems, coarse and fine aggregates were brown and grey coloured with white deposits presumably of calcium carbonate. The physical characters of the samples rendered them the most suitable environmental samples to be investigated for MICP.

Enrichment and isolation of bacteria

All 11 samples were independently enriched in B4 and CCP broth for a period of 10 days. B4 broth and CCP broth owing to their composition and ingredients like calcium salts and urea makes them the

most ideal options for selective enrichment of $CaCO_3$ precipitating bacteria (Vahabi *et al*., 2013; Ojha *et al*., 2021). As the incubation for enrichment progressed, cloudiness was developed inside all sample containing CCP broth flasks. The cloudiness progressively got converted into the white deposits on the inner surface of these borosilaciate Erlenmeyer flasks hinting the effective precipitation of calcium carbonate. Post incubation the enriched broth was processed for isolation of bacteria using B4 agar and CCP agar through spread plate technique. A total of 94 morphologically distinct bacterial colonies were selected and purified on respective media. Out of these 94 isolates, 58 were obtained on CCP agar and 36 on B4 agar. The purified isolates were maintained on respective media for subsequent studies.

Screening of isolates for $CaCO_3$ precipitation

Microscopic observations (Wei *et al*., 2015) of isolated colonies of all the morphologically distinct isolates could help screen isolates possessing ability of $CaCO_3$ precipitation. Presence and abundance of crystals was studied by visualizing the colonies under effective magnification of 450x using light microscope (Figure 1). Furthermore, the same colonies were visualized under stereo microscope to confirm the crystal formation. Out of 94 isolates grown on B4 agar and CCP agar respectively, 27 showed presence of crystal formation on and around the vicinity of the bacterial colonies. 2 isolates on B4 agar and 25 isolates on CCP agar showed visible crystal formation, whereas the same isolates did not show any crystal formation on Nutrient agar. This characteristic difference indicates that the calcium salts from B4 and CCP media have been transformed in to crystals of $CaCO_3$ through MICP process of the respective bacteria.

Urease activity

Total of 14 isolates were capable of hydrolysing urea through the action of urease enzyme. Urease production was confirmed by colour change of the medium from red to pink due alkaline pH. Urea hydrolysis is one of the mechanisms used by bacteria for $CaCO_3$ precipitation. The isolates thus showing capability of urea hydrolysis becomes significant for enhancing $CaCO_3$ precipitation.

Characterization of $CaCO_3$ precipitate

Acid fizz test

The $CaCO_3$ precipitate thus formed were qualitatively analyzed. The precipitates of all 27 isolates were checked for the formation of effervescence (CO_2 formation) using the acid fizz test. The dried precipitate of all the isolates in the conical bottom tubes when flooded with 10% (v/v) HCl, generated CO_2 effervescence. This in turn confirmed the presence of $CaCO_3$ precipitate.

Quantification of $CaCO_3$ precipitates

The quantification of formed $CaCO_3$ in the precipitate is a significant step to determine MICP potential of the screened isolates. Hence the gravimetric quantitative estimation of $CaCO_3$ precipitation by all 27 isolates was done. The potential of $CaCO_3$ precipitate formation by these isolates ranges between 0.0025 g %(w/v) to 0.2 g %(w/v). This accentuates not only the capability of $CaCO_3$ precipitation but also highlights few of these isolates as potential candidates in bioconcrete formation process. The three isolates 15C S9, 19C S5 and 4C S6 were able to precipitate 0.2 g %(w/v), 0.16 g %(w/v) and 0.15 g %(w/v) of $CaCO_3$ respectively. These three are the most promising isolates as far as $CaCO_3$ precipitate

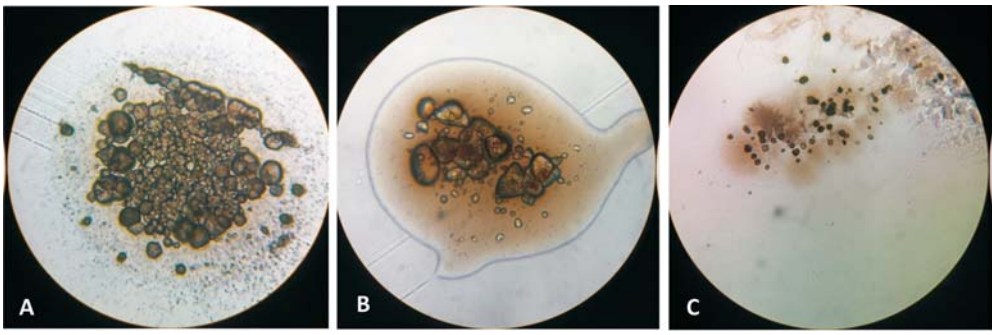

Fig. 1. Crystals observed under light microscope at 450X magnification on bacterial colonies of isolates A) 4C S6 B) 15C S9 C) 19C S5

obtained through MICP is concerned. Thus, they became the most favourable candidates for bioconcrete formulations. Hence the precipitate obtained from these isolates was further subjected to physicochemical characterization using certain analytical techniques.

Physico-chemical characterization of $CaCO_3$ precipitates

Scanning electron microscopy revealed the shape and morphology of the $CaCO_3$ crystals formed. Scanning electron micrographs of all three isolates confirmed abundance of crystals in the precipitates (Figure 2). Majority of these crystals were seen in the form of aggregates. The crystals showed hexagonal or trigonal symmetry. EDX micro analysis of all three precipitates (Figure 3) affirmed the presence of Ca, C and O elements in each of them. The elemental composition of the precipitate formed by 4C S5 showed mass percentage of C, O and Ca to be 16.80%, 35.36% and 47.84% respectively. In the precipitate formed by 15C S9, the mass percentage of C, O and Ca was 19.44%, 42.38% and 38.18% respectively. However, in the precipitate formed by 19C S5, the mass percentage of C, O and Ca was 40.07%, 58.15% and 1.78% respectively. The FTIR spectra of all three isolates (Figure 4) were compared with standard spectrum of calcium carbonate. It was observed that the absorption peaks around 718, 875 and 1425 cm^{-1} of the precipitate resemble to plane bending and asymmetric stretching vibration peaks of O-C-O. Also, the IR spectra of all three precipitates have been found to be in agreement with calcite characteristic vibrations. Accordingly, the scanning electron micrographs, EDX microanalysis and FTIR spectral analysis supports that the prominent MICP activity showed by these isolates makes them the ideal candidates for bioconcrete formulations.

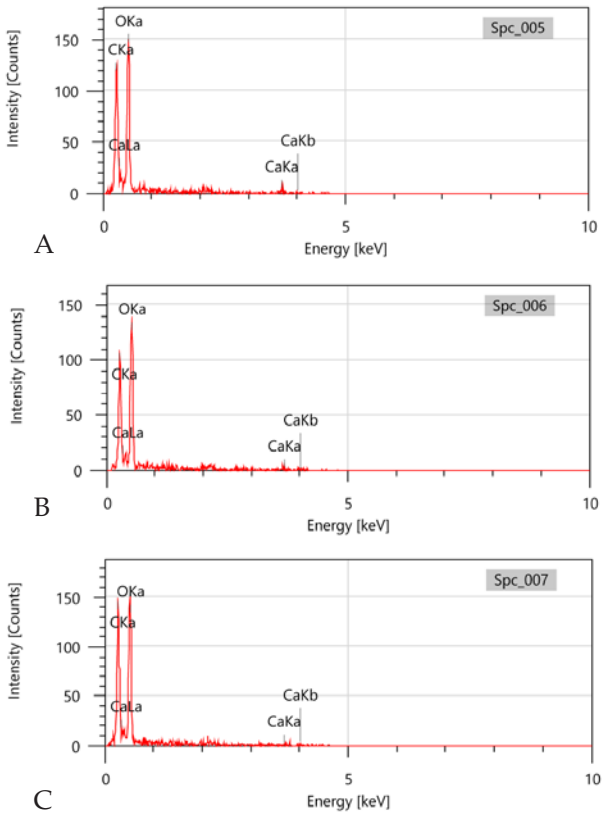

Fig. 3. EDX analysis of isolates A) 4C S6 B) 15C S9 C) 19C S5

Identification of bacterial isolates

Gram staining of all screened isolates showed presence of Gram positive rods. Short rods, long rods and rods in chains were observed. Spore staining of these isolates confirmed the presence of endospores in 22 isolates. Spores of different shapes and positions like – spherical terminal, spherical terminal bulging, and oval central bulging were observed. Based on the microscopy, $CaCO_3$ precipitation, urease activity and spore forming nature, three isolates

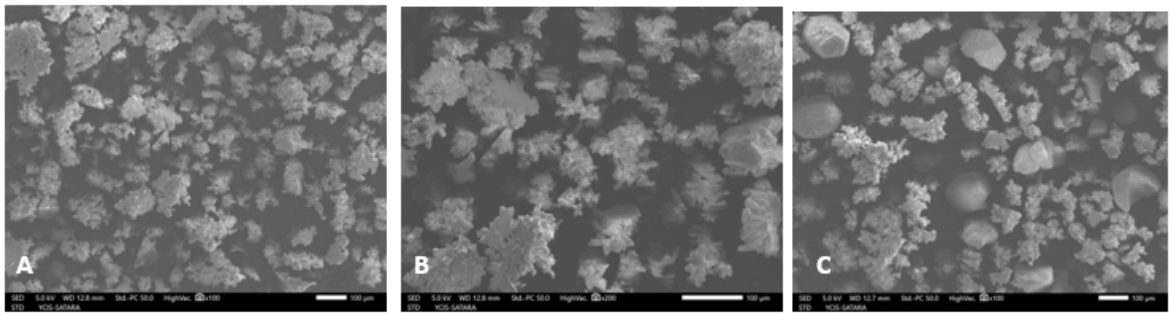

Fig. 2. Scanning electron micrographs showing crystals with hexagonal symmetry for isolates A) 4C S6 B) 15C S9 C) 19C S5

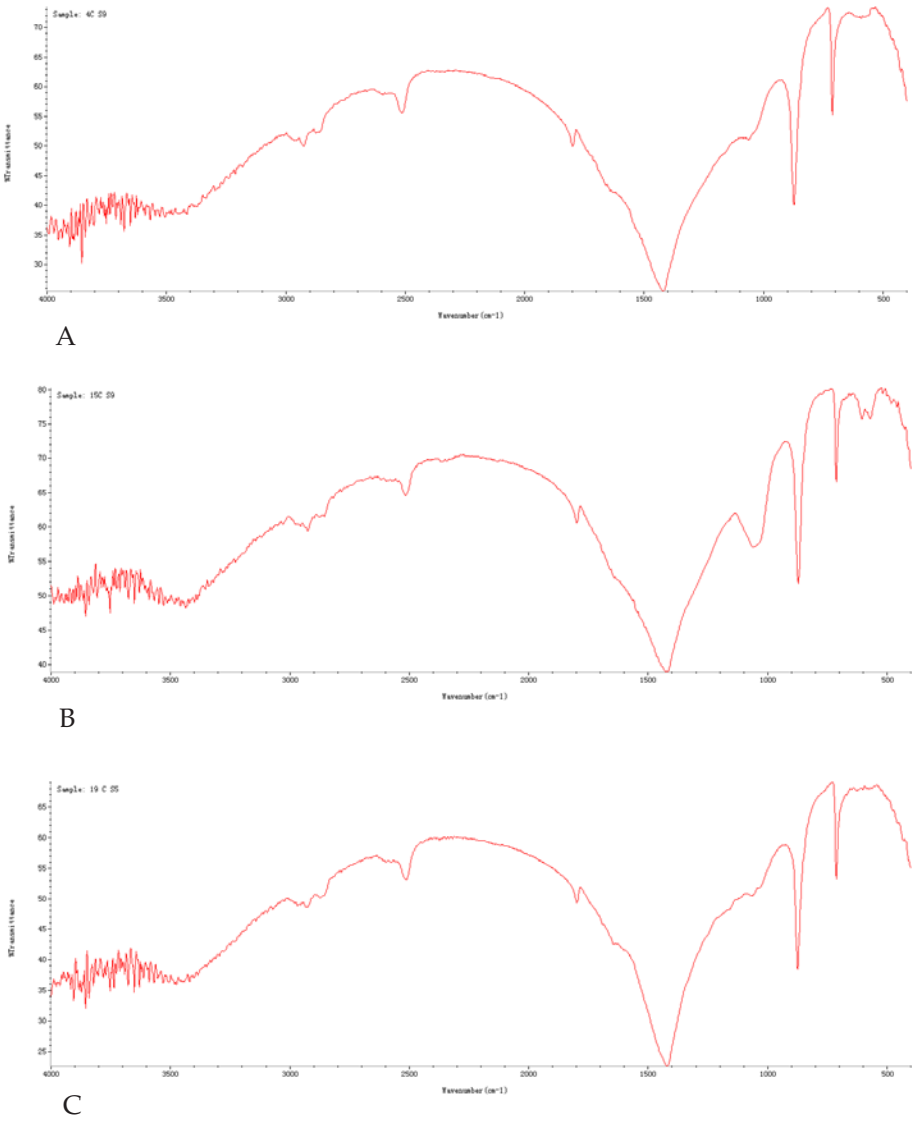

Fig. 4. FTIR spectra of isolates A) 4C S6 B) 15C S9 C) 19C S5

viz. 15C S9, 19C S5 and 4C S6 were shortlisted. These isolates were identified using 16S rRNA gene sequencing. The 16S rRNA gene sequences of both the isolates - 15C S9 and 19 C S5 showed 99.77% similarity with *Sporosarcina luteola* strain Y1^{T}. However, gene sequence of 4C S6 showed 99.41% similarity with *Paenibacillus fonticola* strain ZL^{T}.

Conclusion

This current study mainly aimed to isolate bacteria from different environments thought to harbour microorganisms capable of MICP. The subsequent objective was to assess the ability of these bacteria to independently precipitate calcium carbonate. The bacteria isolated from the environmental samples revealed capabilities of MICP through biomineralization on B4 and CCP medium. A basic technique that involves light and stereomicroscopy of bacterial colonies proved to be helpful in screening of $CaCO_3$ precipitating organisms. The shortlisted isolates having capability to form spores, hydrolyse urea and precipitate $CaCO_3$ has been confirmed for their potential MICP behaviour. Various analytical tests that includes scanning electron microscopy, EDX microanalysis and FTIR spectral analysis established the fact that the shortlisted organisms identified as *Sporosarcina luteola* and

Paenibacillus fonticola are amongst the few that might prove to be promising strains in bioconcrete formation. These findings suggest the possible application of these isolates in the production of bioncrete.

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