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Isolation and Identification of Urolytic Bacteria to Produce Biocement

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ABSTRACT

Application of carbonate precipitation induced by urolytic organisms for improving some properties of cement. Biomineralization is a naturally occurring process in living organism, which induced calcium carbonate precipitation the process of microbially induced calcite precipitation (MICP) is widely used recently in construction engineering in improving compressive strength, durability and self-healing of building materials and cultural heritage. Urease help in mineralization of Calcium carbonate, by hydrolyzing urea present in the environment. It releases carbon dioxide from urease activity, bacteria are able to use urea as sole nitrogen source and produce ammonia, which increase the pH in the proximal environment causing Ca^{+2} and CO^{+3} to precipitate as $CaCO_3$. The microbes isolated from the cemented soil sample are tested for Urea hydrolyzing and tolerance capacities. Urease positive bacteria is used to produce Bio cement in-vitro by incubating it for Three week then the precipitated biomass is filtered and air dried, the biomass is checked for its porosity using Scanning Electron Microscope comparing to the ordinary cement. Multiple applications of MICP such as removal of heavy metal and radionuclides, improve the quality of construction materials and sequestration of atmospheric CO_2.

Key words: Bio cementation, Urea hydrolysis, Scanning Electron Microscopy, Microorganism, Calcium carbonate.

INTRODUCTION

The use of miscellaneous stone materials for artistic terms and as building material ranges from small statues over historic monuments to all kind of different building from the beginning of mankind to all kind of different buildings from the beginning of mankind to the present (Marjadi; 2016). IN the construction sector, concrete is considered as one of the most important building materials around the world. Advancement in concrete

technology is in its strength improvement and its enhancement in durability, using pollution-free and natural methods. This needs to be taken care of at the design stage itself. The general ideas of biomimetics, which can be used for concrete, are biodeposition and Biomineralization (Maheswaran *et al;* 2014).

Biomineralization is defined as a biologically induced process in which an organism creates a local micro-environment with conditions that allow optimal extracellular chemical precipitation of mineral phases. There is great variety of structures and, in nature, more than 60 types of biological minerals have been describes (Arias *et al*; 2017). Bacterially induced and mediated mineralization is a research subject widely studied in the past decades. Due to its numerous consequences, bacterially induced precipitation of calcium carbonate, so-called carbonatogenesis , has attracted much attention from both basic and applied points of view (Dhami *et al*; 2012, Qian *et al;* 2018). The increase in the concentration or decrease in the solubility of the calcium or carbonate in the solution causes the natural precipitation of $CaCO_3$. Abiotic change (e.g. evaporation or change in the temperature or pressure) or Biotic action (microbial action) participates in the natural precipitation of $CaCO_3$. The rate of microbial $CaCO_3$ precipitation correlated with cell growth (Gondaleeya & Marjadi; 2019). These bacteria are able to influence the precipitation of calcium carbonate by the production of urease enzyme. This enzyme catalyzes the hydrolysis of urea to CO2 and ammonia, resulting in an increase of the pH and carbonate concentration in the bacterial environment (Achal et al; 2009, Maheswaran *et al;* 2014).

Calcium carbonate precipitation mediated by microorganisms is basically decided by six factors: (i) Reactive calcium ions available, (ii) Concentration of inorganic carbon in the microenvironment, (iii) pH, (iv) The availability of an appropriate nucleation site, (v) Genetics of urease gene clusters, (vi) Polymorphs of calcium carbonate crystals (Saryu *et al;* 2014, Sensoy *et al;* 2017, Gondaleeya & Mrjadi, 2019). To produce a high urease rate, the bacteria need urea and a protein source to grow. This will be assimilated by the bacteria and used as energy for its metabolism and reproduction. For producing bio-calcite grains, the presence of free calcium ions is compulsory as well (Cuzman *et al*; 2015).

The structure of precipitate produced by bacterial strains was analysed by XRD and SEM (Sensoy *et al;* 2017).

MATERIALS AND METHODS

Isolation of Bacterial Strain:

The organisms were isolated from a cemented soil sample taken from a construction site at saharadarwaja, Surat. Further isolation was carried out by performing serial dilution and spread the diluted sample directly on to sterile Christensen's Urea Agar Plate (1.0 g peptone, 1.0 g dextrose, 5.0 g sodium chloride, 1.2 g disodium phosphate, 0.8 g monopotassium phosphate, 0.012 g phenol red, 15.0 g agar, pH 6.8, 1000 ml distilled water) add 50 ml of 40% sterile filtered urea solution, and incubate at 30°C for 24-48 hours. After that isolated colony was further maintain on nutrient agar plate.

Identification of Bacterial Strain by Biochemical Test

Morphology of microorganisms were identifying by using Gram staining technique. Further Characterization was carried out by various biochemical tests like, Methyl Red test, Voges-Proskaur (V-P) test, Citrate utilization test, Indole production test, Nitrate reduction test, Catalase test, Motility, Starch hydrolysis test. Prepare all the media according to the practical manual and inoculate a 1-2 loop full culture of organism and incubate at 30°C for 24-48 hours and note down the results.

Endospore staining

The selected isolates were further tested for their ability to form endospores by Schaeffer-Fulton endospore staining procedure as per Geeta and Mehrotra (2009). The smears of bacterial isolates were prepared and heat fixed. The smears were covered with a piece of absorbent paper cut to fit the slide and the slides were placed on wire gauze on a ring stand. The paper was saturated with malachite green and the slide was heated until steam could be seen rising from the surface. The slide was removed from heat and reheated to keep the slide steaming for about 3 min. As the paper began to dry, a drop of malachite green was added to keep it moist. The paper was removed with tweezers and the slide was rinsed thoroughly with tap water. The slide was drained and counterstained for 45 second with0.5% safranin. It was then washed, blotted and examined under a

research compound microscope (100×). The vegetative cells will appear red or pink and the endospores will appear green.

Production Media

Bacterial isolate were first enriched in to a nutrient broth (5.0 g peptone, 1.5 g beef extract, 1.5 g yeast extract and 5.0 g sodium chloride per liter) for 24 hr. To carry out the experiments, filter sterilized 2 % (w/ v) urea solution and 25 mM CaCl2 was added to nutrient broth (NBU media) and final pH of the medium was adjusted to 8.0. The culture was incubated at 37°C, under shaking conditions (120 rpm) for 21 days. After 21 days check the pH of the media and EDTA titrimetric method was used to determine the amount of calcium carbonate produced by the urease positive bacterium (APHA, 1989). The amount of calcium carbonate calculated by the formula of [CaCO3 = (V1.M.1000)/V2), V1: consumed EDTA, M: 1 mL EDTA= 0.96 mg CaCO3 V2: sample amount (mL)]

Effect of mutagenic treatment on the Biocement Production

The phenotypic mutants of isolated bacterial strain were developed by UV irradiation. Bacterial strains were grown overnight in nutrient agar plate at 30°C. After incubation plates were exposed to UV using a Philips 20-W germicidal lamp for 20 min, where a less than 10% survival rate was observed. The colonies were randomly selected and transferred onto urea agar base medium (HiMedia, India) to check the production of urease based on the intensity of pink color. Isolated colonies were selected for further studies based on their ability to produce intense pink color. The mutants were cultured at least five times to assure incapability of reverse mutation and compared with wild-type strain for calcite precipitation.

Consolidation of soil cement blocks

For microbial calcification in the soil cement blocks, bacterial cells (OD600 1.0) were grown in NBU media. Sand, soil and cement were sterilized prior to use to eliminate the indigenous microflora by autoclaving at 121°C for 1 h. Soil–cement blocks with density 1.75 g/cm3, soil: sand 1:1 along with 7 % cement content were mixed with 10 % bacterial culture medium. The contents were packed in petriplate lead or base. The specimens treated with bacteria (BBl) and control (C-Bl) were kept at room temperature for 24 h followed by their curing for 28 days by spraying 5ml NBU media/day on the surface of the tubes. After 28 days of curing the specimens were allowed to dry at room temperature. Precipitated CaCO3 crystals on the surface were measured by EDTA titration method (APHA 1989).

Water Absorption Test

For studying water absorption, bacterial treated and control blocks were dried in oven at 50°C and dry weight was measured ($W_{oven\ dried}$) as per IS 3495 (1992). The blocks were then saturated overnight in water and weighed
again ($W_{saturation}$). Water absorption was calculated by using following formula:

$$\%\text{Water absorption} = \frac{W_{saturation} - W_{oven\ dried}}{W_{oven}} X100$$

Molecular identification

Further identification of the isolate was performed using 16SrRNA gene sequencing. The DNA was isolated and the analysis of DNA sequences was performed by using the Blastx software (BLAST), National center for biotechnology information.

SEM analysis

In order to examine MICP in dried samples were collected and mounted on aluminum stubs prior to observation under SEM. The samples on stubs were sputter coated with gold to increase the conductivity and reduce the charge of the specimen. The detector, accelerating voltage and spot size were secondary electron detector, 5–15kV and 3.0, respectively.

X-Ray Powder Diffraction

X-ray diffraction (XRD) was conducted with dried sample after the incubation.

RESULTS

Isolation and Identification of Microorganism

The isolated colonies from the Christensen's Urea Agar were picked out and further maintain on a nutrient agar plate. The isolated bacteria give following test positive /negative after the incubation,

Endospore staining

The endospore designed by green rods within red or pink cells was clearly visible in the results obtained from endospore staining. The endospores in green color are visible as dark black colored rods in Fig. 2 The vegetative cells are visible as light colored rods in

these figures. This indicates that all the selected bacterial isolates are capable of forming endospores. The Fig. 2 which shows the endospore staining picture of isolate 1 may be *Enterobacter spp* .Isolate 2 is *Bacillus spp. and* isolate 3 may be *Proteus spp.* From left to right respectively.

Calcium Carbonate precipitation in the broth state

Directly after inoculation of NB-U/Ca, a white powder appeared in the media and its density increased with incubation. After 21 days of incubation, CaCO3 precipitants were collected and weighed. All the three bacterial isolates precipitated calcite. The highest amount of calcite (1.13 g) was precipitated by *Bacillus. spp.*. The bacterial isolates 1, and BI 3 precipitated 1.05, and 1.0 g of calcium carbonate respectively.

Effect of mutation on Production media

The phenotypic mutants were developed by UV irradiation and compared with the wild-type strain for their ability to grow at high pH and produce urease activity and calcite precipitation. The growth studied up to 168 h showed that all the mutants increased their growth. The maximum growth was observed with mutant Bacterial isolate 1 (Fig. 4). The pH of the medium was slightly increased with the increase in growth of these isolates. The maximum pH increase of 10.5 was recorded in the case of the Bacterial isolate 1 (Fig. 4)

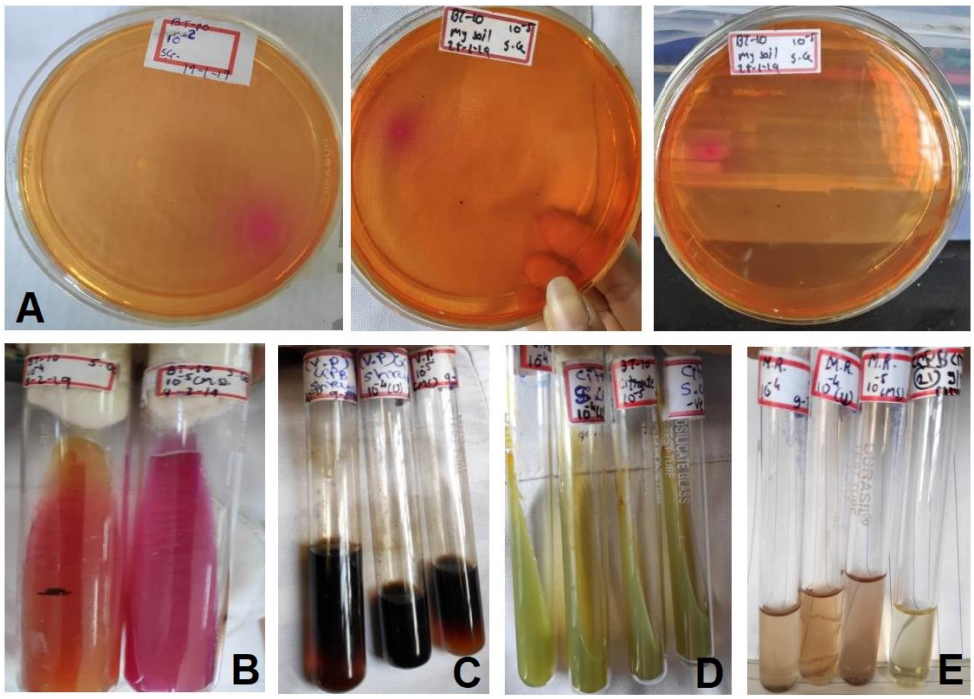

Figure-1 : Isolation and identification of microorganism, (A) isolated colony having pink color zone around the colony, (B) Urease test, (C) V.P. test, (D) citrate utilization test, (D) M.R. test positive and negative with control.

Table 1. Result of Biochemical test by 3 Isolates

NO.	Test	Isolate 1	Isolate 2	Isolate 3
1.	Voges-Proskaur (V-P)	Positive	Positive	Negative
2.	Methyl red (M.R.)	Positive	Negative	Positive
3.	Indole	Positive	Negative	Positive
4.	Citrate utilization	Positive	Positive	Negative
5.	Catalase	Positive	Negative	Positive
6.	Nitrate reductase	Positive	Positive	Positive
7.	Starch hydrolysis	Positive	Positive	Positive
8.	Motility	Motile	Motile	Motile

From the above result the bacterial isolate identify as isolate 1 may be *Enterobacter spp*., Isolate 3 may be *Proteus spp.* and isolate 2 is *Bacillus spp.*.

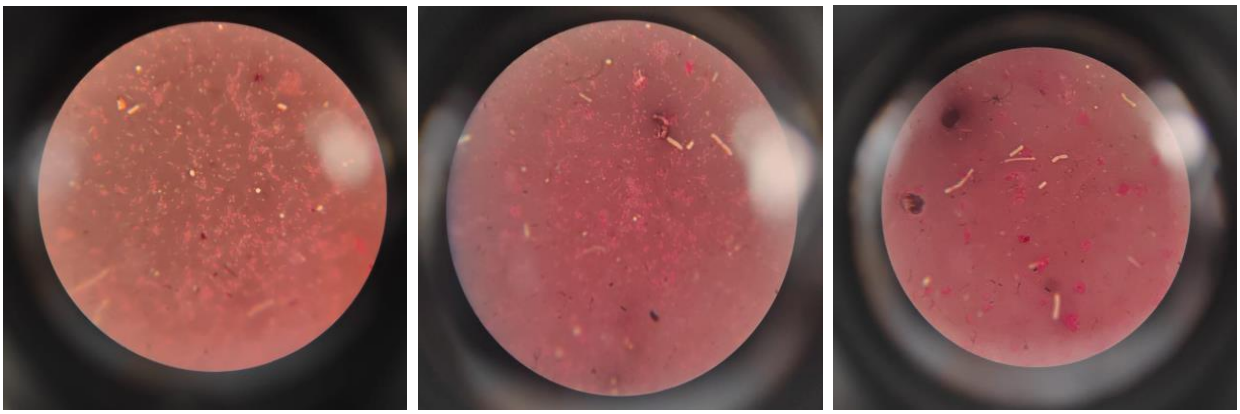

Figure 2: Light endospores appear red in color with colorless cells in all 3 isolates. In Figure Isolate 1, isolate 2, and isolate 3 from left to right respectively.

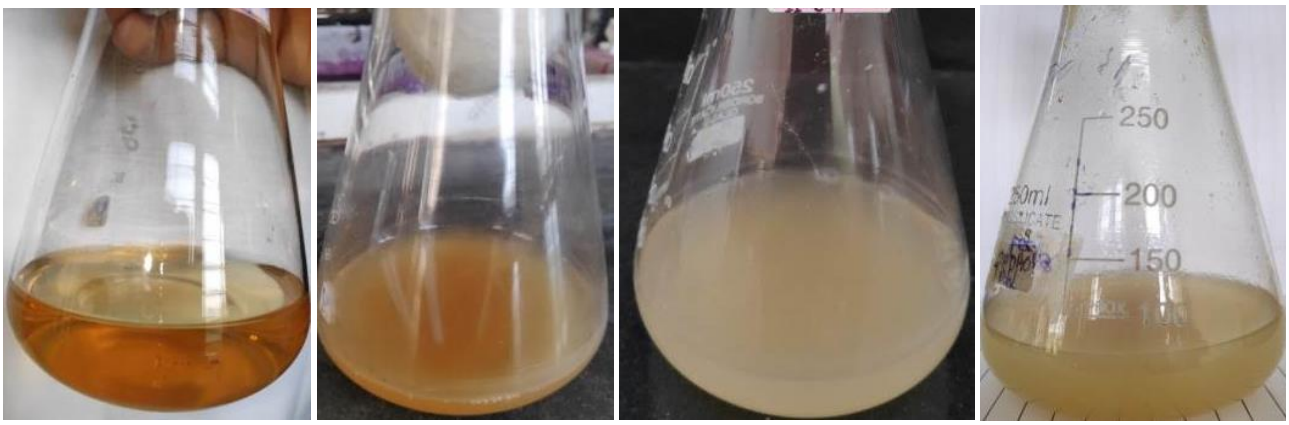

Figure 3: NB-Urea media inoculated with bacterial culture after incubation at day 21 with control. In Figure Isolate 1, isolate 2, isolate 3 and control from left to right respectively.

Figure 4 Production media having mutant bacterial cells after the 21 days of incubation. In Figure Isolate 1, isolate 2, isolate 3 and control from left to right respectively.

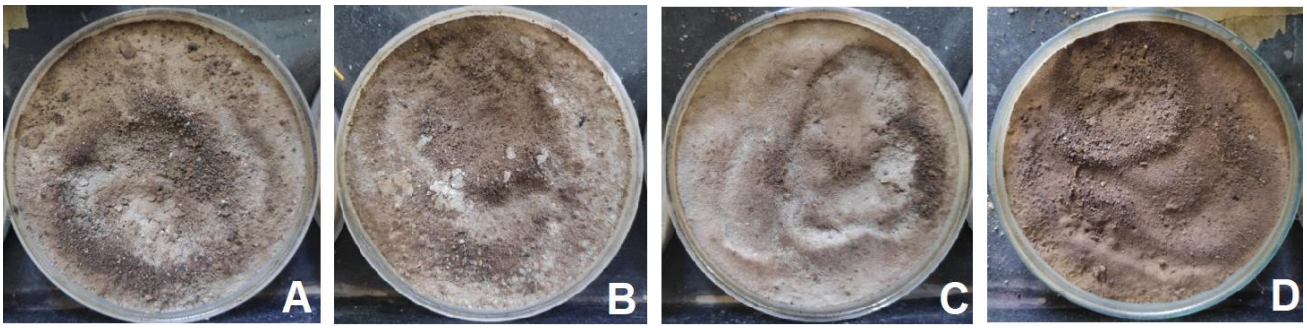

Figure-5 Consolidation block after curing of 21 days by spraying 5ml media/ day showing whitish layer on the block indicate the precipitation. (A) Isolate 1, (B) Isolate 2, (C) Isolate 3 and (D) Control.

Microbial Calcification in soil cement blocks

The biodeposition treatment resulted in the formation of a whitish layer on the surface of blocks which was attributed to the formation of CaCO3 precipitates while in case of control blocks, no whitish deposits were seen. The abundant CaCO3 present on the surface compared to inside is mainly due to higher oxygen availability and active participation of facultatively anaerobic bacterial cells on the surface. The influence of microbial cementation on granular behavior in sand is dependent on the ability of microbes to move freely throughout the pore space and on sufficient particle–particle contacts per unit volume at which cementation will occur (Dhami *et al*; 2013). The crystals on the upper layer completely covered the surface of soil cement grains. The involvement of bacteria in carbonate crystallization was inferred as distinct crystals embedded with bacterial cells (Fig. 5). The presence of crystals associated with bacteria suggests that bacterial cell surface with various ions could non-specifically induce mineral deposition by providing nucleation sites as suggested by other researchers (Dhami *et al*; 2013). Impressions of bacterial cells inside the crystals showed that they had been occupied by bacteria at some stage of crystallization or the cells had completely colonized the crystals.

Water absorption test

The percentage of water absorption in bacterial treated specimens was found in isolate 1 is 14.28%, Isolate 2 is 11.48% and isolate 3 is 18.82, compared to control specimens where it was 27.10 %. This test proved significant decrease in water absorption to reduction in surface permeability and improvement in service life of the specimen.

Molecular identification

The nucleotide sequences were compared with known sequences using the Blastx software (BLAST), National center for biotechnology information. The bacterial isolate 2 was identified as *Bacillus pacificus* MCCC 1A06182 having 95% similarity with *B. paramycoides* MCCC 1A04098 and *B. cereus* ATCC 14579, bacterial isolate 1 and 3 was identified as may be *Enteribacter spp*. And *Proteus spp*. from the biochemical test.

Scanning electron microscopy (SEM) analysis

Calcium carbonate precipitation by *Bacillus pacificus* in the production media was visualized by SEM analysis. The growth of crystalline calcium carbonate was observed in dried media after the production following by filter the media and dry in hot air oven at 60°C for 48 hours.

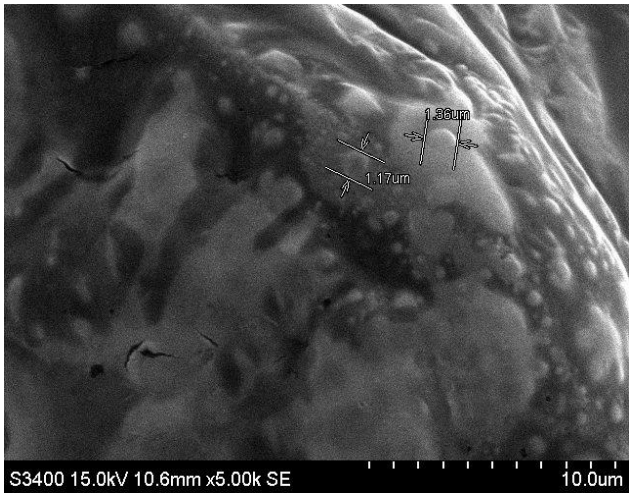

Figure 6 shows Scanning electron micrographs of *Bacillus pacificus* MCCC 1A06182 indicate the presence of Calcium carbonate in the media

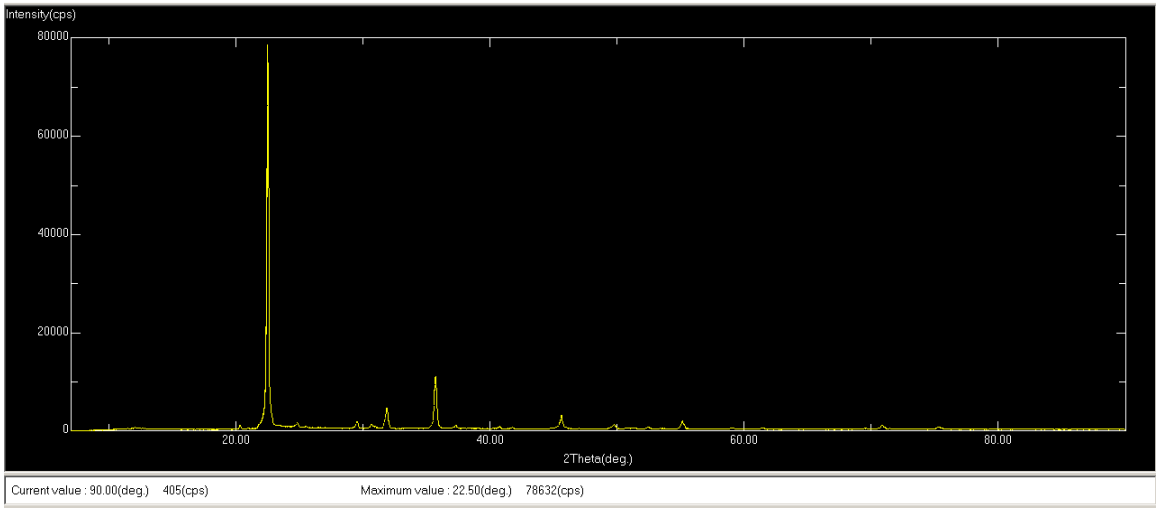

Figure 7: shows X-ray diffraction pattern of crystalline phase present in the production media after the incubation showing the calcite present in the media which is precipitate by the *Bacillus pacificus* MCCC 1A06182.

X-ray powder diffraction analysis (XRD)

The identification of the crystalline phases present in the specimens by XRD analysis was shown in Fig. 7. The major peaks in the XRD pattern were clearly seen at the same degree 2θ which are corresponded to the spectra of calcite in standard sJCPD files. This observation indicated that the microbial carbonate precipitation found in the dried powder was in the form of calcite.

CONCLUSION

All the three isolated bacterial strain exhibited high urease activity. They formed endospores and precipitated calcium carbonate. The newly isolated bacterial strains are identified by performing biochemical test are may be *Enterobacter spp*., and *Proteus spp.* and isolate 2 is *Bacillus pacificus* MCCC 1A06182 identified by 16s rRNA sequencing. Present study has offered new possibilities for using MICP in green building materials through further work need to be done on dealing with the production of ammonia. This method will inspire new perspective for investigating the application of biominerals in green conservation and hence contribute largely to a cleaner environment.

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