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Monitoring biocalcification potential of *Lysinibacillus* sp. isolated from alluvial soils for improved compressive strength of concrete

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ABSTRACT

The present study reports the potential of newly isolated calcite precipitating bacteria isolated from alluvial soil to improve the strength and durability of concrete. A total of sixteen samples of alluvial soil and sewage were collected from the different locations of province Solan (India). For isolation, enrichment culture technique was used to enrich calcite precipitating strains in Urea broth. After enrichment, fourteen distinct bacterial strains were obtained on Urea agar. Based on qualitative and quantitative screening for urease activity, five isolates were obtained possessing higher calcite formation and urease activities (38–77 μmhos/cm) as compared with standard strain of *Bacillus megaterium* MTCC 1684 (77 μmhos/cm). An isolate I13 identified as *Lysinibacillus* sp. was selected for self healing property in the concrete mix of M20. An improved compressive strength of 1.5 fold was observed in concrete samples amended with *Lysinibacillus* sp. over the concrete amended with *B. megaterium* MTCC 1684 after 28 days of curing. The higher calcite precipitation activity was indicated in *Lysinibacillus* sp. by FE-SEM micrographs and EDX analysis.

1. Introduction

Concrete is the most common and cheapest construction material used for building strong structures due to its common ingredients like cement, sand and aggregate (Jonkers et al., 2010). However, deterioration of the concrete by moisture, gases and acids decreases its durability, posing a major concern in its application in the construction (Wiktor and Jonkers, 2011). Any type of crack in concrete structure leads to its deterioration with corrosion of reinforcement bars inside it (Achal et al., 2013). To overcome this deterioration, application of mortar over fracture and application of epoxy gel over the crack has been suggested, however, these methods require proper monitoring and idea of crack (Tittleboom et al., 2010). Alternatively, the immobilization of bacteria in concrete mixture has been suggested as self-healing therapy (Jonkers and Schlangen, 2007; Joshi et al., 2017). The use of this biological repair technique is highly desirable because the mineral precipitation induced by microbial activities is pollution free and natural (Tittleboom et al., 2010, Jonkers and Schlangen, 2007). However, as self healing agent, the bacteria must remain viable in highly alkaline characteristic of concrete and withstand the mechanical stresses during mixing of concrete (Jonkers and Schlangen, 2007). The mechanism for this property has been attributed to enzymatic hydrolysis of the urea into ammonia by bacterial urease, then to carbonate ions resulting in calcite formation (Tittleboom et al., 2010). Previous studies reported that the strains of spore forming *Bacillus* sp. resulted in formation of calcium carbonate due to their urease activities (Krishnapriya et al., 2015). It was found that after 80 days of crack generation, the spore forming alkalophilic unidentified bacteria healed the macro cracks completely up to the crack width of 0.48 mm (Mian et al., 2015). Another strain, *Sporosarcina pasteurii* has also been reported in the micro cracks sealing by filling pores by calcium carbonate, thereby increasing the durability of concrete against water absorption (Chahal and Siddique, 2013) with increased compressive strength (Chahal et al., 2012, Knoben, 2011). It has been suggested that increase in structural life of concrete helps in saving environment due to the emission of harmful gases during the production of cement (Knoben, 2011).

The majority of calcite forming ability has been primarily conducted in model organism of different species of only Bacillus (*B. subtilis, B. cohnii, B. pasteurii, B. pseudofirmus*, *B. alkalinitrilicus*, *B. sphaericus* LMG 22257 and *B. megaterium*) for increasing the durability of concrete (Jonkers et al., 2010; Wiktor and Jonkers 2011; Wang et al., 2017; Huynh et al., 2017; Sharma et al., 2017a). Recently, the urease activity has also been reported in fungi *Pestalotiopsis* sp. and *Myrothecium gramineum* (Li et al., 2015) from calcareous soil, however there are scanty reports on bioprospecting other ureolytic bacterial species from other sources such as alluvial soils for their application as self healing agents

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in the concrete. Therefore, the present study was conducted with an aim to isolate alkalophilic bacteria with an ability to form calcite and to check its efficiency in filling the cracks in concrete autogenously in comparison to standard strain of *Bacillus megaterium* MTCC 1684.

2. Materials and methods

All the chemicals and reagents used in the present study were of analytical grade. The media ingredients were procured from Himedia Laboratory, Mumbai, India. The Portland Pozzolana Cement (PPC) Fly Ash based cement according to IS: 1489-1 (1991) (IS: 1489, 1991) (Jaypee Cement, India) was procured from local market and used for the preparation of concrete and testing of concrete. The oxide composition of Portland Pozzolana Cement (Table S1) was obtained by X-Ray fluorescence and the physical properties of materials used in preparation of concrete cubes are presented in Table S2.

2.1. Isolation of bacteria

A total of sixteen samples comprising eight sample each of alluvial soil (alkaline in nature and rich in Iron oxide and lime) and sewage samples were collected from the different locations of district Solan (Himachal Pradesh, India). For enrichment, 1 ml or 1 g of each sample was added to enrichment urea broth and incubated at 37 °C for 48–72 h. After enrichment, the broth samples were serially diluted and plated on Urea agar (pH 9.4) containing urea (20 g/l), sodium bicarbonate (2.12 g/l), ammonia chloride (10 g/l), Nutrient broth (3.0 g/l), calcium chloride hydrate (25 g/l) (Chahal et al., 2012). The plates were incubated at 37 °C for 48–72 h. The distinct bacterial colonies with appearance of crystalline precipitation were selected and further grown in Urea broth. A standard bacterial species *B. megaterium* MTCC 1684 was procured from Microbial Type Culture Collection (MTCC, IMTech, India) for comparison.

2.2. Identification of bacteria

The isolated bacterial cultures were tentatively identified by standard Gram stain and endospore formation was determined by staining procedure.

2.2.1. Urease activity

2.2.1.1. Qualitative urease assay test. For qualitative urease assay, the urea agar tubes with phenol red (0.018 g/l) as pH indicator was used. The isolated bacterial strains were inoculated in urea agar tubes and incubated at 37 °C for 3-5 days. The tubes were examined daily for change in colour from red to orange indicating positive urease activity (Andalib et al., 2016).

2.2.1.2. Quantitative urease assay test. Electric conductivity test was performed as the quantitative urease activity test (Krishnapriya et al., 2015). An overnight grown culture of all the selected isolates in nutrient broth was centrifuged at 8000*g* for 15 min to obtain the cell pellet. The cell pellet obtained was suspended in deionised water and mixed homogeneously with the deionised water. The Optical Density (OD_{600}) of the suspension was determined and set at 1.0 for conductivity test. The conductivity test was performed in a reaction mixture containing 1 M urea solution (10 ml), deionised water (8 ml) and cell suspension (2 ml). A blank containing 2 ml of deionised water instead of culture was taken as control. The conductivity of 1 M urea solution, deionised water was recorded for the calibration purposes and the conductivity of isolates was compared to that of the *B. megaterium* MTCC 1684. The conductance was recorded at different time intervals from 5 to 90 min.

2.3. Calcium carbonate precipitation test

The quantification of $CaCO_3$ precipitation was determined by inoculating 1%, bacterial culture in urea broth supplemented with calcium chloride. The tubes were incubated at 37 °C for 3–5 days at 130 rpm (Krishnapriya et al., 2015). After 5 days, the broth was centrifuged at 8000*g* for 15 min to get a pellet of the product. The pellet was dried at 80 °C for 24 h and weighed to determine the dry weight of the precipitates (Dhami et al., 2016).

2.4. FT-IR spectroscopy

The Fourier Transform Infrared Spectroscopy (FT-IR) of the precipitates was determined using Agilent Technologies Cary 630 FTIR instrument. The $CaCO_3$ precipitation was confirmed by observation of peaks under 1000–1300 cm^{-1} wave number (Filip et al., 2014). All FT-IR spectra were recorded in the range of 4000–700 cm^{-1} (Sharma et al., 2017a).

2.5. Molecular identification of the isolate

The genomic DNA extraction was carried out using overnight grown cultures of bacterial isolates. The cell pellets from 1 ml culture was mixed with 1 ml of extraction buffer [1 M Tris-HCL (pH 8), 5 M NaCl and 0.5 M EDTA] and DNA was extracted with some modifications (Sharma et al., 2017b). The isolates were identified using 16S rRNA gene sequencing. The PCR reaction was carried with total volume of 25 µl containing 1 µl of DNA, 2.5 µl of 10 × PCR buffer, 0.5 µl of 25 mM $MgCl_2$, 0.5 µl of 10 mM dNTP (Promega) mix, 1 µl each of 10 pmol, 0.25 µl of 5 U Taq DNA polymerase (Intron) using Total Bacteria primers Bact27f (5′-GTTTGATCCTGGCTCAG-3′) and 1492r (5′- CGGCTA CCTTGT TACGAC-3′) (Uphoff et al., 2001). Using reaction cycle with initial denaturation step of 7 min at 95 °C with an amplification of 35 cycles with denaturation step of 1 min at 94 °C then annealing for 40 s at 52 °C with an extension of 1 min at 72 °C and final extension of 10 min at 72 °C. After the complete reaction, the PCR amplified DNA fragments were analysed followed by visualisation in a UV *trans*-illuminator. The amplified 16S RNA gene from different bacteria was sequenced and the sequence data was analysed by using BLAST and Clustal Omega (http://www.ncbi.nlm.nih.gov/). The 16 s rRNA gene sequences have been deposited in gene bank database (Gene Accession No. MF164037).

2.6. Concrete sample preparation with bacteria

Portland Pozzolana Cement (Fly Ash Based) as per IS: 1489-1 (1991) was used for concrete sample preparation. The amount of fly ash in PPC cement was standardised as per Bureau of Indian Standards (BIS) with not more than 5% by weight of grinded clinker. The physical properties of the cement were checked according to the India Standards specifications (Table S2). The sand of zone II (Grade of soil = Medium) conforming to (IS: 383, 1970) along with 10 mm coarse aggregates was used for preparation of concrete mix of M20. The M20 grade mixture comprised of cement (300 kg/m^3), fine aggregate (1172 kg/m^3), Coarse aggregate (1020 kg/m^3) in water 135 l/m^3. The cubes with a dimension of 100 mm × 100 mm × 100 mm were casted using listed M20 grade homogeneous mixture of concrete with and without incorporated bacterial cells. For bacterial culture addition, an overnight grown culture of *Lysinibacillus* sp. I13 and *B.megaterim* MTCC 1684 was centrifuged and the pellet was suspended in buffer. The cell pellet with a CFU count of approx. 10 log CFU/ml was added in the concrete mix with no culture in control.

2.7. Testing layout

The prepared concrete samples, including control sample, were

placed in the separate curing tanks for the purpose of curing. Curing was done for 28 days and then the compressive strength of these samples was recorded by using Compression Testing Machine. Artificial cracks were made by using hexa blade on each concrete cube. The self healing of the cracks was determined by visual as well as FE-SEM inspection of cracked cubes after 7 days intervals.

2.8. Characterization of calcite precipitation by FE-SEM (Field emission − scanning electron microscope) and EDX (Energy dispersive X-Ray spectroscopy)

In order to investigate the specimen's microstructure, some parts of the broken specimens of 28-day compressive strength were chosen to be visualised by FESEM. The calcite precipitation by *B. megaterium* MTCC 1684 and isolate I13 in concrete was determined using Field emission scanning electron microscope (FE-SEM). The energy dispersive spectroscopy (EDX) (FE-SEM Quanta 200 FEG and EDX, Oxford Instruments) was used for morphological and elemental analysis of all the prepared samples. The micrographs obtained from these results helped to get the calcite precipitation results.

3. Results and discussions

The primary objective of this study was to isolate a novel alkalophilic bacterial strain from alluvial soils and sewage samples with calcite forming ability to heal the cracks in the concrete.

3.1. Bacterial strains

A total of fourteen different isolates were obtained on agar plates from sixteen samples (Soil and sewage) are presented in Table 1 with no bacterial growth in two samples. All the isolates were Gram positive rods or coccus in shape. However, the endospore formation was observed in only one isolate later identified as *Lysinibacillus* sp. (Gene Accession No. MF164037). The formation of endospores enables the bacteria to withstand the mechanical as well as chemical stresses encountered during the mixing of concrete (Sagripanti and Bonifacino, 1996). The formation of spores also enables the bacteria to heal the cracks as the water and air enters the concrete (Krishnapriya et al., 2015).

Table 1
Qualitative assessment of bacterial isolates for Urease activity and Calcium precipitation assay.

Isolate id	Source	Phenol red assay	Calcium Precipitation	Dry Weight of Precipitation (mg/g)
Standard	*B. megaterium* MTCC 1684	+ + +	+ +	9.0
I1	Cement	–	+	–
I2	Sandy Soil	+ + +	+	12.0
I3	Sandy Soil	+	+ + +	20.0
I4	Sandy Soil	–	+ +	–
I5	Crusher Soil	+ + +	+ + +	15.0
I6	Natural Soil	–	–	–
I7	Natural Soil	–	–	–
I8	Vegetative Soil	–	–	–
I9	Vegetative Soil	+	+ +	14.0
I10	Vegetative Soil	–	–	–
I11	Waste Water	–	–	–
I12	Waste Water	–	–	–
I13	Sandy Soil	+ + +	+ + +	21.0
I14	Sandy Soil	–	+	–

+ + + Strong positive response, + + Intermediate response, + weak response, – No result

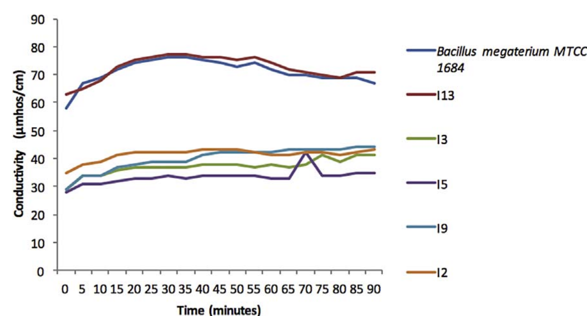

Fig. 1. Urease activity of bacterial isolates in terms of Electric Conductivity (µmhos/cm).

3.2. Urease activity

The results for qualitative and quantitative determination of urease activity are presented in Table 1. Qualitatively, the urease activity is determined in terms of amount of ammonia released from urea termed as the urease activity (Dhami et al., 2016) which changes the colour of the phenol red in the media. The colour of phenol red in urea agar slants changed from pink to orange after 3–5 days. Among fourteen isolates, only five isolates indicated positive urease activity, which were further selected for the quantitative measure of urease activity. Overall, the electric conductance ranged from 28 to 77 µMhos/cm (Fig. 1). The general trend was observed as increase in conductance with time and later on fall in the conductance in all the samples. The isolate I13 resulted in maximum conductance of 77 µMhos/cm in 30 min which was comparable to76–77 µMhos/cm for the *B. megaterium* MTCC 1684. Based on the electrical conductance, the isolate I13 was selected for its potential as healing agent in concrete mix.

3.3. Calcium carbonate precipitation and characterization

The calcium carbonate precipitation (Table 1) weighed in between 9.0–21.0 mg/g dry weight of pellet. The isolate I13 resulted in highest recovery of calcium carbonate precipitation (Fig. 2). The formation of calcite precipitates resulted from the hydrolysis of urea into ammonia and carbonate. The released ammonia increased the pH of the medium therefore favors the binding of carbonate ions to the Calcium. All the calcite forming endospore forming bacteria are reported with this activity (Hammad et al., 2013). The calcite formation helps in healing of the micro-cracks and pores in the concrete. The amount of calcite formation was higher in the five bacterial isolates than the standard strain of *B. megaterium* MTCC 1684 indicating their suitability for use in concrete mix.

The FT-IR Spectra (Fig. 3) indicated the presence of single bond between carbon and oxygen as desired for carbonate group which lies between 1300–1000 cm^{-1} wavenumber. The bands near to wavenumbers of 1716 cm^{-1}, 1122 cm^{-1}, and 846 cm^{-1} indicated the presence of CO_3^{2-} have been reported for confirmation of FTIR spectra of carbonates (Xu et al., 2015). However, in one sample I5, negative absorption was recorded which might be due to presence of water and CO_2.

3.4. Compressive strength

The compressive strength of concrete cubes was determined after 28 days of curing (Fig. 4). The target mean strength was of 24.95 MPa. As it is evident from the compressive strength results, an increase in compressive strength was observed with addition of bacterial culture. An increase of 14.8% in compressive strength was observed in *B. megaterium* MTCC 1684 concrete sample and 34.6% was observed when *Lysinibacillus* sp. I13 was added as compared with control concrete. It is very evident from FE SEM images that calcite formation was clearly noted and as a result pore refine and interface refinement was observed.

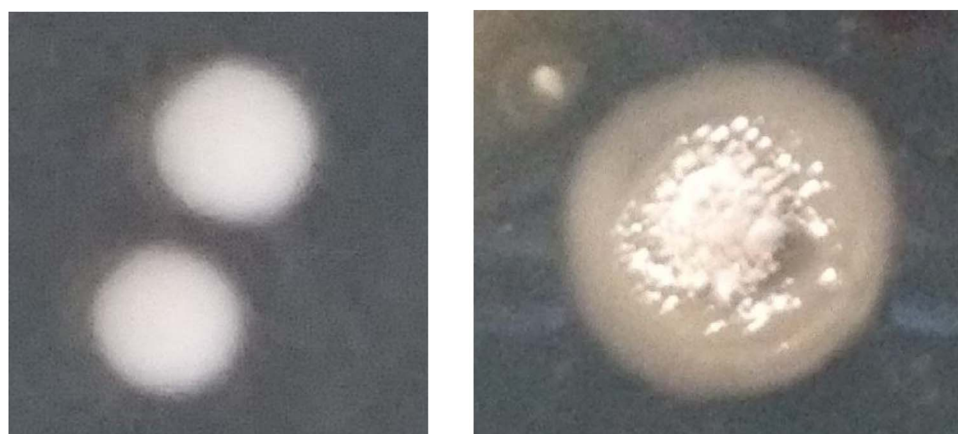

Fig. 2. Calcite Precipitation by *Lysinibacillus* sp. I13 on Urea agar plate incubated for 72 h at 37 °C.

Variable results have been reported on strength improvements (9–25%) obtained by mixing bacteria into concrete. The enhanced compressive strength of the concrete by bacterial strains has been suggested due to the deposition of the calcite of filler materials on the microbial cell surface and within the pores of the concrete (Krishnapriya et al., 2015, Ramachandran et al., 2001). The increased calcite formation could also be due to the nucleation effect of the bacterial cell walls or to the urease enzyme remaining active even in the dead cells. The results indicated that the isolate *Lysinibacillus* sp. I13 resulted in higher compressive strength as compared to the standard strain indicating its potential for use in concrete mix.

3.5. Visual inspection

The photographs of artificial cracked concrete samples (Fig. 5) were taken at regular intervals of 7 days upto 28 days. The calcite formation was clearly seen over the surface of concrete cubes after 7 days and the healing of artificially generated cracks was also visualised. The concrete sample with *Lysinibacillus* sp. I13 resulted in maximum healing as compared to *B. megaterium* MTCC 1684 and control concrete samples. The white coloured layer over concrete cubes confirmed the calcite formation by the bacterial strains.

3.6. FE-SEM and EDX

The microstructure of concrete samples was determined using FE-SEM at different magnifications (500–2500 X) (Fig. 6). The concrete cubes with bacteria resulted in the presence of a crystalline layer on the surface which varied with bacterial culture. The potential calcite formation in each concrete sample was clearly observed in the bacterial incorporated concrete samples at 2500X. The high calcite amount seen in the *Lysinibacillus* sp. I13 amended concrete followed by *B. megaterium* MTCC 1684. However, calcite formation was not observed in control concrete sample. The crystal morphology appeared to be spherical shaped with amorphous. The matrix of the control concrete sample appeared to be amorphous whereas it was heterogeneous for other samples with *B. megaterium* MTCC 1684 and *Lysinibacillus* sp. I13. These results are in agreement of the previous published reports where the matrix of untreated concrete samples appeared to be amorphous without any conspicuous crystal growth and the concrete amended with

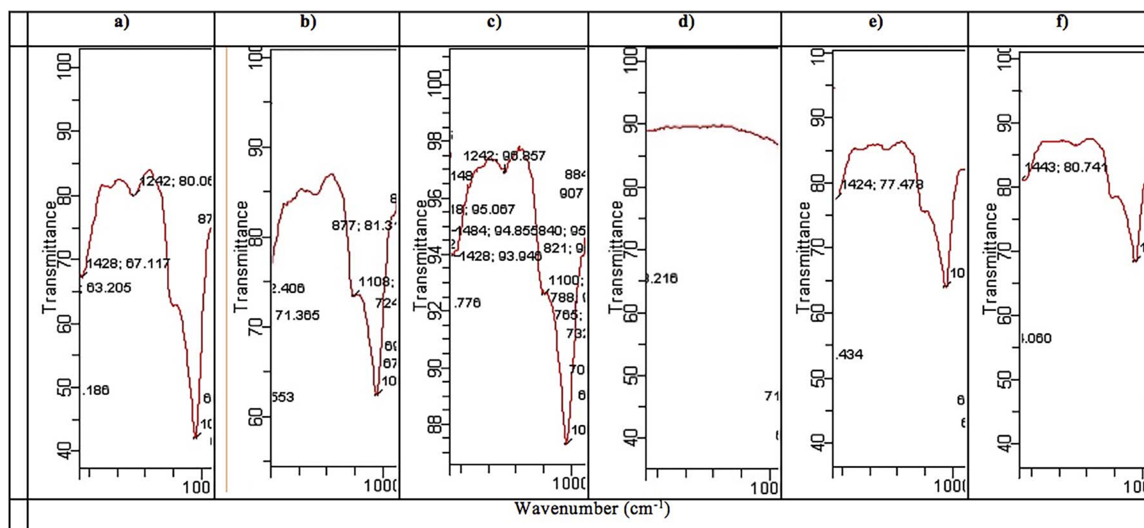

Fig. 3. FT-IR transmittance value for IR wavenumber from 1000 to 1300 cm^{-1} for the precipitated Calcium carbonate by different bacterial cultures. a) *B.megaterium* MTCC 1684, b) I2, c) I3, d) I5, e) I 9 and f) I13.

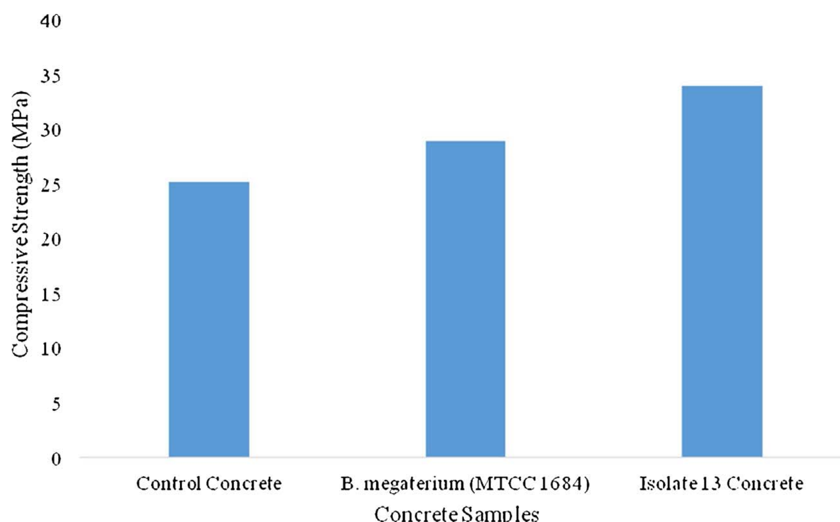

Fig. 4. Compressive strength analysis of concrete mix amended by bacterial culture after 28 days of curing.

bacteria, results in visible individual crystals (Krishnapriya et al., 2015, Ghosh and Mandal, 2006; Siddique et al., 2017). Distinct calcite crystals have also been reported in the studies conducted with *S.pasteurii* (Chahal and Siddique, 2013). The EDX spectra for three concrete samples (control, *B. megaterium* MTCC 1684 and *Lysinibacillus* sp. I13 indicated the increase in Ca deposition in case of *B. megaterium* MTCC 1684 and *Lysinibacillus sp.* amended concrete samples (Table S3) in comparison with control concrete sample. However, a decrease in Al and Si was observed. The decrease of silica is due to the pozzlonic reaction between $Ca(OH)_2$ and SiO_2 forming Calcium silicate hydrate which is the main strength providing compound (Weng et al., 1997). The decrease in Al might be due to the advances in setting of concrete. Al is present in cement in the form of Al_2O_3 which is responsible for the initial and final setting of concrete. As the final setting time of concrete is achieved the rest of the Al_2O_3 is consumed for the formation of ettringite (Brykov et al., 2013). The rod shaped impression in the FE-SEM showed the presence of bacterial mediated crystallization. Nevertheless, based on the observed results, effect of different other factors and their interactions governing the CaCO3 precipitation yield, needs to be optimized, such as bacterial cell concentration, concentration of urea, organic acids or nitrate and calcium concentration in the concrete. The most important part for achieving durable concrete or mortar is to protect the concrete from weathering actions. The resistance towards the penetration of water or any other aggressive chemicals resulted in higher durability. Additionally, to achieve maximum healing characteristics, protection of bacterial strains is suggested via microencapsulation or suitable carrier (Wang et al., 2017)

4. Conclusion

The present study reported the potential of newly isolated *Lysinibacillus* sp. for self-healing of the concrete. The bacterial urease activity and precipitation of calcite resulted in pore refinement and interface refinement, which is being clearly noted in the increment of compressive strength. With the increase of age, this concrete will clearly have reduced permeability to water and other chemicals. The

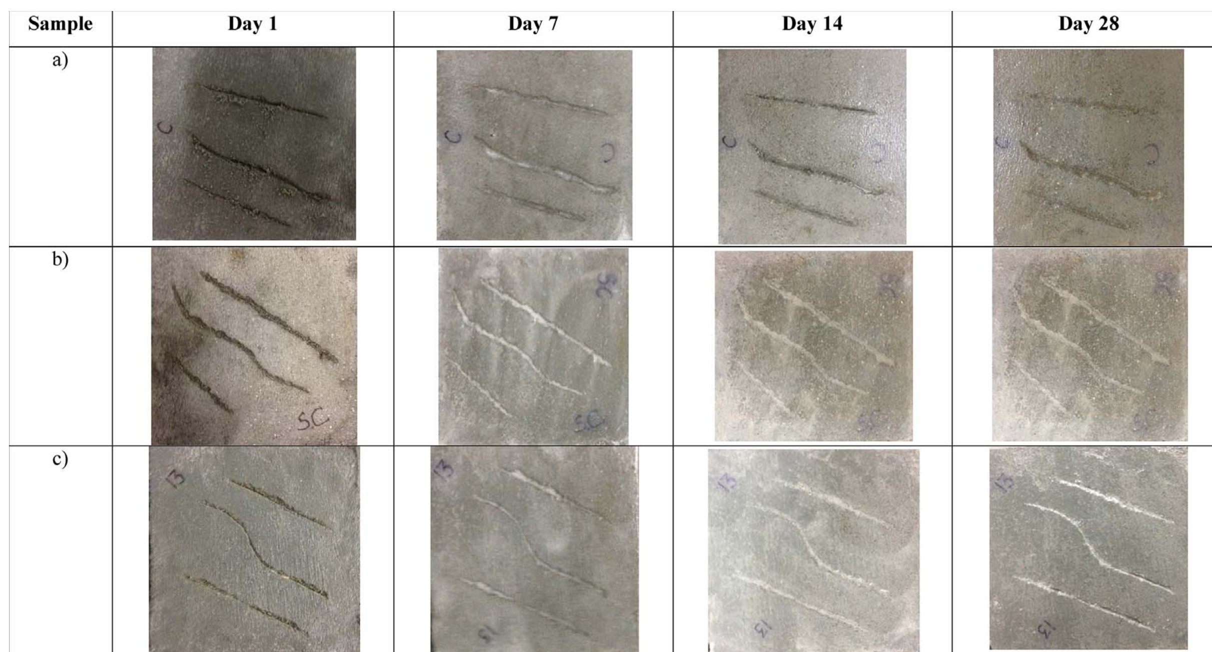

Fig. 5. Visual inspection and quantification of crack healing by bacterial isolates in concrete mix samples at each seven days interval. Control, b) *B.megaterium* MTCC 1684 and c) *Lysinibacillus* sp. I13.

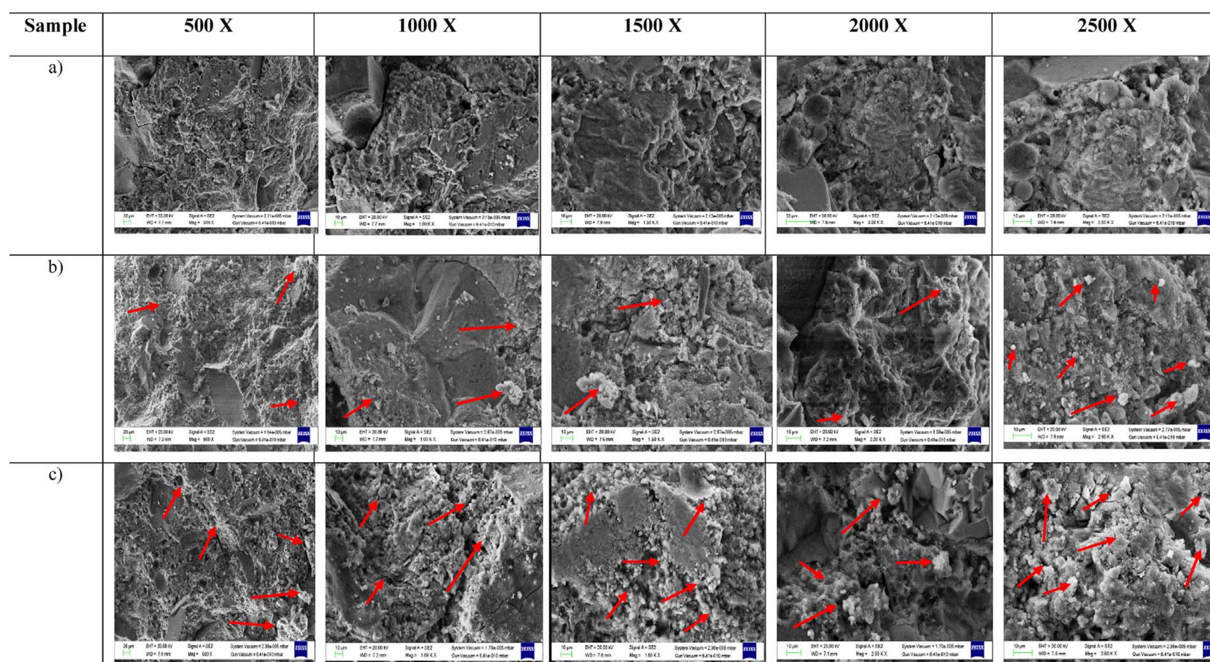

Fig. 6. Microstructure of concrete samples as determined by FE-SEM (Calcite formation shown by red coloured arrows) at each seven days interval (500× at 20 μm; 1000×: 10 μm; 1500×: 10 μm; 2000×: 10 μm); a) Control (No calcite formation found), b) *B.megaterium* MTCC 1684 and c) *Lysinibacillus* sp. I13. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

self healing treatment with bacteria could compete with conventional surface treatments for increased durability of concrete. Further investigations are warranted to study the stress-strain behaviour of the isolate to improve the strength of concrete.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.micres.2017.12.010.

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