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Research Article

POTENTIAL OF UREOLYTIC AND CALCITE FORMING *BACILLUS CEREUS* SSS1 IN BIO-CEMENTATION

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ABSTRACT

Bio-cementation results into formation of deposits of $CaCO_3$ that is essential for the healing of cracks and strengthening the concrete building materials. In this view, 56 ureolytic bacteria were isolated from rhizospheric soil of sugarcane, maize, onion and chickpea on Christensen's agar and screened for calcite formation by growing them on precipitation agar. The culture was inoculated in calcium chloride-urea broth and incubated at 30°C for 7 to 8 days. The calcite crystals were detected by light microscopy, XRD, SEM and EDS. One of the potent isolates, SSS1 was identified by 16S rDNA sequencing as *Bacillus cereus*. XRD analysis revealed the formation of rhombohedral structure of calcite crystals by SSS1. The average size of crystals was 53.75 µm as determined by SEM and EDS. The ability of SSS1 in strengthening of cement sand mortar and healing of cracks was evaluated. The compressive strength of cement sand mortar was 23, 36.2 and 40.6 N/mm^2 on 3rd, 7th and 14th day, respectively and these values were significantly ($p<0.05$) higher than 43 grade cement mortar. Remediation of cracks was experimented by injecting a cementation solution containing urea-$CaCl_2$ and SSS1 cells in the cracks of mortar cubes. Post 14 days incubation, the cracks were found to be sealed indicating filling of void spaces. This potential of SSS1 is advantageous in the making of cement sand mortars with better compressive strength and self-healing property. This study is important towards promoting the use of bacterial concrete in practical engineering applications as compared to conventional ordinary concrete.

Keywords: MICP, Urease, Concrete, Calcite, Calcium carbonate

1. INTRODUCTION

Bio-cementation is a technique that makes use of bacterial activity which leads to the formation of deposit of $CaCO_3$, also called as calcite. Calcite is essential for the joining of soil particles; hence results into strengthening of the concrete building materials and healing of cracks in concrete [1]. Ureolytic bacteria play an important role in bio-cementation. These bacteria produce urease enzyme which is responsible for the formation of $CaCO_3$ precipitate (calcite) through hydrolysis of urea to ammonia and CO_2. This is considered as the most effective microbial reaction used for the microbially induced calcite precipitation (MICP) [2]. MICP is an emerging new sustainable technique that has geotechnical, structural and environmental applications viz., soil strengthening or stabilization, concrete building and crack remediation [3]. Thus, its use in construction materials rather than ordinary cement is highly recommended.

Calcite forming bacteria (CFB) are important in relation to soil stabilization prior to tunneling construction and improvement in the stiffness or strength of sandy soil. Study of cementitious materials is essential to improve the mechanical properties of such materials. The construction sector around the world requires concrete as one of the most central building ingredients [4]; however, formation of cracks in concrete generates complications. Cracking of concrete is an unavoidable phenomenon. Percolation of cracks may lead to leakage problems, causing deterioration of the concrete matrix or corrosion of embedded steel reinforcement [5]. Many times manual inspection and repair of the crack is not possible. Nowadays, bacteria based concrete preparations have being developed in order to extend the shelf life. Thus, self-healing property in the concrete can be achieved with the help of such CFB [6]. Based on continuous research a number of innovations have been made from time to time to improve strength and durability performance of cement concrete. Several ureolytic bacteria have been experimented for calcite formation and their utilization in MICP. *Sporosarcina pasteurii* is one of the model bacteria used at laboratory scale studies [7]. Several species of *Bacillus* have been utilized in these applications. *B. pasteurii, B. subtilis, B.*

sphaericus and *B. lentus* are usually used to initiate and stimulate the calcite precipitation due to urease mediated reaction between $CaCl_2$ and urea [8].

In the view of this background, the major objective of this study was to isolate the CFB and testing their potential in improving the strength of concrete and remediation of concrete cracks.

2. MATERIAL AND METHODS

2.1. Isolation of ureolytic bacteria

The rhizosperic soil samples were collected from the fertilized farmlands of sugarcane, maize, onion and chickpea. The samples were collected from Kolhapur and Baramati area, India, during December 2018. The enrichment was done by inoculating soil samples in Christensen's urea broth followed by incubation at 30°C for 48 h. The enriched broth was inoculated onto Christensen's urea agar followed by incubation at 30°C for 24 h. Pink colored colonies of urease producers were detected on Christenson's agar plates.

2.2. Screening of calcite forming bacteria

For screening of CFB, ureolytic bacterial cell suspension was inoculated onto precipitation agar and incubated at 30°C for 48 h. After incubation the colonies were observed for the formation of precipitate in agar using inverted eyepiece. These bacterial isolates were inoculated into urea-$CaCl_2$ broth followed by incubation at 30°C for 3 to 7 days. A loopful from it was streaked on urea-$CaCl_2$ agar and incubated at 30°C for 24 hrs. The pure culture was maintained at 4°C.

2.3. Detection of calcite formation

Calcite formation by CFB was also detected in urea-$CaCl_2$ broth. The selected CFB isolates were cultivated in urea-$CaCl_2$broth. Following inoculation, broth was subjected to constant shaking for 7 days at 120 rpm at 30°C. After incubation, broth was centrifuged at 10,000 rpm for 5 min. Supernatant was removed and $CaCO_3$ precipitate was harvested and dried in oven for 24 h at 65°C.

2.4. Analysis of calcite crystals

Crystals were observed under light microscope at 40X magnification. Surface structures of crystals were examined by using scanning electron microscopy (SEM) followed by determination of elemental composition by energy dispersive spectroscopy (EDS), and crystalline phase or crystalline structure was characterized by wide angle X-ray diffraction (XRD). All these analyses were carried out at National Chemical Laboratory, Pune, India.

2.5. Phenotypic and molecular characterization of ureolytic bacteria

The isolates were characterized phenotypically according to Bergey's manual of determinative bacteriology. Molecular characterization was based on 16S rRNA gene sequencing [9].

2.6. Potential of calcite forming bacteria in cementation

2.6.1. Improvement of compressive strength of mortar cubes

The cement mortar cubes of size 70mm×70mm×70mm were casted in a hoop. For casting of three cubes 2200 gm of fine sand, 800 gm cement were measured [10]. The cement used for the preparation of mortar was 43 grade Ordinary Portland Cement (OPC) [11]. A volume of 150 ml solution containing bacterial cells (10^6 cfu/ml) and cementation solution consisting of urea-$CaCl_2$ medium was added per mortar cube. Three CFB, namely, MSS1, OSS4, and SSS1, isolates from rhizosphere of maize, onion and sugarcane, respectively, were added to cement-sand mixture. All cementing reagents were properly mixed and casting was done on a vibrating table. Three cubes were prepared for each type of CFB isolate. All the mortar cubes were allowed to set for 24 h. The mortar cubes were demolded thereafter and cured by submerging in water at room temperature. All the cubes provided with sufficient time for hardening. The cubes were then tested on 3^{rd}, 7^{th}, 14^{th} day for its maximum load in the compression testing machine. Compressive strength of cement sand mortar was calculated by following formula: Load / Cross sectional area

2.6.2. Remediation of concrete cracks

An injection method was used to test the potential of bacterial culture in healing of cracks [12]. A two-phase injection strategy was used. For this, bacterial cells grown in nutrient broth were harvested. The bacterial suspension was diluted to a final concentration of 10^6cfu/ml and injected into the cracks. After 2 h the cementation solution consisting of urea-$CaCl_2$ medium was injected continuously for at least 18 h. After that a new batch of inoculation was repeated. Each crack of concrete mortars received an injection volume of 2-5 ml

of cementation solution. This strategy was applied to prevent crystal accumulation around the injection point and led to a more homogeneous distribution of $CaCO_3$. A set of mortars that did not receive any injection served as control. The mortars were observed for the healing of cracks as compared to control mortars after 14 days.

3. RESULTS & DISCUSSION

Concrete is the most widely and globally used material in the construction. The good quality concrete has the properties such as high compressive strength, stability, cost-effectiveness, design flexibility and resistance to fire [7]. However, one of the major problems that affect these properties of concrete is formation of cracks in its structure that may leads to the collapse of construction building materials. Thus, concrete cracks must be repaired immediately to avoid the structural damages and big losses. Attempts should be made in order to prolong the service life of the building materials. There are many conventional methods that have been used for the repairing of concrete cracks viz., used of sealing agents (epoxy or latex binding agents), stitching, overlay and grouting. However, these techniques have several disadvantages such as high cost, modified aesthetic appearance, laborious and environmental pollution. Also, the cement industry generates large amount of CO_2 and there is a huge requirement of energy. Several chemical binding materials viz., acrylic, polyvinyl acetate and butadiene styrene are toxic to animal life [5]. Considering the negative side effects of this chemical approach, there is requirement of safe eco-friendly strategy of remediation. Biological approach through the use of the biochemical reaction of microbial induced calcite precipitation (MICP) is one of such recent eco-friendly technique for the self-healing or remediation of concrete [13].

MICP is performed by many urease producing bacteria or alkalophilic bacteria due to their ureolytic activity [14]. During this process, bacteria generate CO_2 and ammonia from the breakdown of urea through urease enzyme. These carbonate ions react with calcium ions forming $CaCO_3$ crystals. The ureolytic CFB can be isolated from various habitats. For example, *B. subtilis* has been isolated from rice leaves [15]; *B. megatherium, B. licheniformis* and *B.flexus* have been recovered from cement factory soil sample [16]. Kim and Youn [17] have isolated ureolytic *Staphylococcus saprophyticus* subsp. *saprophyticus* from calcareous sand and *Sporosarcina globispora* and *B. lentus* from limestone cave soils. A strain of *Lysinibacillus* sp. has been reported form alluvial soil [18]. Anitha et al. [19] have reported the isolation of ureolytic *B. cereus* from urea-rich paddy soil.

On the similar lines, herein, the urease producing bacteria were screened from soil samples of fertilized farmlands. These samples were collected from the rhizosphere of sugarcane, maize, onion and chickpea where urea is frequently used as a fertilizer. In all, 56 urease producing bacterial isolates were obtained from rhizospheric soil samples by enrichment in Christensen's broth (Fig. 1a) followed by isolation on Christensen's agar plates. Out of these, four potent urease producers were tested for the induction of crystallization in urea-$CaCl_2$ broth (Fig. 1b). The formation of irregular shaped crystals of $CaCO_3$ was detected by light microscopy.

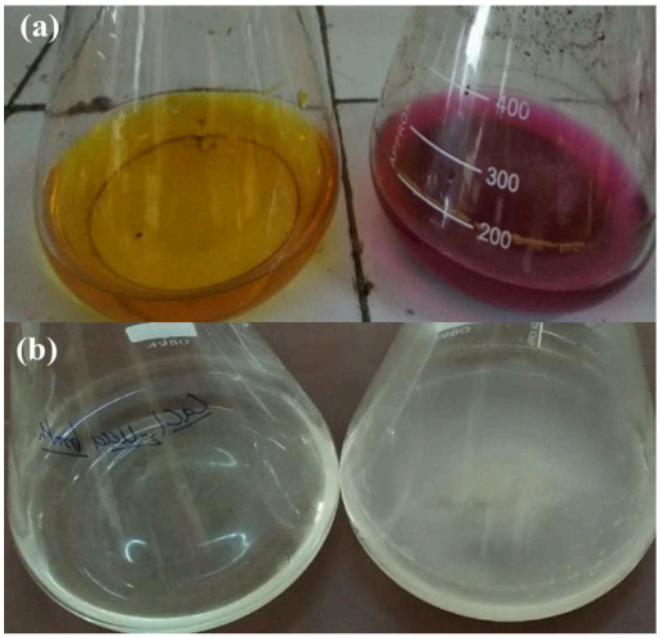

Fig. 1: (a) Enrichment of urease producers in Christensen's urea broth. Uninoculated medium (left) and after inoculation of soil sample over a period of 48 h (right); (b) Test for calcite formation in urea-$CaCl_2$ broth by bacterial isolate. Uninoculated broth (left) and after inoculation of isolate over a period of 7 days (right)

The selected ureolytic bacterial isolates were characterized phenotypically according to Bergey's manual of determinative bacteriology. All the isolates were Gram-positive, spore producing and rod shaped. Based on the preliminary examination the bacterial isolates were found to belong to genus *Bacillus* and one

of the isolate was confirmed as *B. cereus* by partial 16S rDNA sequencing. The sequence was submitted to NCBI GenBank database and the accession number is MN052963. BLAST analysis of this nucleotide sequence revealed close match with *Bacillus cereus*. Using Neighbour-Joining method, a phylogenetic tree was constructed along with different representatives of *Bacillus* genus as shown in fig. 2. There are several reports on the use of several bacteria in MICP. However, due to the very high alkaline pH of the concrete, the use alkalophilic or alkali-tolerant bacteria is recommended. This advantage can be gained from endospore producing bacteria whose spores may survive such extreme conditions due to higher resistance. Ureolytic *Bacillus* strains have been used widely on cementous materials for the concrete surface treatments and management of cracks [20]. Many of the studies reported self-healing of cracks by strains of *Bacillus*, *Pseudomonas* and *Sporosarcina* [21] however, other bacterial species have been also utilized for this purpose such as *Myxococcusxanthus*[22], *Microbacterium* sp. [23] etc. In this study, the selective bacterial isolates were Gram-positive and spore formers that belong to genus *Bacillus* as determined on the basis of phenotypic characterization as stated earlier.

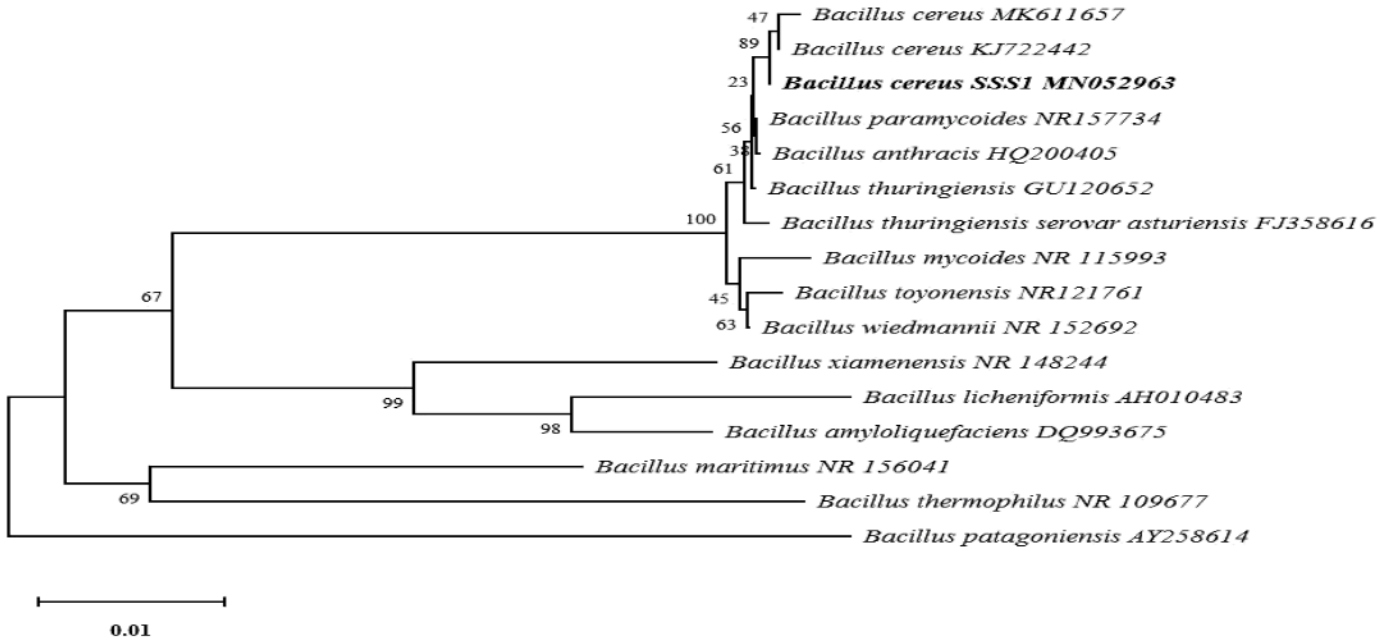

Fig. 2: Phylogenetic relationship of *Bacillus cereus* SSS1 along with representative *Bacillus* sp. based on partial 16S rDNA sequences. MEGA X software was used for the construction of tree

All the isolates were responsible for calcite formation as determined through light microscopic examination. The formation of calcite crystals by selective bacterial isolates was determined by XRD analysis. From graphs and data, it can be concluded that maximum orientation towards 104 plane is observed for all the samples tested. XRD pattern in case of SSS1 sample is shown in fig.3 that confirms the rhombohedral structure of crystals. Further, calcite crystals from a representative sample, SSS1, were analyzed by SEM and EDS for the authentication. The size of crystals produced by isolate SSS1 ranged from 39.55 µm to 69.61 µm with the average size of 53.75 µm. The SEM and EDS pattern of the crystals formed by SSS1 is shown in fig. 4.

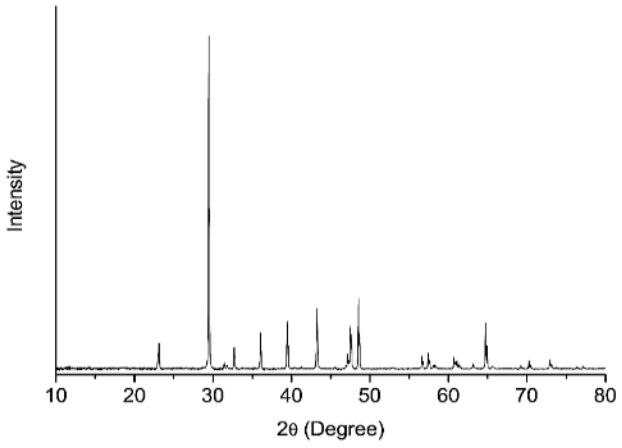

Fig. 3: XRD analysis of calcite from samples treated with *Bacillus cereus* SSS1

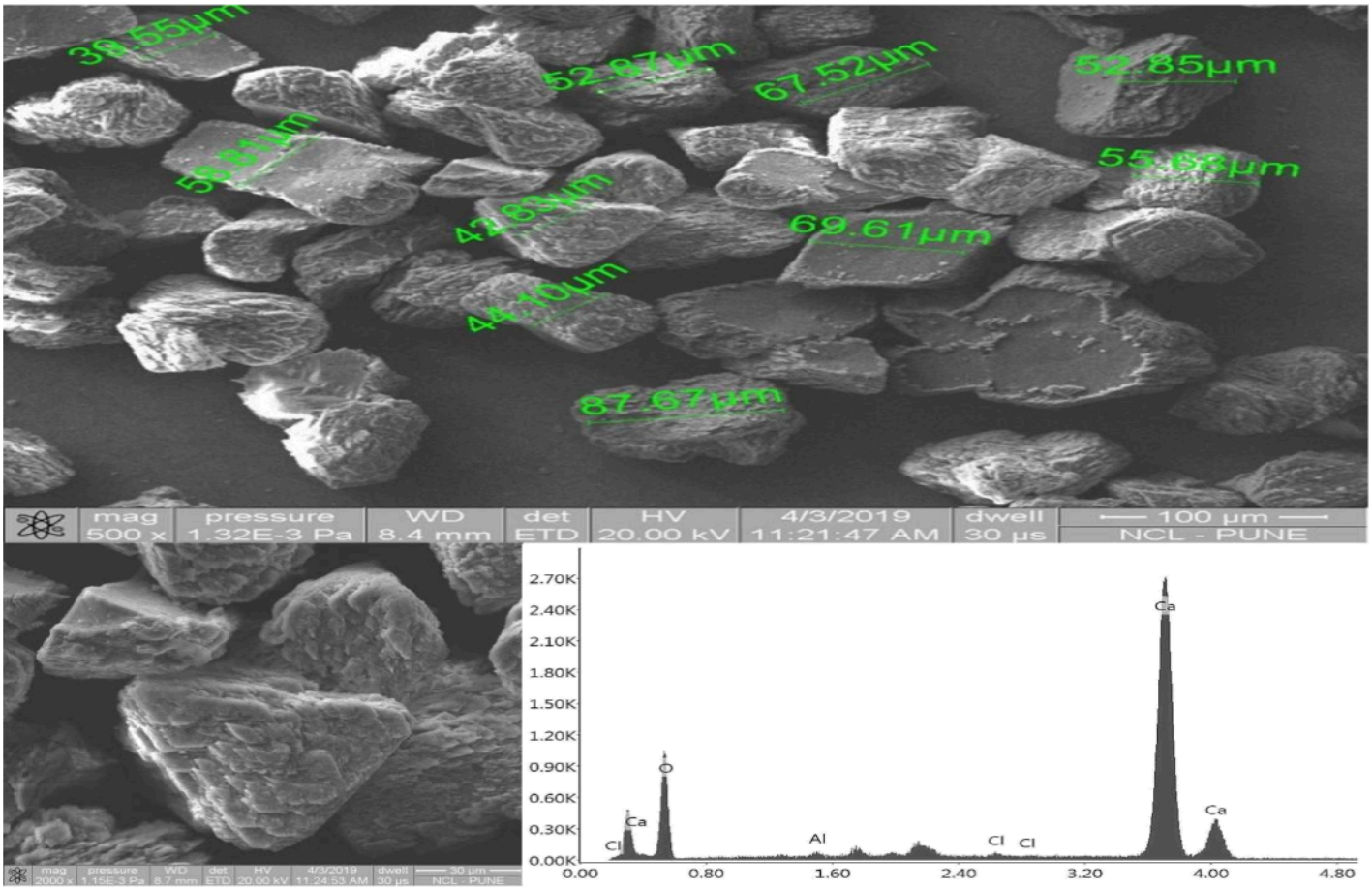

Fig. 4: Scanning electron micrographs of calcite crystals formed through the action of *Bacillus cereus* SSS1 and its EDS spectrum

Further, the potential of ureolytic bacteria in the improvement of compressive strength of cement mortars was determined. The compressive strength is the ability of material or structure to carry the loads on its surface without any crack or deflection. The use of three bacterial cultures viz., MSS1, OSS4, and SSS1, separately, was found to be effective in strengthening of compressive strength as compared with control (43 grade OPC) following 7th day post inoculation (P<0.05). Among bacterial cultures, SSS1 inoculated mortars showed the most significant compressive strength values as compared to those of OSS4 and MSS1 (P<0.05) on all post inoculation days. Thus, this signifies the potential of SSS1 in improving of the compressive strength of mortars as compared with other isolates (Table 1). Thus, through the action of ureolytic bacteria, calcite formation helps in the knitting of sand and cement particles in the mortars increasing their compressive strength.

Table 1: Effect of use of ureolytic bacterial inoculum on strength of cement mortars

Post inoculation period (days)	Compressive strength (N/mm^2)†			
	43 grade Ordinary Portland Cement	Biocement prepared with inoculation of ‡:		
		OSS4	MSS1	SSS1
3	23.0±1.09^{a}	23.5±1.96^{a}	23.0±0.97^{a}	25.0±1.12^{b}
7	33.0±1.33^{a}	35.9±1.33^{b}	36.2±1.87^{b}	38.5±1.88^{c}
14	36.0±1.51^{a}	37.9±1.29^{b}	40.6±1.77^{c}	42.0±1.74^{d}

† Values in a row with different letters indicate significant difference (P<0.05) according to Tukey's HSD; ‡ Mortar cubes casted by the inoculation of bacterial isolates viz., OSS4, MSS1 & SSS1and cementation solution, separately

In addition to this, the potential of ureolytic bacterial isolate SSS1 in the healing or remediation of cracks in the cement mortars was determined using two-stage injection strategy. Bacterial suspension and urea-$CaCl_2$ medium injections were given simultaneously into the cracks of mortars. Following 14 days post inoculation, the mortars were observed for the remediation of cracks in cement mortars. The mortars that received the treatment showed the formation of a homogenous calcite fill along the crack. The figure 5 shows the presence mortar cracks in (a) part on 0 day (or before treatment) and part (b) shows the crack fill area marked by arrows. A significant reduction in the crack width (mm) in the treated mortars after 14 days post inoculation was recorded as shown in table 2. Thus, presence of ureolytic bacterial culture in the treatment area forms calcite crystals through the action of urease enzyme using urea-$CaCl_2$ medium. These calcite crystals are responsible for the filling up of gaps in the cement mortars.

Table 2: Effect of microbial inoculum on healing of mortar cracks

Mortar type	Crack width† (mm) on	
	0 day	14th day
Untreated Control	1.33±0.12	1.32±0.09
Treated with urea-$CaCl_2$ medium with *B. cereus* (SSS1)	1.43±0.11	0.03±0.15

†The data shows mean of three replications ±SD

Fig. 5: Remediation of concrete cracks in the cement mortars. (a) mortar cracks seen on 0 day (before treatment); (b) calcite filled cracks indicated by solid arrows 14 days post-injections with cementation solution containing *Bacillus cereus* SSS1 bacterial suspension

The continuous deposition calcite during MICP process leads to its accumulation which is advantageous in the filling of cracks present in the cement mortars or several such materials. Thus, MICP is most widely used method for the treatment or remediation of damaged stone surface and concrete. Another vital application of microbial carbonate precipitation is that it improves the quality of recycled aggregates for sustainable concrete manufacturing. The calcite deposition on the surface and in the openings of the recycled aggregate obstructs the penetration of water thereby decreasing water absorption and improving the concrete strength [24].

Calcite precipitation treatment is effective in protecting and knitting of porous ornamental limestone materials due to newly formed calcite crystals by ureolytic bacteria [22]. Nowadays, bioconcrete is one of the most viable approaches due to its self-healing properties, improved mechanical strength and durability properties [21]. During the process of remediation of cracks in the concretes or related materials, or strengthening of concrete, the treatment solution containing bacterial endospores and nutrients, $CaCl_2$ and urea are added to cracks or concrete during the mixing process. When the cracking occurs in concrete mortars, the endospores embedded in the cracked area get exposed to moisture and oxygen leading to their activation. The metabolic activities as mentioned above leads to the formation of calcite that heals the cracks [25].

Species of *Bacillus* are reported for their use in the preparation of concrete to increase their shelf-life and healing of concrete cracks. A report by [16] suggests that *B. megaterium* and *B. licheniformis* enhances the compressing strength of mortar and cracks healing. *B.subtilis* satisfactorily increases the concrete strength up to 31% [15]. According to a recent investigation, *B. megatherium* treatment successfully brought the bioremediation of cracks at low temperature [26]. Bacteria other than *Bacillus* sp. such as urelytic *Lysenibacillus* sp. shows self healing property in the concrete and improves the compressive strength of mortars by 1.5 fold [18]. *Sporosarcina pasteurii* is one of the well known calcite forming bacterium used for the improvement in compressive strength of mortars [1]. In the same vein, present investigation reports that addition of spore culture of *B. cereus* SSS1 during preparation of concrete mortars improves their compressive strength.

In general there are two injection strategies utilized for the treatment of cracks in mortar cubes viz., parallel injection method and two-stage or staged injection method. In parallel injection technique, the ureolytic bacterial suspension and cementation fluid are injected at the same time. In latter technique, the bacterial suspension is first inoculated followed by cementation solution. However, it is noted that use of two-stage injection method leads to formation of homogeneous calcite fill as compared to other technique [27]. Choi et al. [28] have demonstrated the bioremediation of mortar cracks of an average width of 0.15 to 1.64 mm were repaired using MICP. Cracks in the cement mortars of width 0.15 mm can be cured effectively using *S. pasteurii* mediated MICP treatment [29]. In like manner, in present investigation, two-stage injection strategy was used that brought the healing of cracks in mortars of width 1.43 mm through formation of calcite fills.

Thus, *B. cereus* SSS1, an ureolytic strain isolated from onion rhizosphere is found to be a potent candidate for improvement of compressive strength of concrete mortars that may increase their shelf-life. Additionally, the injection of cementous solution containing *B. cereus* SSS1 bacterial culture seals the cracks in the concrete mortars and these characteristics appears to be promising in the development of structural engineering strategies in future.

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