Isolation, molecular characterization and self-healing capability of some native isolates of Bacillus sps

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Abstract

The paper describes characterization and sustainable concrete development, by using native isolate of alkaliphilic bacteria *Bacillus stratosphericus*. The isolate has high urease activity, continuous formation of dense $CaCO_3$ crystals and a very negative zeta potential and also persists in extreme environments. The self-healing of experimentally induced cracked concrete varying different widths 0.1 to 0.55mm with different bacterial cells was studied. The bacteria were contemporaneous in the concrete and form calcium carbonate healed the cracks up to 0.50mm. These precipitated crystals can thus fill the cracks. The calcite crystal formation was observed by scanning electron microscopy.

Keywords: Crack-healing, *Bacillus stratosphericus*, compressive strength, SEM analysis.

1. Introduction

Most of the civil engineering structures are made of concrete. After construction of concrete structure, cracks are formed due to the weathering, heat of hydration process, different settlements and uneven distribution of loading on structures. These cracks create major problems such as decreases the durability of structures, waste of money, material and man power. To prevent this crack, apply extra coating of material which further increases the dead load of the structure. To overcome the above drawbacks, the self-healing type of concrete is introduced which increases the strength and decreases maintenance work for the concrete structure [1]. The self-healing property is obtained with a new type bacteria named as *Bacillus stratosphericus.*Excrete of the bacteria helps in filling the cracks [2].

The independent healing of cracks in concrete is one of the thrust areas in the concrete research [3]. The usage of bacteria for ecological engineering purposes is becoming increasingly popularand it was reflected by recent studies on filling of pores and cracks in concrete which has been recently investigated [4]. Barget 2001, Ramachandran 2001[5]; DM Muynck et al., 2008 a[6],b[7]; Ramakrishnan 2007[8]; Henk et al.,2010[3]; Jngxu, Wuyao, (2014)[9]; M.Roigflores et al.,; 2015[10], M.Guadalupe Sierra-Beltran et al (2014)[5], S.KrishnaPriya et al., (2014)[12]. Main Luo, Chung Qian et al., (2015) [13]. For crack repair, a variety of techniques are available, but the traditional repair system has number of disadvantageous aspects Kim et al., (2010) [14]. Therefore bacterially induced calcium carbonate precipitation has been proposed as

an alternative and environmental friendly crack repair technique. Gollapudi et al., Bang et al., (2001)[15,16]. The microbial precipitation of calcium carbonate is determined by the concentration of dissolved inorganic carbon, pH, concentration of calcium ions and the presence of cell wall in bacteria which act as nucleation site. (Hames and W. Verstrate – 2002)[17], Dick et al 2006[18], Jhonkers et al., 2008[19].The bacterially induced $CaCO_3$ precipitation is used to heal cracks in mortar cubes. The following *Bacillus species* are used to know the healing ability of concrete.

Bacillus strainB2-E2-1[20],B.pasteurii[21-28],B.sphaericus[21-24,28-31,14],B.subtilis [21,22,24,28,32],*B.cohnii[22,24,33,34], B.pseuodofirmis[22], B.balodurars*[22,24], *B.subtilis JC3*[33,35,36],*B.pseudo films*[24,37], *B. cohniidsm 6307[38], B.balodurars DSM 497 [38], B.pseudofilmsdsm 8715*[34,38,39], *B.megaterium*(34,41), *B. flexus[28,12], B.sps.CT-5*(40), *Streptococcus*(41), *B.alkalinitrilicus*(39),*B.licheniformis[12].*

2. Materials and Methods

2.1. Characteristics of organism:

Bacillus stratosphericus is a genus of Gram-positive, motile rod-shaped bacteria and a member of the division *Firmicutes*. It is a spore-forming bacterium. Under appropriate environmental conditions, it permit the spores to undergo germination to obtain metabolically active vegetative cell. *B. stratosphericus* determination in adverse conditions include confrontation to UV radiation (42, 43).

Phylogeny

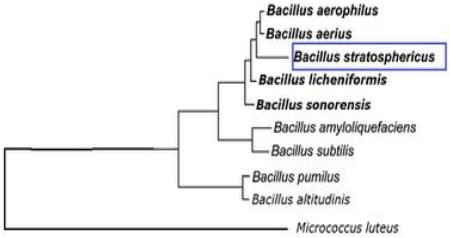
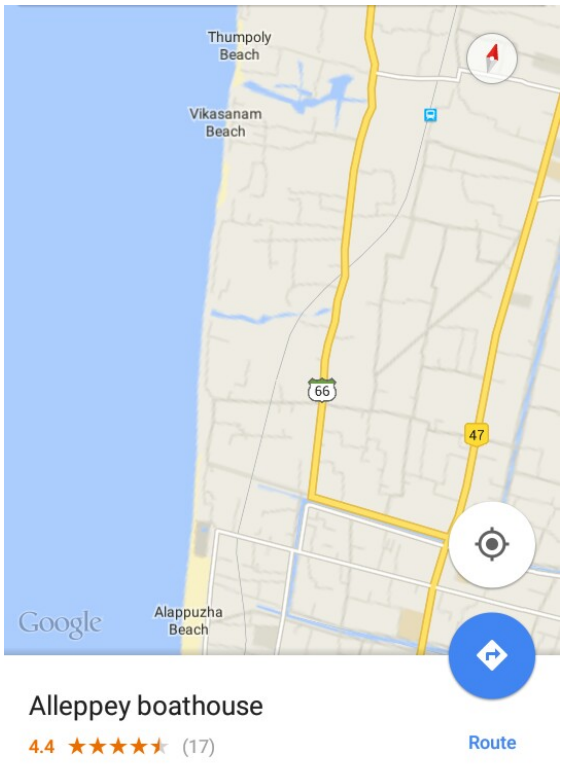


Figure 1: A phylogenetic tree showing relationships between a few closely related *Bacillus* species, including *B. stratosphericus*. The complete genome sequence of *B. stratosphericus* has not yet been determined. Phylogenetic examination founded on the 16S rRNA gene sequence shows a common ancestry with two other novel *Bacillus* species.

2.2. Bacteria strain

Pure alkali resistant bacterial strain was isolated from marine soil samples of Alleppey, Kerala, India. 1 mg of soil sample was suspended in 10ml of sterile distilled water and was subjected to serial dilution. The serially diluted soil suspension was spread on the Petri plates containing LB (Luria Bertani) agar medium of the composition Tryptone 10g, Yeast extract 5g and NaCl 10g in 1 liter of sterial distilled water and incubated at 37^{o}C placed in an orbital shaker for 48 hours while pH was maintained at 12.The purification of the colonies developed during the process was done by a streak plate method and further sub cultured. The selected isolate was identified as *Bacillus stratosphericus* by 16SrRNA sequencing. They were maintained on nutrient agar slants. The collection of soil sample as shown in map below.



2.3. 16S rRNA gene sequence Analysis

For the sequence investigation, bacterial genomic DNA was extracted and filtered using CTAB method. Two primers annealing at the 5´ and 3´ end of the 16S rDNA were Forward: 5´-AGAGTTTGATCCTGGCTCAG-3´, Reverse: 5´-TACCTTGTTACGACTT-3´21 PCR amplification was performed in a finalreaction volume of 100 µl. The PCR reaction was run for 34 cycles in a DNA thermal cycler. The amplifiedPCR products were then analysed in a (1.0) % (w/v) agarose gel, excised from the gel, and purified. Theamplified DNA sequence was then sequenced. The 16S rRNA gene sequence of the isolates was aligned withreference 16S rDNA sequences of the GenBank using the BLAST algorithm available in NCBI.

2.4 Electro conductivity studies for quantitative urease assay

Urease activity assay by conductivity method (Hammad et al. 2013 and Marien et al. 2010[44-45]) was performed. For enzyme assay 1.0 ml of bacterial nutrient growth was added to 9.0 ml of 1.11 M urea solution. Final conductivity was taken after 5 minutes of incubation at 20 C by electrical conductivity meter. Urease activity presented by rate of conductivity increase as mS/minute. Table 1 gives the electrical conductivity (EC (mS/m)) of urease assay mixture at different time intervals.

Table 1 Electrical conductivity (EC (mS/m)) of urease assay mixture at different time intervals.

Time (minutes)	EC(ms/m)
0	128.5
05	139.4
30	145.1
60	151.7
90	157.2

2.5. Bacterial culture and $CaCO_3$ formation

Prepared media was inoculated into LB (Luria Bertani) broth with Calcium lactate of concentration ranging from 2mg, 5mg, 7mg, 10mg, 15mg and 20mg separately in boiling test tubes and incubated for 48 hours at 37^0 C.

2.6. Materials for Mortar *specimens*

2.6.1. Cement and sand

Ordinary Portland cement of grade 53, available in the local market was used for the preparation of concrete specimens. The cement was found to be conforming to various specifications of IS: 12269 (1987) [46] after being tested for various properties conforming to IS: 4031 (1988) [47]. Ennoresand is used according to BIS specifications.

2.7. Preparation of CementMortar *specimens*

The bacterial concentration of 10^5, cells/ml was taken for the preparation of the specimens. Mortar cubes of dimensions

70.7mm × 70.7mm × 70.7mm were casted conforming to IS: 4031 (2000) [52] part 6. The weight ratio of cement and sand was maintained as 1:3. The samples were removed from moulds after 24 hours and cured in water for 3 days, 7 days and 28 days. Control concrete specimens were also casted and cured similarly without adding any bacteria.

2.8. Creation and Incubation condition of cracks

According to the slight modification of Kim et al. 2010 [14], after curing of the concrete specimens for the specified duration. The cubes were taken out of the curing tank after 3 days and left to dry for an hour before it's test. Gradual increase of the load was applied on the cube by compression strength machine until a realistic crack was visible. The maximum strength obtained at the moment of crack formation was observed and load is released. The crack formation was carried out for the cubes, after curing of 7 days and 28 days following the similar procedure.

2.9. Evaluation of crack healing

The healingof cracked specimens was observed after a couple of days. The crack was completely filled by the bacterial spores.

2.10. Scanning Electron Microscopic analysis

The morphology of the calcium carbonate precipitation by *Bacillus stratosphericus* was studied in a FEI QUANTA 200F SEM. Samples were completely dried in the oven at 50^{o}Cfor 48 hours and were gold coated by a Baltec SCD30 sputter coater before examination.

3. Results

3.1. 16S rRNA gene sequence

Based on 16S rRNA test, we had obtained the following sequence.

TGCAGTCGAGCGGACAGAAGGG
AGCTTGCTCCCGGATGTTAGCGGCGGA
CGGGTGAGTAACACGTGGGTAACCTGC
CTGTAAGACTGGGATAACTCCGGGAAA
CCGGAGCTAATACCGGATAGTTCCTTG
AACCGCATGGTTCAAGGATGAAAGACG
GTTTCGGCTGTCACTTACAGATGGACCC
GCGGCGCATTAGCTAGTTGGTGAGGTA
ACGGCTCACCAAGGCGACGATGCGTAG
CCGACCTGAGAGGGTGATCGGCCACAC
TGGGACTGAGACACGGCCCAGACTCCT
ACGGGAGGCAGCAGTAGGGAATCTTCC
GCAATGGACGAAAGTCTGACGGAGCAA
CGCCGCGTGAGTGATGAAGGTTTTCGG
ATCGTAAAGCTCTGTTGTTAGGGAAGA
ACAAGTGCAAGAGTAACTGCTTGCACC
ATGACGGTACCTAACCAGAAAGCCACG
GCTAACTACGTGCCAGCAGCCGCGGTA
ATACGTAGGTGGCAAGCGTTGTCCGGA
ATTATTGGGCGTAAAGGGCTCGCAGGC
GGTTTCTTAAGTCTGATGTGAAAGCCCC
CGGCTCAACCGGGGAGGGTCATTGGAA
ACTGGGAAACTTGAGTGCAGAAGAGGA
GAGTGGAATTCCACGTGTAGCGGTGAA
ATGCGTAGAGATGTGGAGGAACACCAG
TGGCGAAGGCGACTCTCTGGTCTGTAA
CTGACGCTGAGGAGCGAAAGCGTGGGA
GCGAACAGGATTAGATACCCTGGTAGT
CCACGCCGTAAACGATGAGTGCTAAGT
G

3.2. Bacterial culture and precipitation of $CaCO_3$

The bacterial isolate was identified by 16SrRNA sequencing method. The Sequence was compared to BLAST and bacterial isolate was identified as *Bacillus stratosphericus*, as per the similarity sequence. The physical verification was also done by bacterial shape and colonies characteristics. The bacterial colonies were small, dull, light creamy and were about 2 mm in diameter. After incubation, a white precipitate was formed on the surface of the tubes (Fig. 2). EDTA (EthyleneDiamineTetraceticAcid) test was performed for the calcite identification. The test sample was alkalized by using ammonia buffer. By using the EBT (Erichrome Black T) indicator, an end point was achieved having the colour changed from reddish pink colour to steel blue colour (Fig. 3) and the precipitate was identified as Calcium Carbonate. The precipitate was collected from each sample for a specific amount of nutrient added and the weight of each specimen was measured

accordingly. The increased amount of the precipitate was observed by adding the nutrient concentration to the solution as shown in Fig 4.

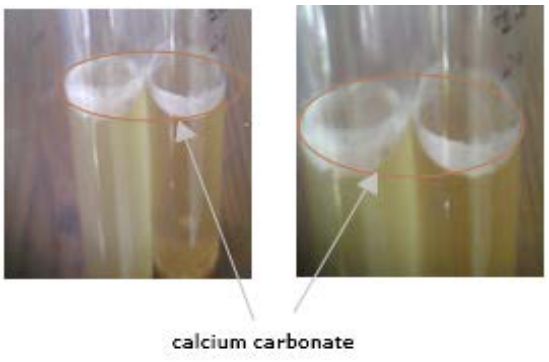


Fig.2 clear precipitate of calcium carbonate

Fig.3 Colour reaction of EDTA test.

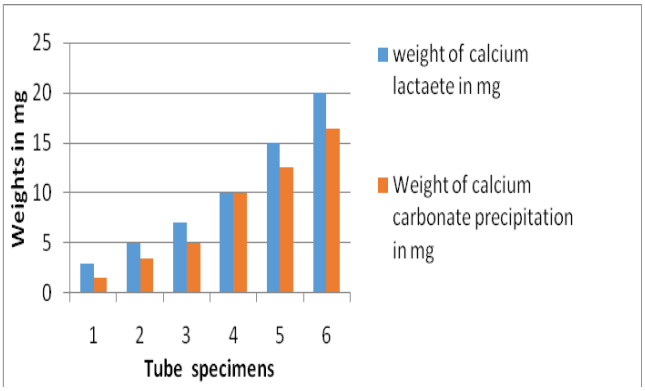


Fig.4 Weight of calcium carbonate precipitation

3.3. Strength studies

The main objective of the present experimental investigation is to obtain specific experimental data, which help to understand the bacterial concrete and its strength characteristics. In the present experimental investigation, studies have been carried out on the behaviour of new and tough property of ordinary grade concrete with and without addition of bacteria. The hardened properties like compressive strength of cement mortar are examined.

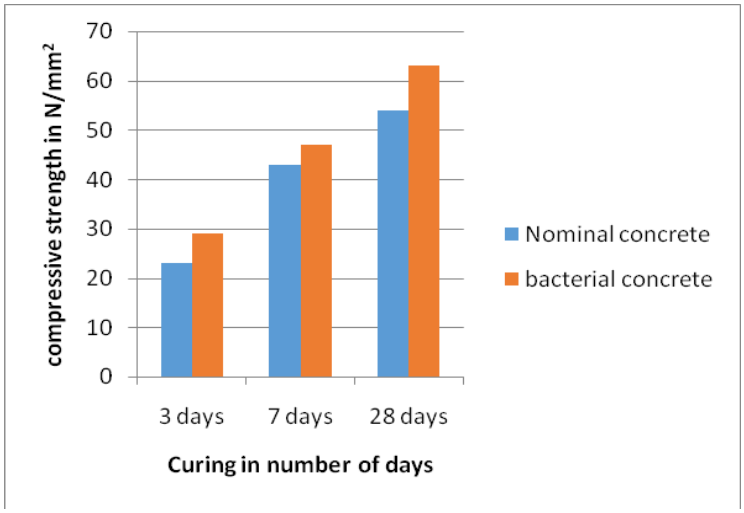


Fig.5 Compressive strength of mortar cubes

3.4. Crack formation and healing

Cracks were formed in the specimens during the compressive test. The healing of cracks was observed after for 28 days of cracking.

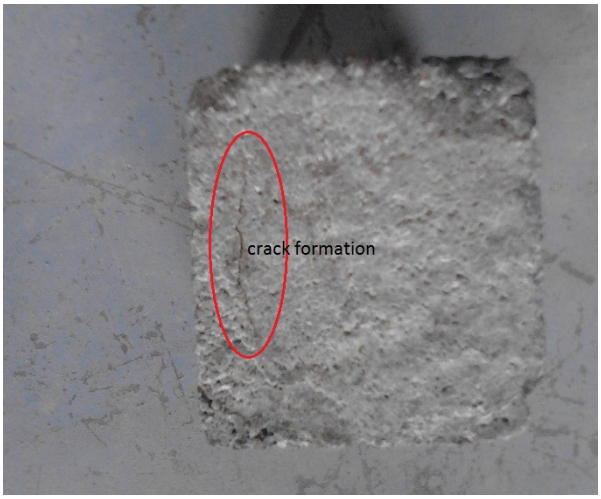


Fig:6 Before crack formation

Fig.7After crack healing

3.5. Scanning Electron Microscopic analysis

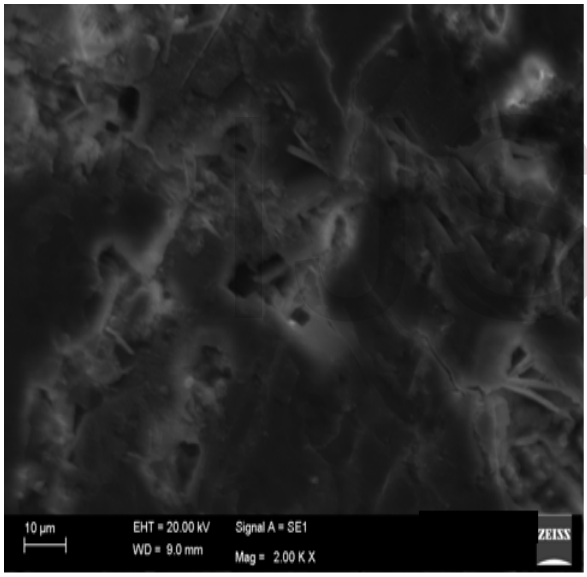


Fig.8Control specimen

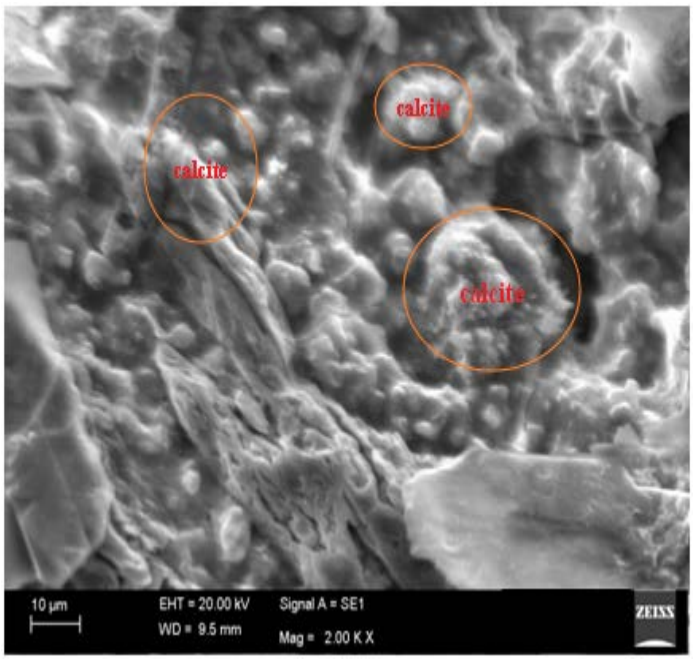


Fig.9.Bacterial concrete

4. Discussion

The bacterial isolate from native marine soil sample identify as *Bacillus stratosphericus* is capable of forming endospores. The endospores forming nature of this species is very special character and also capable of serving in hostile condition. These species were identified from marine. So, bacteria collected from the marine sample which can sustain in the alkaline substance of the similar pH value is considered for the experiment. Due to the similar pH values of the media from which the bacteria are isolated and the concrete, the bacteria can survive in the similar environmental conditions. Spores are metabolically inactive and dehydrated. When favourable conditions occurs, it takes water which can germinate into vegetative cells. (Geeta and Mehrota 2009[48], Micheal et al 1989[49]). Endospores withstand mechanical stress and chemical induced stress during a mixture of concrete (sagripanti and Bonifacino 1996)[50] and can remain variable for 200 years (Schlegel 1993)[51]. When

water and air enters through micro cracks, endospores can germinate into vegetative cells with in concrete and start precipitating calcite to heal micro cracks in concrete. *Bacillus stratosphericus* produce more number of endospores after mixing in concrete.

Bacteria can survive in the nutrient by providing calcium lactate as food.. The bacterial cell wall contains calcium compounds. Normally enzymes degrade complex molecules to simple molecules. Urease is an enzyme that degrades non-ionic urea into ionic compounds. Calcite is one among those products [52-53]. EDTA test confirms the precipitation obtained was calcium carbonate. When EBT indicator is added to the solution, it forms wine red colour, unstable complex at pH 12 with Ca^{2+} ions. When this solution is titrated against EDTA solution, the colour of the complex changes from wine red to deep blue, which indicates the end point i.e. EDTA formed a stable complex with Ca^{2+} ions. The significant improvement in compressive strength of bacterial concrete is the formation of calcite by *Bacillus stratosphericus* within thepores of matrix, which fill the pores. Similar results also represented by other *Bacillus species* Ramachnadran et al. 2001[54], Ramakrishna et al 1999[55], Navneet et al.2012 [56])

The average compressive strength of specimens as shown in the fig.5 from the results observed that the compressive strength of a specimen with bacterial concrete was increased. Moreover, Calcium lactate which was added to the nutrient to feed the bacteria helps in developing $CaCO_3$. So, the increased amount of Calcium adds strength to the concrete after adding the bacteria without affecting any other properties. The compressive strength was increased as shown in figs5, along with the control specimen cured for 3, 7 and 28 days. The healing of the cracks represented from the Fig.7 after the formation of cracks. When bacterial isolate was added to the concrete, the strains are developed and remain immobilized after the reaction with the available oxygen and water in the atmosphere. When the cracks are formed, bacteria start reacting with oxygen and atmospheric moisture, which forms the precipitation of calcite. The precipitated calcite that fills the cracks can be understood as crack healing.

A SEM micro graph for the control specimen without having any bacteria is shown in Fig.8.in which no crystals are observed from which it can be understood that no precipitation is formed. It is the reason for the low compressive strengths of the specimens. Fig.9. show the SEM micrographs for the specimens after crack healing. Crystals were noticed in the pictures which are formed due to the calcite formation by the bacteria during healing. Since the deposited calcite fills the cracks, the strength is increased for the specimens.

5. Conclusion

In conclusion, the results presented in this study shows that native isolate of alkali-resistant spore forming bacteria *Bacillus stratosphericus* represent promising microbial source for application as self-healing agent in concrete. The experimental study proves that incorporated calcium lactate to calcium carbonate upon activation by crack ingress in atmospheric condition. The crack was more and more difficult to repair with increase of average crack width and repair ability of *Bacillus stratosphericus* was limited for specimen with crack width up to 0.50mm, In future more influence factor and molecular mechanism of concrete crack self-healing under laboratory, construction site condition need to be researched for better practical applications can be considered. Self-healing of concrete results in crack free and durable concrete structures.

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