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APPLICATION OF BACTERIAL BIOMASS IN BIOCEMENTATION PROCESS TO IMPROVE THE STRENGTH OF CEMENT CONCRETE

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ABSTRACT

Application of bacterial biomass in biocementation process to improve the compressive, split-tensile strength of cement concrete is considered as the primary objective of the present study. Bacterial concrete was prepared using M-Sand isolate, *Bacillus megaterium.* Compressive strength and split-tensile strength of the developed concrete was tested using Bureau of Indian Standards (IS: 516-1959). During the analysis, the ability of *Bacillus megaterium* that act as a potential agent in increasing the compressive and split tensile strength in developed concrete was highly evident. A significant increase in compressive strength of 31.5MPa and 30.1MPa was observed for the concrete developed using 10^5 cells and 10^6 cells of bacteria after 28 days. Similarly, increase in split-tensile strength of 2.92MPa and 2.78MPa was also observed for the developed concrete using 10^5 cells and 10^6 cells of bacteria. Scanning electron microscopic images revealed that more calcite was produced in the mortar developed with 10^5 Cells/ml of bacteria. Energy Disperse X-Ray studies confirmed the increase in elemental composition of calcium upto 32.5% for the concrete mortar developed using 10^5 cells/ml. It is concluded that bacterial cells/spores aided in the deposition and precipitation of calcite minerals in the concrete matrix which influences the strength of bioconcrete.

Keywords: bacillus megaterium, bioconcrete, compressive strength, split-tensile strength, calcium precipitation.

INTRODUCTION

Concrete is considered as the highly essential materials for constructing all types of buildings world-wide [1]. Even though concrete is strong and highly rigid in nature it is highly susceptible to crack formation [2]. Cracks occur due to different physical, mechanical and environmental factors. Freeze-thaw reactions, shrinkage, tensile force and compressive forces are considered as significant method earlier. Cracks and micro-pores thus formed reduce the concrete strength and its durability which is considered undesirably significant [3]. Crack repairing in the constructed structures of concrete commonly done by applying the mortar of concrete at the damaged or cracked space. After normal curing process, bonding takes place naturally at the concrete mortar applied surfaces. Apart from this common method, different other chemical and mechanical methods were used for repairing the cracks and micropores. The chemical methods were reported as expensive, incurs more cost for repair process; causes environmental and health hazards [4].

Hence, microbiologically induced calcite precipitation has been studied and proved an effective alternative technique to repait the micro cracks and pores in concrete [5]. Bacteria have the ability to produce secondary metabolites like enzymes in the natural environment. Urease is one such enzyme produced by soil bacteria considered as confirmatory organisms for the selection in the concrete studies. Urease positive bacteria have been found to influence the precipitation of calcium carbonate (calcite) by the production of urease enzyme which catalyses hydrolysis of urea to produce carbon di oxide and ammonia. This results in increase of pH and calcite precipitation in the bacterial environment [6].

Based on the ability of bacteria to improve the repair mechanisms, few numbers of innovations have been reported to improve the strength and durability of cement mortar and concrete. Van Tittelboom *et al*. [7] (2010) developed bioconcrete using *Bacillus sphaericus* and *Bacillus pasteruii*. Their research analysis reported the increase in compressive and tensile strength of bioconcrete which influences the crack healing abilities of developed bioconcrete. Seshagiri Rao *et al*. [8] (2013) investigated the durability properties of concrete developed using *Bacillus substilis* by performing acid test on the bacterial concrete. In another similar kind of work reported by Ramachandran et al. [9] (2001) increase in compressive and other durable properties were evident when the bioconcrete samples were exposed to harsh conditions like alkalinity, sulphate contents and freeze-thawing. The bioconcrete was developed using *Bacillus pasteruii*. Maheswaran et al. [10] (2014) compared the compressive strength of bioconcrete developed from the wild strain of Bacillus pasteuri and Bacillus Cereus.

Limited research work on concrete developed using the cells and spores of bacterial species has been reported during the literature survey. Hence, *Bacillus megaterium* was isolated from the M-Sand samples and used to develop bioconcrete for the first time in the present research. The ultimate aim of the present study is to use *Bacillus megaterium* as test bacterial species for the development of bioconcrete. This is significantly involved in the deposition and precipitation of calcite minerals (as calcium carbonate) in the concrete matrix. Thus formed calcite results in remediating or repairing the cracks and

fill up the pores in the concrete. The influence of bacterial cells or spores on increasing the compressive strength, split-tensile strength emphasizing the crack healing properties is investigated.

Calcium carbonate as calcite precipitates thus produced considered as a significant binding agent acts as pore-filling medium for the improvement of concrete strength. Calcite emphasizes the adhesive property within the concrete matrix and the resultant factor reduces the capillary pores, increases the durability and strength of concrete. The calcite or calcium carbonate in different crystal shapes formed in the concrete mortar was visually examined using Scanning electron microscope/Energy dispersive X-ray studies (SEM/EDX).

MATERIALS AND METHODS

The present research work was carried out in the Department of Civil Engineering and Department of Microbiology, Karunya University, Coimbatore, Tamil Nadu, India.

Isolation of Calcite Producing Bacteria from M-Sand Samples

M-Sand samples were collected from the construction material agents, Coimbatore, Tamil Nadu, and India. Samples were serially diluted using microbiological standards and spread plate method was adopted to isolate the bacterial species. The samples were cultured to check their morphology on nutrient agar (NA), which contained peptic digest of animal tissue 5g/l, sodium chloride 5g/l, beef extract 1.5g/l, yeast extract 1.5g/l, and agar 15g/l, and the final pH of the medium was found to be 7.4 ± 0.2 at 25°C. The culturing was done by spreading the stock culture of the bacteria onto the plates and allowing it to be incubated for 24h at 37°C. The plates with pure culture of isolate was stored at refrigeration temperature and used for further studies.

Potential for spore-formation and calcite production of these strains was tested by cultivation in specific media. Basic medium was composed of 0.2 g NH4Cl, 0.02g KH_2PO_4, 0.225g $CaCl_2$, 0.2g KCl, 0.2g $MgCl_2{\cdot}6H_2O$ per liter of Milli-Q ultrapure water. For sporulation (spore-formation) experiments, 50mM $NaHCO_3$, 50mM Na_2CO_3 and 20mM sodium citrate was added to the basic medium. To investigate calcite production potential of these bacteria in liquid media, basic medium was amended with 50mM $NaHCO_3$, 100mM sodium citrate and 25mM $CaCl_2$.

Identification of Bacterial Isolate Using 16srRNA Sequencing Method

Extraction of Bacterial Genomic DNA

Bacteria isolated from M-Sand samples were inoculated in Luria Bertani broth (10mg tryptone, 5mg yeast extract, and 5 mg NaCl, pH-7.0). Inoculated broth was incubated at 25°C in the dark. The bacterial mycelium were harvested after 36h of incubation and transferred into sterile freeze dry bottles. After 2 days of freeze dried, the dried mycelium was ground using liquid nitrogen into fine powders. In this study, extraction of DNA of bacterial samples was performed using the bacterial genomic DNA isolation kit RKT13 (Chromous Biotech, Bangalore, India).

Molecular Identification of Bacterial Strain

The most powerful tool to identify the unknown microorganism is to sequence the gene (DNA) coding for 16S rRNA .This was carried out by standard protocols as follows (Chromous Biotech pvt Ltd, Bangalore, India);
Requirements for the genomic DNA amplifications are,

DNA: 1 µl (100 ng), forward primer (5'AGHGTBTGHTCMTGNCTCAS3'): 400ng, reverse primer (5'TRCGGYTMCCTTGTWHCGACTH3'): 400ng, dNTPs (2.5 mM each) 4µl, 10×Taq DNA polymerase assay buffer 10 µl, Taq DNA polymerase enzyme (3U/µl) 1µl, water X µl to make up the total reaction volume: 100µl. The following cycle times were set for the different processes: Initial denaturation at 94°C for 5 minutes, followed by a 35 cycles of denaturation at 94°C for 30 seconds, annealing at 55°C for 30 seconds, extension at 72°C for 1 min, and a final extension at 72°C for 7 minutes. The PCR amplified product was subjected to 1.2% agarose gel (with ethidium bromide) electrophoresis for bp size analysis (Chromous Biotech, Bangalore).

Sequence and Phylogenetic Analysis

Sequencing of the PCR amplified product was performed on ABI 3500 × L Genetic Analyzer of Applied Bio system Micro Amp, USA, using cycle sequencing kit and using Big Dye Terminator Version 3.1. 10 µl of the sequencing analysis mixture contained 4 µl of Big Dye Terminator Ready Reaction Mix, 1 µl of PCR amplified product (100 ng/µl), 2 µl primer (10 pmol/λ) and 3 µl Milli-Q Water. Analysis conditions for sequencing was programmed to - denaturation at 96°C for 1 minutes, followed by 25 cycles of denaturation at 96°C for 10 seconds, hybridization at 50°C for 5 seconds and elongation at 60°C for 4 minutes. The resultant nucleotide sequence was analyzed using the software Seq Scape version 5.2, which follows analysis protocol of BDTv3-KB-Denovo_v 5.2. Jukes–Cantor corrected distance model was used to generate a distance matrix. A minimum comparable position of 200 ignoring alignment insert was used. The phylogenetic tree was created using Weighbor with alphabet size 4 and length size 1000 using utilizing the sequences aligned with a system software aligner Seq Scape_v 5.2.

Concrete Studies

The cement used for mortar/concrete was 53 grades Ordinary Portland Cement (Zuari OPC) conforming to IS: 12269-1987 [11]. Crushed angular aggregate (grading Zone III of IS: 383-1970) with the material size of less than 4.5mm was used as fine aggregates. The locally available coarse aggregate with equal proportion of 12mm size [12] (conforming to IS: 383-1970) was used. Potable water has been used for casting concrete specimens with cemet at a ratio of 0.45.

The concrete was prepared using the proportions of cement, fine and coarse aggregate as per the units presented in Table-1.

Table-1. Proportions of concrete ingredients used for the cube and cylinder preparation*.

Cement	Fine aggregate	Coarse Aggregate	Water
450	597.55	1135.29	203.6
1	1.32	2.52	0.45

*Mix proportion as per IS: 10262-1982

Scanning Electron Microscopy (SEM) Studies of Developed Concrete Samples

The Scanning Electron Microscope is essentially a high magnification microscope, which uses electron beam to produce images of the sample, both top-down. The morphology and chemical constituents of the concrete samples were analyzed with SEM. SEM is a powerful instrument which permits the characterization of heterogeneous materials and surfaces. Powdered sample is used for this study.

Energy Disperse X-Ray (EDX) Analysis

Energy Dispersive X-Ray Analysis (EDX) referred to as EDS or EDAX, is an x-ray technique used to identify the elemental composition of crystals in powdered mortar on the point of spectrum. SEM-EDX analysis was carried out on the powdered samples of the mortar cubes after 28 days of curing. SEM used for the scanning the image of specimen by the help of electron beam. EDX is used to provide elemental identification and quantitative compositional information. The scanning electron micrographs of freshly crushed cement specimens after 28 days curing were taken with Oxford Instrument equipped with an Energy Dispersive X-ray (EDX). The powder of crushed cement mortar was placed on the carbon tape to attach to the sample. High vacuum was pumped with gun vacuum of 1.22 E-9 Mbar. SEM/EDX was done at Nano Science and Technology Laboratory, Karunya University.

Preparation of Concrete Samples for Compressive and Split Tensile Strength Test

The cubes were prepared for concrete mix with bacterial cultures and without bacterial cultures (conventional concrete). Concretes were prepared using three different bacterial biomass cultures using calcite-producing *Bacillus megaterium* as per Indian specifications [13] (IS: 10262-2009). Three different bacterial biomass were further named as B_1 (with 10^4 cells/ml), B_2 (with 10^5 cells/ml) B_3 (with 10^6 cells/ml),,. Thus prepared cubes were tested for its compressive and cylinders for split-tensile strength to differentiate the conventional concrete from the bacterial concrete.

Compressive Strength Test

The compression test was used to determine the hardness of cubical specimens of the prepared concrete. The strength of a concrete specimen depends upon cement, aggregate, bond, water-cement ratio, curing temperature, and age and size of specimen. Mix design is the major factor controlling the strength of concrete. Cubes of size 15cm x 15cm x 15cm (IS: 10086-1982) were casted in the present study. All the specimens were provided with sufficient time for hardening and cured for 28 days. After the specified period (28 days) all the specimens were tested for its maximum load in the compression testing machine. Compressive strength of the test specimens were calculated by dividing maximum load by the cross-sectional area [14] (IS: 516-1959).

Compressive Strength (N/mm^2) = Ultimate load/Cross sectional area (1)

Split-Tensile Strength Test

Split- tensile strength is indirect way of finding the tensile strength of concrete by subjecting the concrete cylinders to a compressive force. Cylinder of size 150mm diameter and 300mm long were casted. After 24 hours the specimen were demoulded and subjected to water curing. After 28 days of curing the cylinders were taken allowed to dry and tested in compression testing machine by placing the specimen horizontal. The tensile strength is calculated from the formula as given below [14] (IS: 516-1959):

$$Ft= 2P/(\pi dl) \quad (2)$$

where, P- is the maximum applied load to the specimen, D- is the diameter of the specimen, L- is the length of the specimen.

RESULTS AND DISCUSSIONS

Identification of Bacterial Isolate using 16srRNA Sequencing Method

Identification of genome was done 16s rRNA sequencing, and the sequence was obtained. This was blasted against the microbial genome database in National Center for Biotechnology Information (NCBI). The BLAST result showed only 98% similarity with other existing bacterial species and thus was found to be *Bacillus megaterium*. Phylogenetic tree was constructed from neighbor-joining program, using bootstrap consensus test with 100 in Phylogenetic Tree Builder and the branch lengths are in the same as those of the evolutionary distances used to infer the phylogenetic tree. The closest neighbours of the isolate was *Bacillus megaterium* strain zjzl-1 16S ribosomal RNA gene, partial sequence (Sequence ID: gb|KF658192.1|) and *Bacillus megaterium* strain EB55 16S ribosomal RNA gene, partial sequence (Sequence ID: gb|KC311342.1|). Based on this similarity the isolated new bacterial strain was identified as a *Bacillus megaterium*.

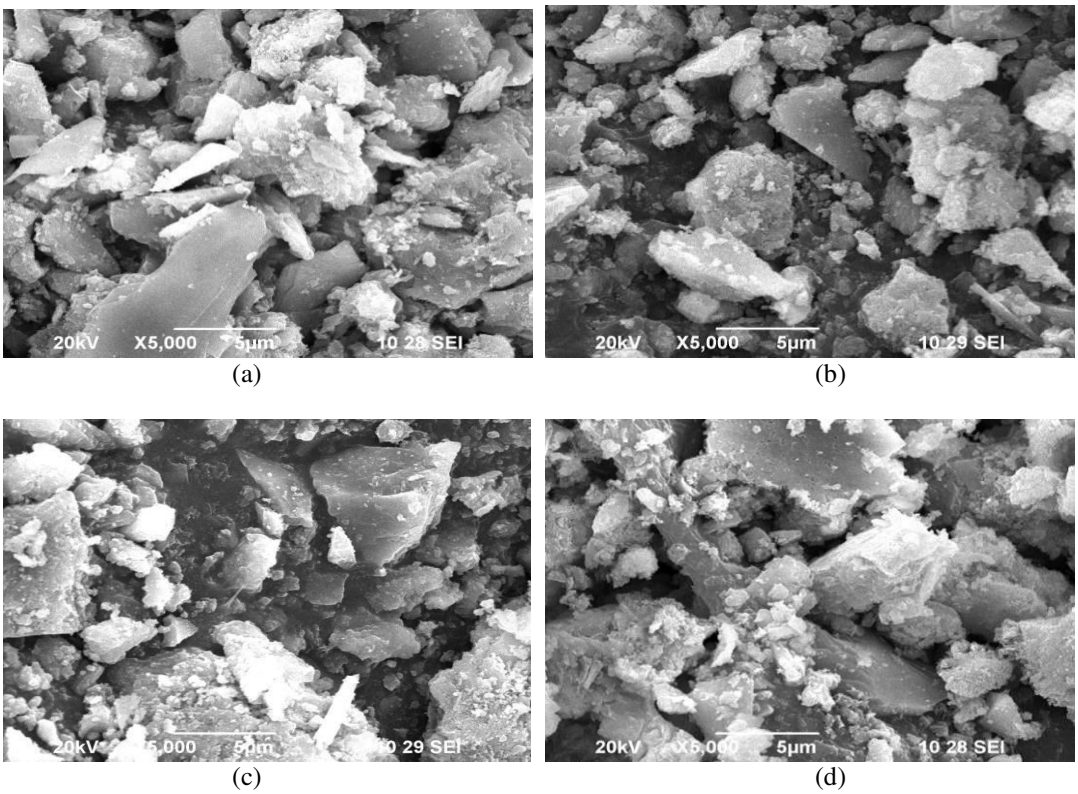
Concrete Studies

The cubes have been tested as per IS specifications. The compressive strength test and split

tensile strength test were carried out both on conventional and bacterial concrete specimens. The conventional and bacterial concrete cube specimens after casting were cured for 28 days in the water bath and were tested in compression testing machine.

Scanning Electron Microscopy (SEM) Studies of Developed Concrete

Scanning electron microscopy (SEM) images of the bacterial free (control) and bacterial mortar samples showed significant difference in the formation of calcite. The microstructure of the developed concrete was observed on the fractured surfaces after mounting on the SEM stubs with gold coating. The strength behaviour of the concrete was mainly depends on calcium–silica–hydrate (C–S–H) phase present in hardened concrete. Different factors influences calcium–silica–hydrate (C–S–H) phase. These factors are size and shape of the particles (topology), distribution of the particles and structure of pores. Increase in compressive strength of bacteria incorporated mortar specimens could be attributed due to the microbial calcite precipitation. The SEM micrograph was captured on powdered sample of 28 days cured concrete mortar under different fields (Figure-1). SEM images give the scanned images of specimens showing the presence of calcite precipitates visualized from SEM microgram. From this observation more calcite was present in the mortar developed with optimum concentration of bacterial cells (B_2 - 10^5 Cells/ml). A network of fine mesh was formed inside the concrete pores with some rod shaped biomineral on the pore surface. These meshes are the calcified filaments which were formed due to the metabolic actions of the bacteria. The biomineral plugs thus formed would act as a filling agent in the cracks of concrete and hence decreased the water absorption and porosity.



(a) (b)

(c) (d)

Figure-1. Calcite precipitation image of SEM (a) Control (b) B_1 - 10^4 Cells/ml (c) B_2 - 10^5 Cells/ml and (d) B_3 - 10^6 Cells/ml

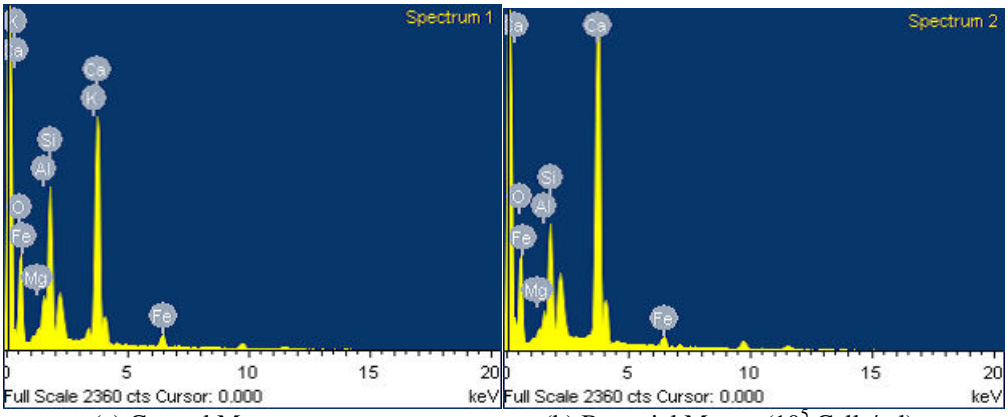
Energy Disperse X-Ray (EDX) Analysis

The elemental composition present in the bacterial and bacterial free mortar samples was determined using this method. In Table-2 it was evident that concrete mortar developed using 10^5 cells/ml provided significant levels in the elemental composition of calcium (32.5%) when compared to that of bacterial free (control) mortar samples (25.9%). The result indicates optimum number of bacterial cells could precipitate more amounts of calcium compounds. *Bacillus megaterium* used in the study produces calcite in the developed mortar emphasizes the metabolic activity of bacteria to promote calcium carbonate precipitation. Calcite or calcium carbonate is considered as a by-product of urea hydrolysis. Earlier, urease activity of *Bacillus megaterium* was determined based on the urease enzyme production. In contrast, the bacterial concrete mortar showed less silica composition (8.5%) compared to control samples (13.12%). The difference in silica composition indicated that the element was not consumed due to absence of bacterial cells in the control mortar samples. EDX spectrum of control and bacterial (10^5 cells/ml) mortar samples showing difference

in the elemental compositions was presented in Figure- 2(a) and (b).

Table-2. Peak values of percentage weight of elemental constituents.

Element	Weight (%)			
	Control	B_1 (10^4 cells/ml)	B_2 (10^5 cells/ml)	B_3 (10^6 cells/ml)
O	51.66	56.4	55.91	53.63
Mg	0.87	1.37	0.72	0.41
Al	3.33	1.03	1.49	2.49
Si	13.12	3.15	8.55	10.03
K	1.28	11.74	-	0.78
Ca	25.95	26.77	32.55	28.42
Fe	3.8	2.53	2.77	2.24



(a) Control Mortar (b) Bacterial Mortar (10^5 Cells/ml)

Figure-2. Energy-dispersive X-ray spectrum

Compressive Strength Test

The compressive strength test results revealed that there is an increase in strength for the bacterial concrete when compared to conventional concrete (Table-3). A significant increase of 31.5MPaand 30.1MPa was observed for respective B_2 and B_3 cell concentrations after 28 days. After 28th day of analysis, bacterial concrete showed significant increase in ultimate compressive strength than the conventional concrete (28.3MPa). Among the three bacterial concentrations used, 10^5 cell concentration of *Bacillus megaterium* culture proved to increase the compressive strength of prepared bacterial concrete (Figure-3).

Table-3. Compressive strength of conventional and bacterial concrete (MPa).

S. No	Concrete samples	Compressive strength (MPa)
1	Control (without bacteria)	28.3
2	B_1 (with 10^4 cells/ml)	29.7
3	B_2 (with 10^5 cells/ml)	31.5
4	B_3 (with 10^6 cells/ml)	30.1

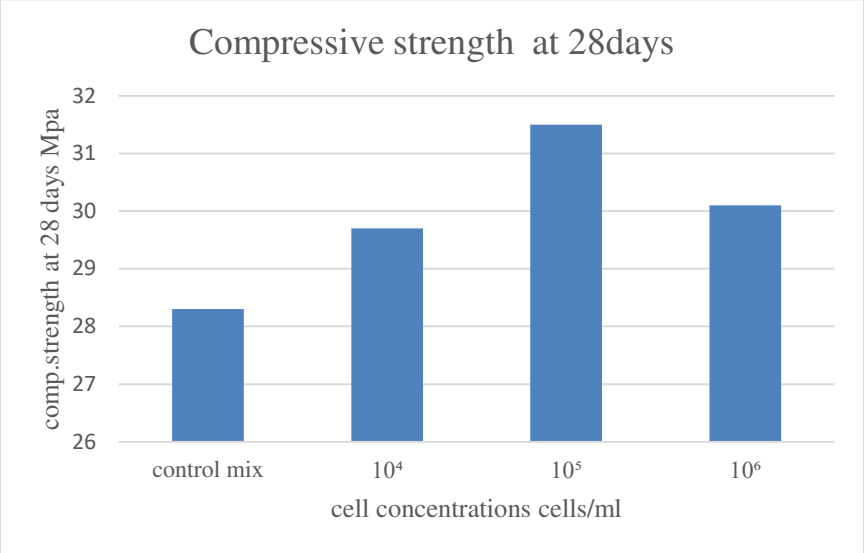


Figure-3. Compressive strength of conventional and bacterial concrete (N/mm^2).

The improvement in compressive strength by the bacterial cells could be attributed to bio-mineralization of $CaCO_3$ on the cell surfaces and within the pores of the cement - sand matrix; pore filling effect within the mortar specimens [15]. Increase in compressive strength after 28 days may be due to increase in pH level. Phosphate buffered saline enabled pH level increase by providing good nourishment and buffering action to microbial cells within the cement–sand matrix. High pH in the cement mortar, improves the growth of microbial cells which increases calcite precipitate formation. The precipitates involved subsequently in filling the pores, which results in reducing the porosity [16].

This enhanced variation in compressive strength confirms the chemically produced urease in the form of $CaCO_3$ precipitation between cement and sand matrix of the cement mortar specimen by the *Bacillus megaterium.* Because of persistence of nutrition in bio-curing process, the bacterial concrete specimen showed higher compressive strength than conventional concrete specimens [17].

Split-Tensile Strength Test

The bacterial culture treated concrete specimens, B_2 and B_3 are tested and split-tensile strength is given in Table-4. It can be observed that the split-tensile strength is increasing in the bio-cured concrete than the conventional concrete specimens. A significant increase of 2.92MPa and 2.78MPa was observed for respective B_2 and B_3 cell concentrations after 28 days; but the control concrete samples exhibited only 2.58MPa. The obtained results emphasized significantly that bacterial culture in B_2 and B_3 concrete specimens biochemically induced calcium carbonate precipitation between cement sand matrix. The microbiological action thus in turn increased the load resisting capacity. Among the three bacterial concentrations used, the higher concentration of *Bacillus megaterium* culture proved to increase the tensile strength of prepared bacterial concrete (Figure-4).

Table-4. Split-Tensile strength of conventional and bacterial concrete (MPa).

S. No	Concrete samples	Split-tensile strength (MPa)
1	Control (without bacteria)	2.58
2	B_1 (with 10^4 cells/ml)	2.69
3	B_2 (with 10^5 cells/ml)	2.92
4	B_3 (with 10^6 cells/ml)	2.78

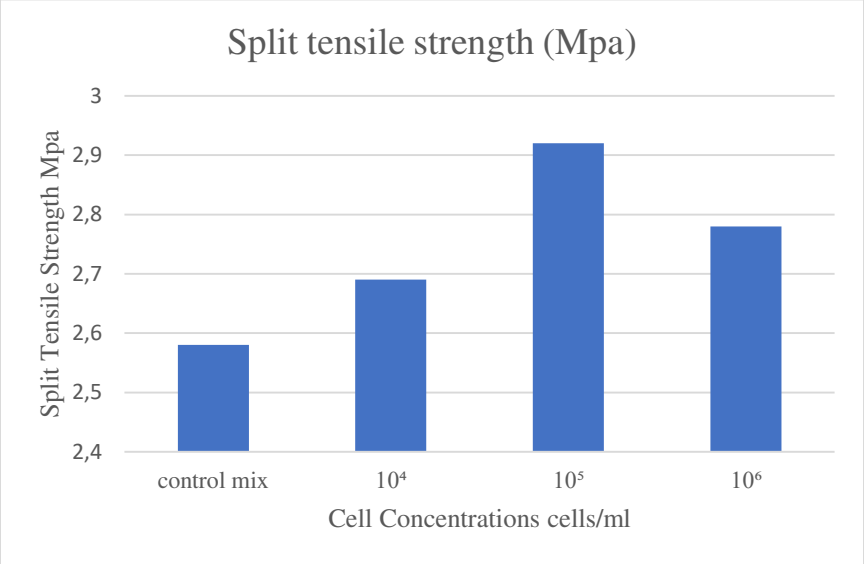


Figure-4. Split-Tensile strength of conventional and bacterial concrete (MPa).

CONCLUSIONS

Ability of *Bacillus megaterium* that act as a potential agent in increasing the compressive and split tensile strength in developed concrete was highly evident. A significant increase in compressive strength of 31.5MPa and 30.1 MPa was observed for the concrete developed using 10^5 cells and 10^6 cells/ml of bacteria after 28 days. Similarly, increase in split-tensile strength of 2.92MPa and 2.78 MPa was also observed for the developed concrete using 10^5 cells and 10^6 cells/ml of bacteria. Scanning electron microscopic images revealed that more calcite was produced in the mortar developed with 10^5 Cells/ml of bacteria. Energy Disperse X-Ray studies confirmed the increase in elemental composition of calcium upto 32.5% for the concrete mortar developed using 10^5 cells/ml. It is concluded that concrete-immobilized bacterial cells in the form of spores could able to seal cracks by biomineral along with improving the strength and durability of cement concrete. The obtained results confirmed the self-healing characteristics of bacterial concrete which would be highly promising in near future in the field of structural engineering.

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