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Development of improved *E. coli* bacterial strain for green and sustainable concrete technology

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ABSTRACT

Development of smart bio-concrete materials has become an emerging area of research for constrictions recently. Here silica leaching attribute transformed to *E. coli* bacterial strain has been utilized for higher strength and more durable concrete structures. The silica leaching gene was fish-out from BKH2 bacterium (GenBank accession no.: KP231522), amplified by PCR technique and cloned into *E. coli* bacteria via suitable T-vector to develop bio-engineered *E. coli* strain. The transformed bacterial cells when incorporated directly to mortar specimens produced high performance bio-composite material. Improvement of compressive strength (> 30%), ultrasonic pulse velocity (> 5%), and decrement of water absorption capacity were noted in the bacteria amended mortars. FESEM analysis revealed rod like crystalline structures within the mortar matrices and XRD analysis confirmed the development of new silicate phase (Gehlenite). The bioengineered *E. coli* cells can be explored directly for green and sustainable high performance composites in near future.

Key words: Bacteria, Bio-concrete, Compressive strength, Durability, Protein.

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1. INTRODUCTION

Increments of compressive strength, rigidity and longevity of concrete structures with the aid of bio-engineered techniques now have gained a momentum in construction field. Due to the heterogeneity of the composition of its principal components, the properties of the final composites can extensively vary.[1] Concrete is typically characterized by a high-compressive strength but low-tensile strength possessing material. The permeability to liquid, consequent corrosion of reinforcement and susceptibility to chemical hazards are the major limitations of concrete structures. Various chemicals, nano-material, steel or other reinforcement materials are widely used in the concrete to increase its strength and durability.[2] In recent times, different types of microbes and their specific enzymes are used in cementitious material to increase the compressive and tensile strengths of the concrete though no reports are available on most commonly used *E. coli* based bioconcrete material.[3-10] Sometimes, this methodology generates self-healing attributes within the concrete which have added advantages for remediation of the cracks and fissures inside the concrete structures.[11-13]

Previous studies have reported that compressive strength and durability of the concrete could be increased by using an extra cellular bioremediase protein (~28kDa) isolated from a hot spring bacterium BKH1.[7, 11] The bacterium BKH1 (Gene Bank accession no.: FJ177512) is a facultative anaerobe and grows gradually (6 -7 days old growth culture contains 10^7 cells/ ml) in a semi-synthetic medium at 65 ^{o}C and pH 8.[14] The bacterium BKH1 is closely related (99.8% homologous) with *Thermoanerobacter thermohydrosulfuricus*. The bioremediase protein is a unique thermo stable and high pH tolerant protein secreted by the bacterium in the growth medium and possesses silica leaching activity.[14] The amino acid sequence of the protein shows 78% homology with bovine carbonic anhydrase II but there is no functional similarity between them (Communicated).

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The aim of the present investigation is to explore the most common *E. coli* bacterial strain having similar characteristics like hot spring microbial consortium for industrially viable and commercially applicable in the development of high performance concretes. This study will also facilitate to develop a modified bacterial strain which will be grown quickly at ambient temperature and normal pH (7.0) in a simplified cost effective medium. Here genetic manipulation technique has been utilized to develop new character within the *E. coli* bacteria (JMJ107) by transferring the silica leaching gene from BKH2. This gene was transferred into a host *E. coli* strain by an appropriate T vector and the transformed cells were incorporated in cementitious mortar to observe its usefulness against the compressive strength and durability of the incorporated material. It has been already observed that *E. coli* itself does not possess the compressive strength increment attribute [7]. This easy and comparatively low cost technology for developing high performance concrete material might be useful for the construction industries where high quality constructing material are needed for buildings, bridges and saving of historical monuments.

2. MATERIALS & METHODS

2.1. Ingredients:

All the analytical grade chemicals used in this investigation were purchased from Sigma Chemical Company of USA, Merck of Germany and Spectrochem Pvt., Ltd. of India. For concrete/mortar sample preparation ordinary Pozzolana Cement 43 grade [15] and standard Ennore sand [16] were used. The E. coli bacterial strain (JMJ107; MTCC No. 1669) received from ICGEB, New Delhi, India was used in this study.

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2.2. Isolation and Molecular Identification of thermophilic microorganism for biosilicification

Mixed population of thermophilic microorganisms were collected from the soil of one of the hot spring (temperature 65 ^{o}C) at Bakreshwar, West Bengal, India (23.8800° N, 87.3700° E). An enrichment culture was developed by growing the mixed population culture in a semi synthetic growth medium containing 0.1M $Fe(OH)_3$, 0.6 g/L Na_2HPO_4,0.33 g/L KCL, 2.5 g/L Na_2CO_3, 0.02% yeast extract and 0.5% peptone at pH 8 and 65°C in anaerobic condition for 7 days as described earlier.[7] Isolation of the pure bacterial strain was done by serial dilution technique from the enrichment culture. The photomicrograph of the isolated bacterium was taken by High Resolution Transmission electron microscope (HRTEM; JEOL, JEM 2100). Gram staining was performed to identify the nature of the bacterium. Silica leaching attribute of the isolated strain was confirmed by biosilicification assay of the supernatant (taken from 7 day old cultured medium) against tetra-ethyl-orthosilicate (TEOS) as described previously.[14] The 16S-rRNA gene sequence primers (Forward-5′CAGGCCTAACACATGCAAGTC3′ and Reverse-5′GGCGGTGTGTACAAGGC 3′) were used for molecular characterisation. The partial sequence of 16s RNA was obtained and submitted to the Gen Bank. For the strain identification phylogenetic tree of the bacterium was developed and named as BKH2.

2.3. Strength measurement

Mortar samples were prepared by incorporating different cells concentration (10^1-10^7 cells/ml water used) of the isolated strain BKH2 to the cement sand mixture to observe the strength increment property. Water vs. cement ratio was used as 1: 0.4 and cement vs. sand ratio was used as 1: 3. Compressive strength of the bacteria amended samples were measured by a digital compression test machine after different days (3, 7, 14 and 28) of air curing and water curing (deionized water) as described earlier.[17]

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2.4. Protein isolation and purification

The bacterium BKH2 secretes few proteins in the growth medium which were purified individually by Sephadex G-100 column chromatography and analyzed for bio-silicification activity.[14] A protein having silica leaching activity like bioremediase was targeted for the gene of our interest. The protein was purified and further applied in mortar samples to observe the strength increment attribute.

2.5. Cloning of the gene that codes for silica leaching protein

Genomic DNA was isolated from the culture pellet of the BKH2 bacterium. Two sets of primers including cloning (restriction) sites for NdeI and XhoI were designed for the study. One set of primer was designed decoding the Amino acid sequence with codon preference for the *Thermoanerobacter thermohydrosulfuricus* which was closely related to BKH1 bacterium.[14] The Primer sequences:

Forward Primer: AGCTCA**CATATG**TCTCATCATTGGGGATATGG

Reverse Primer: AGCACT**CTCGAG**TTGTGCAGGTCTCCAATTTGCTAAC

The second set with respect to Carbonic anhydrase-II of *Bos Taurus* because bioremediase protein was found to be 78% homologous to it [communicated].

The Primer Sequences:

Forward Primer: AGCTCA**CATATG**TCCCATCACTGGGGATACGGC

Reverse Primer: AGCACT**CTCGAG**TTTGGGGAAACCTCTGACTTGTC

The following conditions were maintained for the Polymerase chain reaction (PCR) in this study:

Initial denaturation: 94 ^{o}C - 5′

Denaturation: 94 ^{o}C - 30″

Annealing: 50 ^{o}C - 30″

Extension: 72 ^{o}C - 1′

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Final extension: 72 °C - 15′

The gene was amplified with both sets of primers. Two clones were obtained from two primers but the clone (~800 bp) obtained from the second set of primers (FPO/RPO of Carbonic anhydrase-II of *Bos Taurus*) and whose product showed biosilicification activity was processed for cloning into T vector. Further the positive clones were screened by digesting with NdeI and XhoI to see a release of 800 bp. The clone DNA was purified and given for sequencing. The sequence data obtained from the clone was analysed and found match with the outer membrane porin protein of *Cupriavidus metallidurans*. The clone was ligated with T vector and transformed into *E. coli* (JMJ107) strain through standard transformation process.[18]

2.6. Expression and identification of cloned gene for biosilicification

The transformed JMJ107 and host cell JMJ107 (*E. coli* strain) were grown in Luria Bertani (LB) medium at 37 °C for overnight using ampicillin wherever necessary. The whole cell bacterial proteins were isolated by lysing the cells from both cultures (host and transformed) and run in different lane of SDS page electrophoresis gel along with the Sigma protein marker. The desired protein of interest was purified using Sephadex G-100 gel filtration chromatographic technique. The activity of the purified enzyme was confirmed by biosilicification assay as described by Biswas et al. [14]

2.7. Mechanical characterization of bio-mortar samples

Control mortar samples (dimension 70.6 mm x 70.6 mm x 70.6 mm) were prepared by mixing of Ordinary 43 grade Portland Cement, Ennore sand and deionised water as per IS 4031-1988. The cement: sand and cement: water ratios were similarly maintained as described earlier in section 2.3 of Materials and methods. The LB medium, host cell JMJ107, transformed JMJ107 and BKH2 were used in the cement, sand and water mixture in the same ratio mentioned earlier for preparing test mortar specimens. The concentration of BKH2 cells

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in test mortar specimen was varied from 10^1 to 10^8 cells/ml water used. Whereas three different cell concentrations (10^7, 10^8 and 10^9 cells/ml water used) of host JMJ107 and transformed JMJ107 were used for test samples preparation. The bacterial cell concentrations were determined directly by noting the optical density (OD) of the bacteria grown cultured medium at 620 nm and then by comparing the observed OD with the pre calibrated OD vs. bacterial cells number calibration plot. Serial dilution for required bacterial cell concentrations was done by adding required volume of deionized water to the bacteria containing growth medium. The required volume of water was replaced by the LB medium only in case of LB medium amended sample preparation. The mortar samples were cured under air and water for 3, 7, 14, 28 days at room temperature to observe the gradual development of various mechanical properties within the bio-mortar samples. Digital compressive strength testing machine (rate 0.5 KN/sec) and Pundit plus PC1007 UPV meter were used to determine the compressive strength and ultra-pulse velocity of those samples as per ASTM C597-02.[19, 20] Water absorption capacity of mortar samples was also determined as per Neville's method. [21]

2.8. Microstructure analysis of bio-mortar samples

After 28 days water curing, the control and transformed bacteria amended mortar samples were broken and crushed into fine powder individually by pestle-mortar. Little amount of powder sample was taken and dispersed in absolute ethyl alcohol and dried on the carbon tape in vacuum desiccators. The dried powder sample was examined by Field emission scanning electron microscope (INSPECT F50 SEM, FEI Europe BV, The Netherlands) and Energy Dispersive Spectra (EDS) coupled with FESEM for the structural and elemental analysis.

A pinch of dry powder samples taken from JMJ107 and transformed JMJ107 bacteria incorporated mortar specimens were sieved to achieve the uniform particles size (~5 μm) for XRD (Bruker AXS, Inc., Model D8, WI, USA) analysis. The experiment was conducted at

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40 kV with a scan speed 0.5 s/step. The XRD spectrum was taken from $2\theta = 10$ to 80°. The peaks in the new positions of the spectrum were marked and analysed from the JCPDS data file.

2.9. Statistical analysis

For each category of testing, five samples were tested. Each experiment was repeated at least three times. Data were presented as average (over 15 samples) with ± SD.

3. RESULTS

3.1. Identification of bacterial strain

A novel anaerobic gram negative and rod shaped (2-3 µm long and 0.6 – 0.7 µm width) bacterium (BKH2) was isolated from the hot spring water of Bakreshwar, West Bengal, India and morphologically identified as small rod shaped by HRTEM (Figure 1). This bacterium was found to grow well at 65 ^oC (optimum temperature) in a specified enrichment medium. The strain was also processed for 16S based bacterial identification to confirm its authenticity with primers used for 16S-rRNA amplification. Partial 16 S-rRNA gene sequence (961 nucleotides) of the bacterium suggested that the isolate belong to '*Burkholderiaceae*' family with 97% identity to bacterium '*Cupriavidus metallidurans*' (Figure 2) as analysed by NCBI BLAST. The phylogenetic tree was developed on the basis of BLAST result. The bacterium was submitted to NCBI and named as BKH2 (Gen Bank accession no.: KP231522). The silica leaching activity of a protein secreted by BKH2 was confirmed by biosilicification assay (Table 1).

It was observed that the compressive strength of the BKH2 bacterial incorporated mortar samples was increased with the increasing concentration of cells and time as compared to control (without bacteria) sample (Figure 3). The maximum increment of strength was achieved at a bacterial cells concentration of 10^5 cells/ml water used at all ages (3, 7, 14, 28

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days) when cured under water. More than 30% increment in compressive strength was recorded at 10^5 bacterial cells/ml of water used under water curing for 28 days.

3.2. Isolation and cloning of silica leaching gene

Total genomic DNA of BKH2 bacterium was isolated and purified. The corresponding gene of the bioremediase like protein was fish out from the whole genome of BKH2 by using the primers constructed from the sequence of carbonic anhydrase-II *of Bos Taurus* (Figure 4). The DNA fragment obtained by using the primers was amplified by PCR method. The PCR product (clone) was detected in 1.0% agrose gel containing ethdium bromide and observed by UV transluminatior, Kodak EDAS 290 (Figure 4). The genomic sequence of the clone (774 bp) was determined by standard sequencing procedure and presented in the Figure 5. The isolated clone was then transferred in *E. coli* bacterial strain (JMJ107) through T vector and the transformation was confirmed by growing the bacterial cells in ampliciline containing LB-agar plate as shown in Figure 6.

3.3. Expression of bioremediase like protein

The expression of bioremediase like protein (M. W. ~28 kDa) in transformed JMJ107 strain was shown in Figure 7A. The protein was purified through Sephadex G-100 column chromatographic technique both from the medium of BKH2 grown culture as well as whole cell lysate of transformed JMJ107 (Figure 7B).

3.4. E**valuation of mechanical properties of bacteria incorporated mortar**

Transformed JMJ107 cells showed positive strength increment property in mortar specimens when incorporated to the samples (Figs.8A, B). The best results of compressive strength was achieved when the transformed cells was incorporated at a concentration of 10^8 cells/ml of water used (Figure 8). Figures 9A, B showed the compressive strength of the mortar samples incorporated by BKH2 and transformed JMJ107 bacterial cells under air and water curing respectively. The transformed JMJ107 strain showed better compactness of the

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mortar samples compared to control and also BKH2 strain as revealed by the ultra-pulse velocity test (Figure 10). There was 6% increment of ultrasonic pulse velocity noted in transformed JMJ107 cells incorporated mortar samples compared to the control samples. Least water absorption capacity (6.57% increment) was found in JMJ107 bacteria modified mortar samples compared to the control samples (Table 2). The biosilicification activity of the novel protein expressed by transformed was confirmed and shown in Table 1.

Microstructures of the different mortar matrices were analysed by FESEM which confirmed the formation of novel irregular nano rod shaped material within the transformed JMJ107 and BKH2 incorporated mortar matrices (Figure 13). The new phase appeared within the mortar matrices were confirmed as calcium-aluminium silicate phase (Gelhenite) by EDS (Figure 14) and XRD (Figure 15) analyses.

4. DISCUSSION

The isolated hot spring bacterium (named as BKH2) is small-rod shape and gram negative microbe. The phylogenetic analysis on the basis of 16 S rRNA gene sequences of the newly isolate suggests that the bacterium is a novel and anaerobic by nature. The bacterial strain is closely related with *Cupriavidus metallidurans* sp. under phylum Proteobacteria. The optimum growth condition of BKH2 has been observed at 65 ^{o}C at pH 8.0 in the specified semi-synthetic medium.[14] The bacterium BKH2 is found to increase the compressive strength and durability of mortar samples when incorporated at different cells concentrations to the samples. It secretes few proteins in the medium during its growth. One of the proteins possesses biosilicification activity like bioremediase which has been confirmed to leach silica from silica-rich substrate (Table 1). The silica leaching behaviour of the protein is responsible for higher compressive strength as well as more durability as reported earlier. [11, 14]

E. coli is the most common and widely used bacterium. It can be cultured easily at short duration and ambient temperature in cost effective manner. This bacterium does not have any

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strength increment property like BKH1 or BKH2 that can be used for development of high performance bio-concrete material. Though the bacteria BKH1 and BKH2 show positive strength increment attribute for concrete/mortar samples [7, 22], yet maintenance and growth of these bacteria is a tedious job due to their anaerobic and thermophilic natures. Also large scale production of these bacteria or its protein needs sufficient time as they require 6 -7 days incubation to reach the cells concentration of 10^7/ml of culture. On the other hand, one can get sufficient *E. coli* cells (10^7/ml of culture) within 4 - 6 h by growing the bacterium in a suitable growth medium (LB medium). Keeping all these in mind, genetic transformation process has been explored to develop silica leaching character within the *E. coli* bacterial strain JMJ107 so that the transformed bacterium can be used in the preparation of bio-concrete material. The responsible gene that encodes bioremediase like protein has been identified from BKH2 bacterial genome. The gene is then transferred into *E. coli* bacterial strain JMJ107 via a suitable T vector. The antibiotic (ampliciline) resistant nature of the T vector has been used to ensure the required transformation event. The host *E. coli* strain fails to grow in ampliciline containing LB-agar plate whereas large numbers distinct colony forming unit (CFU) are observed for transformed JMJ107 strain at the similar condition (Figure 6). Positive expression (in terms of protein) of the inserted gene has been seen in the transformed cells (Figure 7B). The expressed protein similarly shows biosilicification activity as observed from Table 1. The expressed protein (2 μg/g cement) similarly shows the compressive strength increment property of the mortar samples when amended to it (data not presented). Hence it is possible to get sufficient amount protein in shorter duration for using large amount of cementitious material in construction technology.

Compressive strength analysis of the transformed JMJ107 incorporated mortar samples clearly shows that the transformed bacterial cells can directly be used to increase the compressive strength significantly of the material (Figures 8, 9). Maximum strength

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increment (> 30%) is noted at the cell concentration of 10^8 cells/ml water used. Above which the strength is slightly lowered which may be due to the disruption of matrix integrity in presence of excess bacterial cells or protein as explained by Ghsoh et al. [22] Interestingly the transformed JMJ107 strain exhibits better performance against compressive strength than BKH2 strain as revealed by the results of Figures. 9A and 9B. In fact, E. coli cannot survive inside the concrete/mortar matrix because of the high alkalinity and anaerobic condition inside the matrix. At high alkaline environment the bacterial cell wall is disrupted and the protein that is expressed by the inserted gene within the cell against stress comes out inside the matrices. This protein possesses silica leaching activity and remains active at very high pH environment similar to bioremediase.[14] The protein is able to produce new silicate phase that fills the micro pores inside the matrices resulting the enhancement of the compressive strength. The microstructure analysis of the mortar matrices clearly shows the development of novel structures in transformed JMJ107 and BKH2 bacteria amended samples. The formation of gehlenite (calcium-aluminium-silicate) has been confirmed by EDS and XRD analyses. This finding corroborates the result of Sarkar et al. [11] where they have explained the strength increment phenomenon of BKH1 incorporated mortar specimens. Least amount of water absorption (Table 2) and the greater ultra-pulse velocity (Figure 10) is also demonstrated that the porosity of the transformed JMJ107 incorporated cementitious matrices is reduced resulting in increase of durability of the material.

5. CONCLUSION

Uses of microbes have been suggested to enhance the strength of concrete and lower the cement content for making the concrete structure of similar strength. It is established that microbiologically induced new phase formation or growth inside the cement-sand mortar/concrete increases its strength and durability. This means in practice that a substantial part of the cement of the mortar/concrete mixtures can be left out while still obtaining needed

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final strength. This would substantially improve the ecological footprint as cement causes massive CO_2 emission during its production what negatively affects the global climate. Exploration of lucrative and easy methodology for development of bioconcrete material by using the mostly common *E. coli* bacterial strain is therefore highly impressive for commercial purposes which creates a new hope in construction technology.

ACKNOWLEDGEMENTS

The financial assistance to this investigation received from Department of Biotechnology, New Delhi, Government of India, and their R&D Grant and file number BT/PR-11289/BCE/08/707/2008, Dated 30 May 2011 is gratefully acknowledged. The PI is also grateful to DBT for continuing the work in two successive projects.

CONFLICT OF INTEREST None.

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Table 1 Biosilicification activity of transformed bioremediase protein

Samples	Total protein (mg)	Activity (Units)	Sp. Activity (Units/mg)
BKH2 Crude Protein	5	142	28.4
Purified BKH2 protein	2	412	206
JMJ107 Crude Protein	5	---	---
Transformed JMJ107 Crude protein	5	267	53.4
Transformed JMJ107 Purified Protein	2	562	281

One unit activity of bioremediase protein is expressed as µg of silica released/mg of protein

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Table 2 Water absorption test of different category mortar samples

	Mass (g)				
Sample	Initial	after 30 min	% increment	after 24 h	% increment
Control	682.17 ± 0.7	700.10 ± 0.2	2.63	736.17 ± 1.0	7.92
LB	681.83 ± 0.8	698.83 ± 0.6	2.49	735.17 ± 0.6	7.82
JMJ107	682.93 ± 0.3	701.47 ± 0.5	2.71	736.80 ± 0.8	7.88
T-JMJ107	664.17 ± 1.9	673.14 ± 0.2	1.35	707.94 ± 1.0	6.57
BKH2	662.74 ± 0.4	672.00 ± 0.3	1.39	708.94 ± 1.2	6.97

Where T-JMJ107 is the transformed bacterium and N = 6

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FIGURE LEGENDS

Figure 1: TEM view of BKH2 bacterium.

Figure 2: Phylogenetic tree of BKH2 based on 16S rRNA (961 nucleotides) sequence.

Figure 3: Compressive Strength of BKH2 (10^1-10^6 cells/ml water used) amended mortar sample at different days of water curing.

Figure 4: Gel electrophoresis images of genomic DNA

(A) The purified genomic DNA of BKH2

(B) The PCR product of the isolated gene (clone) DNA.

Figure 5: The sequence of isolated gene (clone).

Figure 6: (A) Growth of *E. coli* (JMJ107 strain) in LB-agar plate with ampicillin.

(B) Growth of transformed *E. coli* (JMJ107 strain) in LB-agar plate with ampicillin.

Figure 7: SDS-PAGE image of the protein profile

(A) Lane 1- *E. coli* (JMJ107), Lane 2- transformed *E. coli* (JMJ107), Lane 3- Marker (Sigma).

(B) Lane1- purified protein of transformed JMJ107, Lane2- purified protein of BKH2; Lane3- Marker (Sigma).

Figure 8: Compressive strengths of transformed JMJ107 amended mortar sample in different days: (a) air curing; (b) water curing.

Figure 9: Compressive strengths mortar sample incorporated with different bacterial strain under (A) Air curing; (B) Water curing.

Figure10: Ultra-pulse velocity test of mortar samples.

Figure 11: Microstructure images of mortar samples by SEM

(A) Control sample.

(B) LB treated mortar sample.

(C) JMJ107 treated mortar sample.

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(D) Transformed JMJ107 treated sample.

(E) BKH2 treated sample.

(F) Elemental analysis of transformed JMJ107 amended mortar matrix by EDS.

Figure 12: XRD analysis of transformed JMJ107 bacteria amended mortar with respect to host JMJ107 bacteria amended mortar samples.

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FIGURES

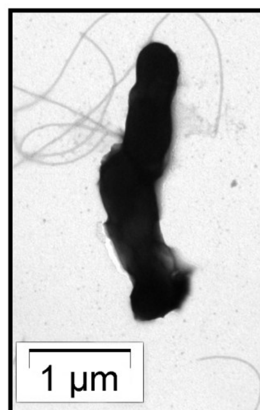

Fig. 1

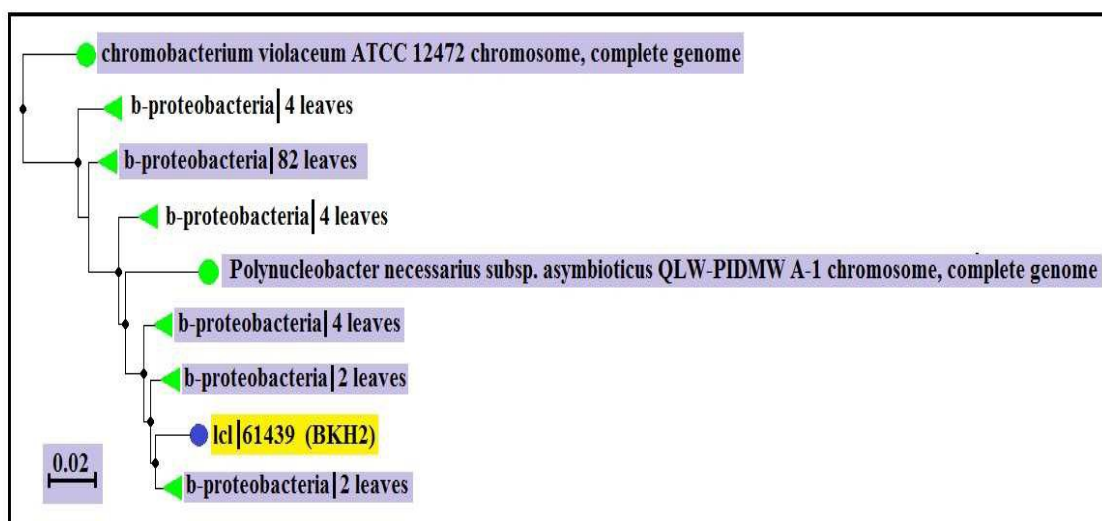

Fig. 2

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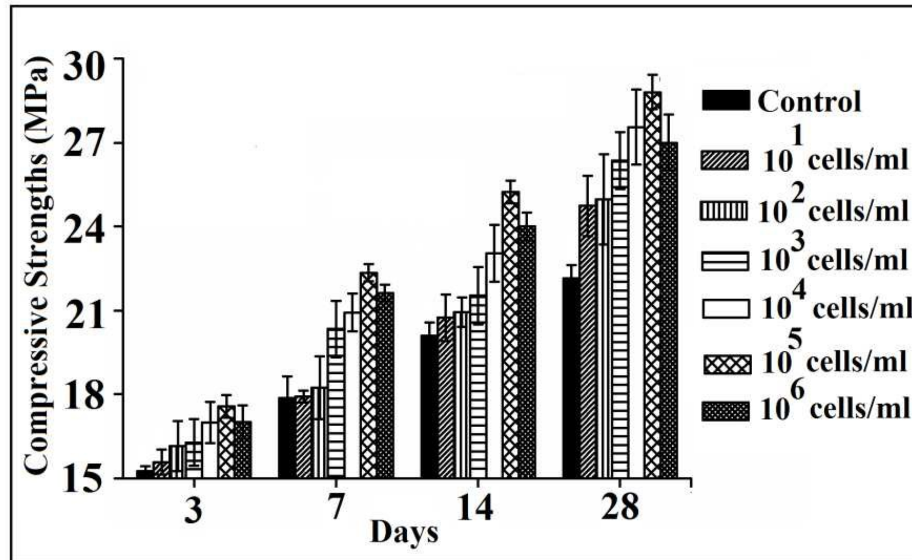

Fig. 3

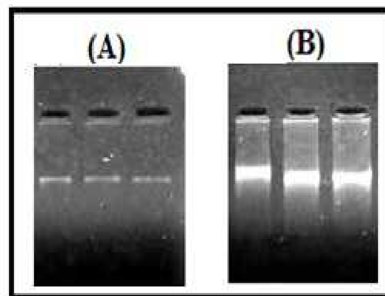

Fig. 4

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TCCCATCACTGGGGATACGGCGCCGGCCTCCACGCCCAGGC
CGTTGATGGCGAAGTCATAGAGGGCGTCCCGCTTGTTGTTGA
TCCGGCTGTAGTAGGTATAGATCGACGTGCGCTTG GAAAGC
GGATAGTCGTAGCCAACAGTGATCTGCGTGGCGCCGGTTTC
GGGGCCGGCATGGAATGAACCGATTGTCTCGGTCGCATTGC
CCGTGCCGTTGCTGGCGAACGTGAAGCCGATCTTCACGCTG
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Fig. 5

Fig. 6

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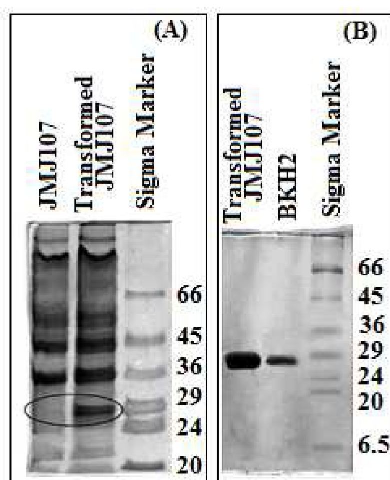

Fig. 7

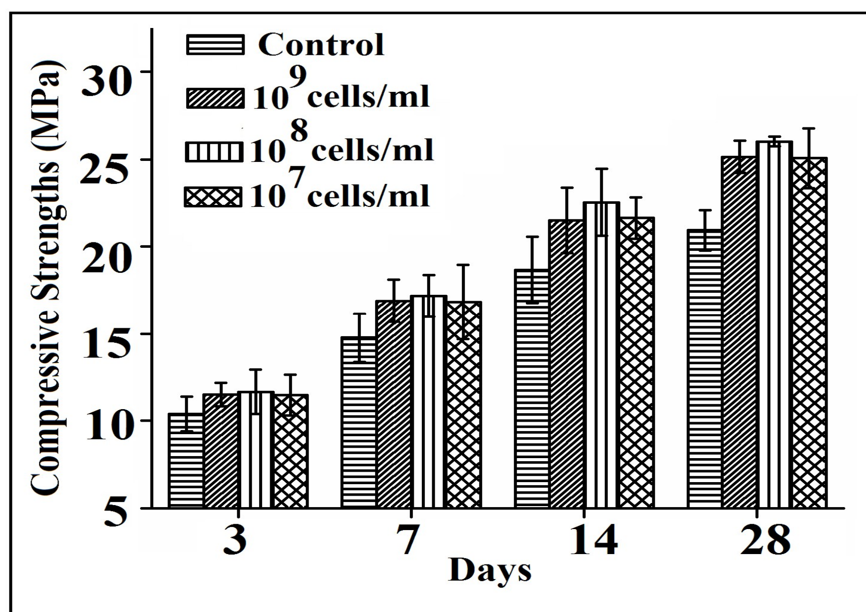

Fig. 8A

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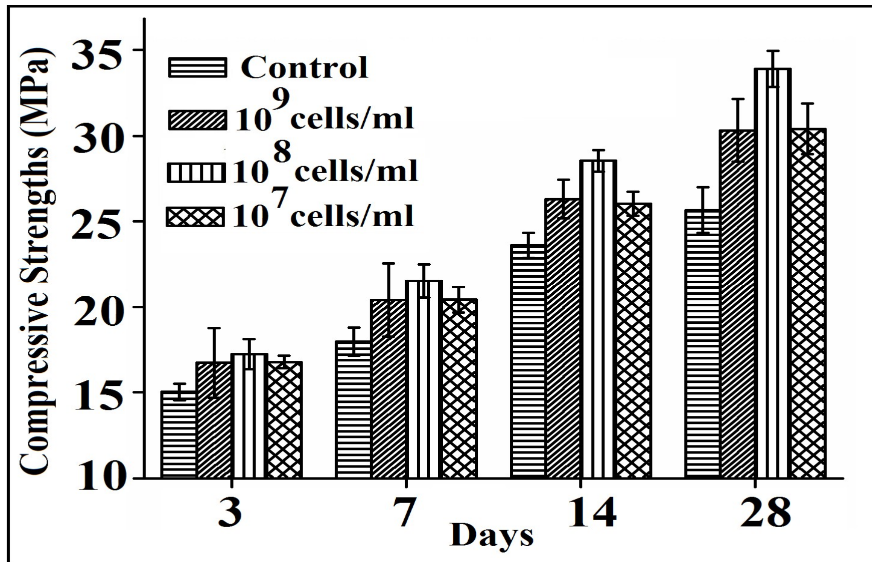

Fig. 8B

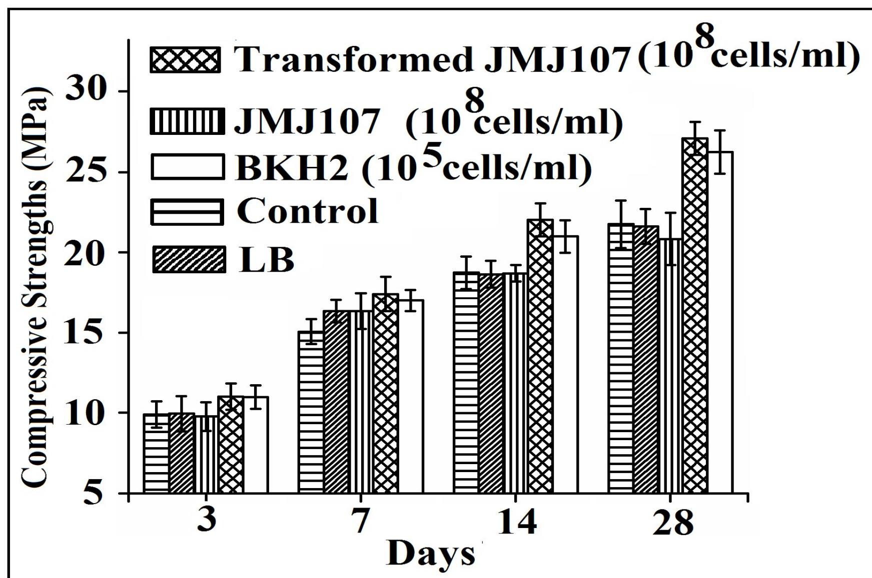

Fig. 9A

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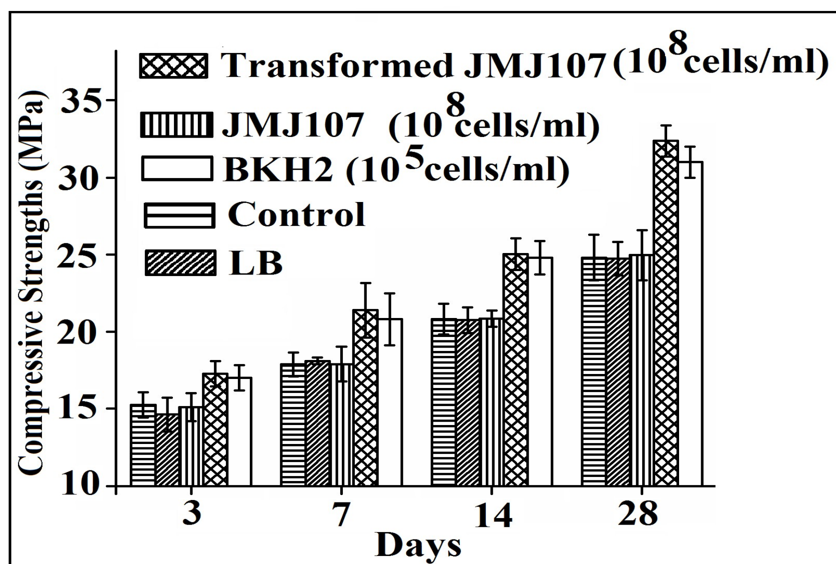

Fig. 9B

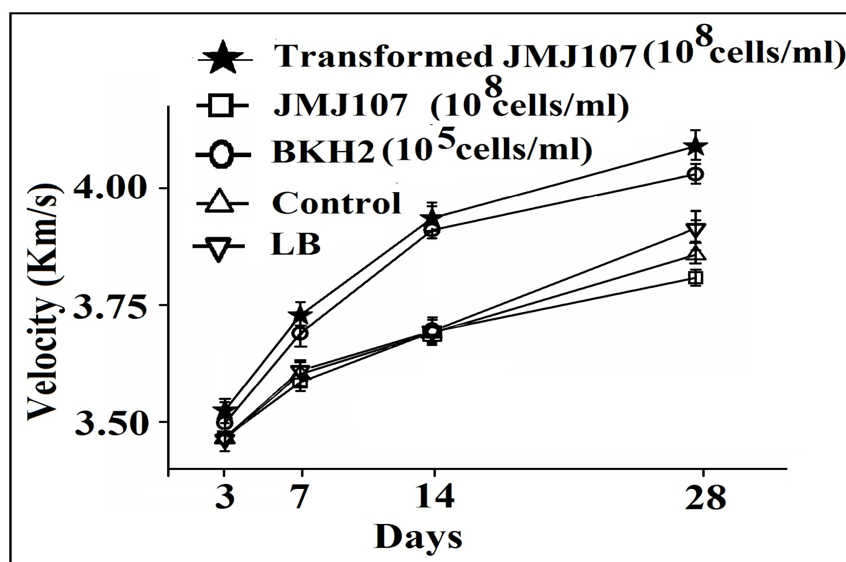

Fig. 10

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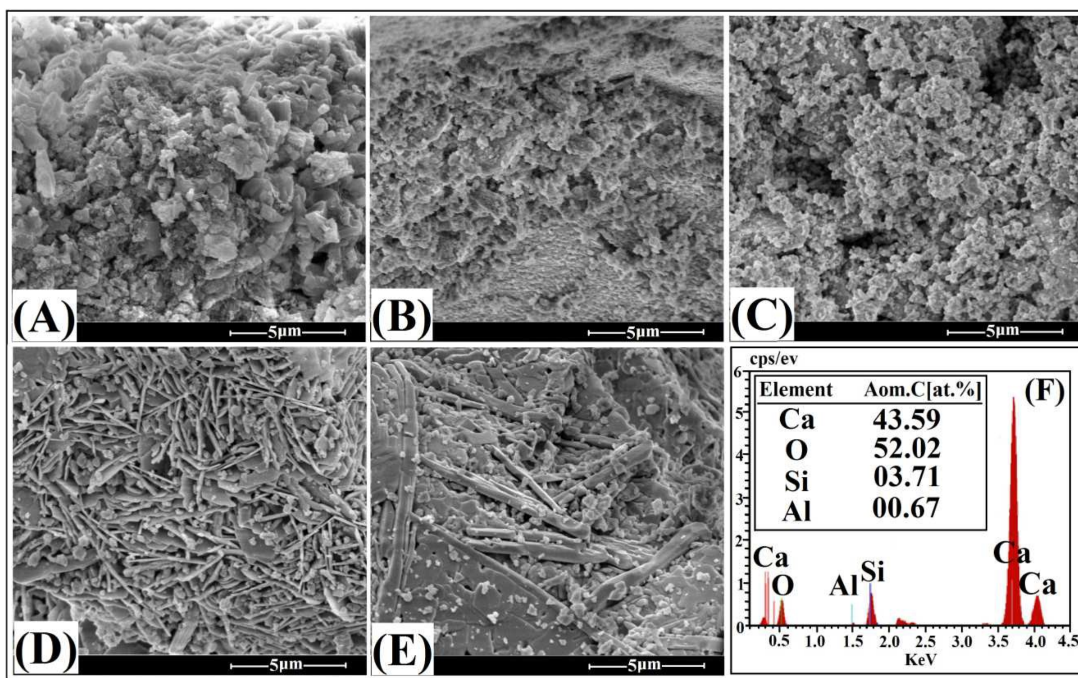

Fig. 11

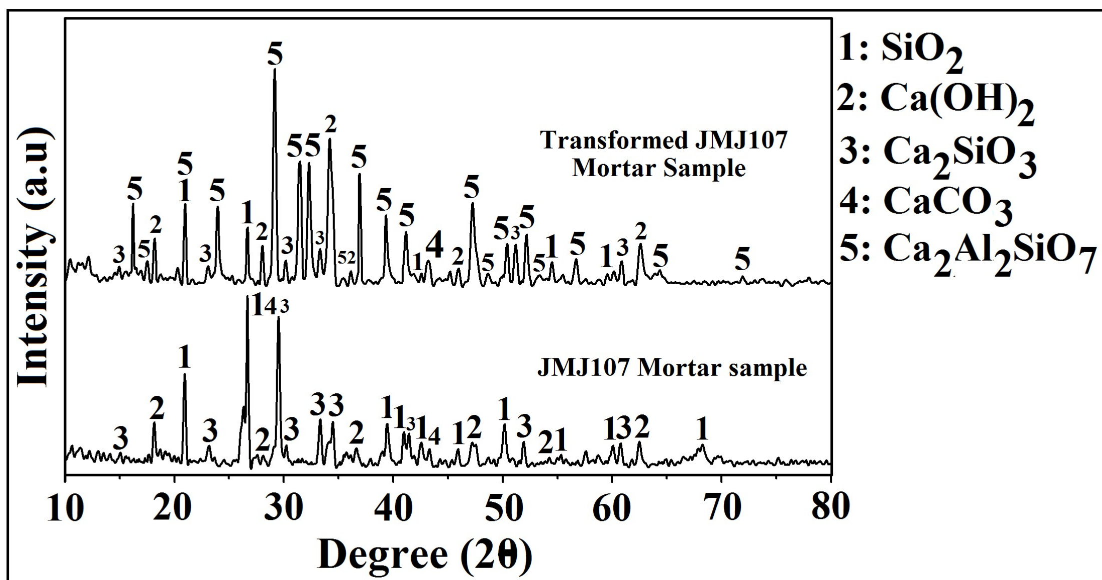

Fig. 12

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