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Modified Bacteria Incorporated Geopolymer - A Qualitative Approach for an Eco-friendly, Energy-efficient and Self-healing Construction Material

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Abstract: Cement production consumes huge energy and creates environmental pollution. Cracks present in the cement-based concretes, deteriorate the structural longevity and requires costly repair. An eco-friendly and energy-efficient geopolymeric material is developed by incorporating modified bacterium cells, assuming that the developed material will be a cement-alternatives in construction industries in near future. Transformed *Bacillus subtilis* cells is incorporated to the alkali-activated fly ash only (100%) for making the geo-polymeric material. The mortar samples prepared by geopolymeric material are cured under various conditions to achieve the best possible energy-efficient curing process. Simulated cracks on mortars are developed by applying 50% (half) of predetermined breaking load for studying the self-healing phenomenon. Artificial cracks on mortars are created by introducing steel bar for studying crack-repairing activity. Mechanical strengths (compressive, tensile and flexural), water permeability, sulfate and chloride resistant activities along with the crack-repairing and the self-healing efficacy of the samples are characterized. Higher mechanical strengths and better longevity in terms of decreased water and chloride ions permeability and increased sulfate resistant activity are noted in the bacterium amended mortars. Ambient temperature modified heat curing process reveals the best possible energy-efficient curing condition. Images and micro-structures analyses show that several new phases (e.g., silicate, mullite, albite and alite etc.) are developed within the bacteria-amended mortars. Eco-friendliness of the bacterium is confirmed by toxicity study against rats models and human cell lines. We hypothesize that the developed geo-polymeric material is a suitable cement alternative in construction industries as well as an eco-friendly and energy efficient material.
Keywords*:* Biomaterials; Cracks; Eco-efficient; Geopolymer; Self-healing.

1. Introduction

Cement manufacturing and transport processes release CO_2 (approximately 5% of global CO_2) and various particulate matters to the atmosphere which are causing several diseases [1, 2]. Cement production is increasing with increasing the demand of concreted structures. Scientists, therefore are trying to find out greener alternatives of cement by using fly ash, blast furnace slag, metakaolin etc. industrial wastes, additives and microbes to reduce cement-related problems [3, 4].

Fly ash, partially used in cement, is an industrial by-product of coal-fired power station, whose disposal significantly increases several ecological problems [5]. Use of 100% fly ash in construction industries will certainly reduce the ecological pollution to a great extent. Alkali-activated fly ash geopolymer needs some extra energy for activation of polymerization that provides mechanical strength of the material [6, 7]. High concentration of sodium/potassium hydroxide helps to gain higher mechanical strength and durability of the geo-polymeric material [7, 8]. Strength and other structural properties of fly ash geopolymer also vary with the curing conditions widely. Though initial curing at elevated temperature (40 ^oC to 95 ^oC), improves the geo-polymerization process leading to a high compressive strength of the material, yet it consumes more energy [9, 10. 11]. Similarly, steam curing at 60 to 80 ^oC for a day leads to the satisfactorily improvement of compressive strength at the cost of more energy consumption [12, 13]. Geopolymer cured in saline water is seem to have improved properties due to reduced leaching of reactants from the samples instead of ingress of saline water into the samples [14].

Development of tiny cracks in concrete reduces it strength. It allows water and various detrimental ions inside the structures which corrodes the steel reinforcement and decreases the lifetime of the structures. Scientists are showing their interest on the recovery of mechanical properties of damaged concrete structures by self-healing manner to extend its longevity. Use of various microorganisms for self-healing is an unique development in this

field prevailing the other existing techniques because of its reduced cost and friendliness [15]. Also, self-healing efficacy of the material contributes to the performance of crack repairing activity by reducing the crack widths without any external intervention to the material [15, 16]. It occurs due to formation of calcite (calcium carbonate) [17] or gehlenite (calcium-aluminium silicate) crystals inside the matrices by the action of some specific incorporated-bacteria [18, 19]. Though self-healing of bacteria in concrete is considered as eco-friendly, still no straight forward experimental evidences are available to support the eco-friendliness.

Here, an approach was taken to develop an alkali-activated only fly ash (100%) based geo-polymeric material, by using a transformed *Bacillus* cells. The bacterium amended geopolymer showed higher mechanical strength and better longevity compared to ordinary cement-based concrete or fly ash geopolymer. The eco-friendliness of the transformed *Bacillus* was established by toxicity study of the bacteria against rats and in two different human cell lines. The promising results from bacterium-amended geopolymer established that the eco-efficiency and energy-efficiency geo-polymeric material would be a good replacement of cementitious material in near future.

2. Materials and methods

Standard Ennore sand [20] and low calcium Class-F fly ash (National Thermal Power Corporation Ltd; Farakka Plant) were used for geopolymer [3]. Commercial grade sodium hydroxide pellets (NaOH, 99 % purity) was mixed with commercial grade liquid sodium silicate (Na_2SiO_3, 45% solid, specific gravity: 1.53 gm/cc) in 1.0:1.75 (v/v) ratio to make activator solution [14]. Transformed *Bacillus subtilis* cells (Laboratory stock culture), grow in a specific mineral media (pH 10.0), were used as bacterial agent [14].

Sufficient numbers of mortar samples for control (without bacterial cells) and experimental (with bacterial cells) were prepared for 8 different curing conditions. The fly ash and bacteria-incorporated activator fluid (10^5 cell/ml activator fluid used) were mixed (at 1.0: 0.4 w/w) properly and heated at 60 ^oC for 45 minutes before addition of sand [14]. The heat cured activator-fly ash mass mixed with sand (at 1.4 : 3.0 w/w) and used for experimental mortar sample preparation. The prepared samples for all categories were initially cured for 28 days in 8 different curing conditions as shown in Table 1.

Table 1. Curing conditions of different category samples

Sample Category	Curing Conditions
1C - Control geopolymer mortar	Air curing (37 ± 3 ^oC)
1S - Bacteria amended geopolymer mortar	Air curing (37 ± 3 ^oC)
2C - Control geopolymer mortar	Water curing (37 ± 3 ^oC, pH 7.0)
2S - Bacteria amended geopolymer mortar	Water curing (37 ± 3 ^oC, pH 7.0)
3C - Control geopolymer mortar	Air curing (50 ^oC Temperature)
3S - Bacteria amended geopolymer mortar	Air curing (50 ^oC Temperature)
4C - Control geopolymer mortar	Air curing (90 ^oC Temperature)
4S - Bacteria amended geopolymer mortar	Air curing (90 ^oC Temperature)
5C - Control geopolymer mortar	Acid curing (5% sulphuric acid)
5S - Bacteria amended geopolymer mortar	Acid curing (5% sulphuric acid)
6C - Control geopolymer mortar	5% Saline water curing
6S - Bacteria amended geopolymer mortar	5% Saline water curing
7C - Control geopolymer mortar	Steam curing (60 ^oC)
7S - Bacteria amended geopolymer mortar	Steam curing (60 ^oC)
8C - Control geopolymer mortar	Cold curing at 8 ^oC
8S - Bacteria amended geopolymer mortar	Cold curing at 8 ^oC

The samples used for heat and steam curing, were first kept for 72 h to their respective curing conditions and then kept in air for another 25 days. After 28 days, 5 samples from each category were used for the measurement of average breaking load. Rest of the samples were used for the self-healing study and crack repairing activity. The Ultrasonic-pulse velocity (UPV) of the samples were determined prior to the measurement of average breaking load by using PUNDIT plus PC 1007 UPV machine, UK and as per ASTM C597-02 [21].

2.1 Self-healing study

Standard mortar cubes (70.6 mm x 70.6 mm x 70.6 mm as per IS 4031-4, 1988 standard) were casted for each category [22]. After initial curing (28 days), the average Ultrasonic pulse-velocity (UPV) and the average (5 samples in each) breaking load of each category samples were determined. Samples used for self-healing event, were employed to 50% average corresponding breaking load to the respective category to make artificial micro-cracks [14]. The samples were then kept for further curing for different days (3, 7, 14 and 28 days respectively) at their respective curing conditions. After that, UPV and compressive strength of the samples were determined.

Some artificially crack-created mortars were kept under water at ambient temperature for 60 days to view their crack healing efficacy.

Mortar cylinders of different categories (100 mm diameter × 200 mm height) were casted for split tensile strength measurement. The flexural strength of different categories samples were determined on mortar bars (50 mm × 50 mm × 200 mm). After initial 28 days curing, the average UPV and average breaking load of the samples of all categories were determined respectively. Rest of the samples used for self-healing study, were employed to 50% of average breaking load (respective category) to make artificial micro cracks and kept at their respective curing condition for 28 days. After the curing period, split tensile strength of mortar cylinders and flexural strength of mortar bars were determined as per standard protocols [23, 24].

2.2 Crack repairing study

Similar mortar samples (70.6 mm x 70.6 mm x 70.6 mm) were prepared for crack repairing study. A small bar (length 68 mm × breadth 15 mm × thickness 5 mm) was introduced on the top surface of the mortar sample during casting to create artificial fissure on the samples. After 24 h of casting, the bar was removed and the mortar was kept in water for 28 days for curing. After that, the artificial fissures were repaired by normal geo-polymeric material for control specimens and bacteria cells incorporated geo-polymeric material for experimental samples and cured under water for another 28 days. Finally, UPV and compressive strength of the samples were determined as described earlier. Similar crack repairing study was done on the samples used for split tensile strength and flexural strength measurements.

2.3 Durability study

Durability of the materials was performed by water absorption test, sulfate resistance test and chloride permeability test.

For water absorption test, the as prepared mortar samples (self-heal and crack-repair both) were air dried for 24 h at room temperature after their respective 28 days curing and weights were recorded. The samples were then immersed in deionized water for 30 min. After which, the samples were removed from water, cleaned properly with tissue paper and their weights were recorded again. The samples were then further kept in deionized water for another 24 h. Finally, the samples were taken out, cleaned and their weights were taken similarly. The water absorption capabilities of the samples were determined as per Neville's Method, 1986 [25].

For sulfate resistance test, the initial masses of the geo-polymeric mortars (self-healed and crack-repaired samples) cured for 28 days were determined. The samples were then immersed in 5% $MgSO_4$ solution (pH 6.0) for 90 days. After curing period, the specimen were taken out from solution, air-dried and followed by the measurement of their masses. Sulfate resistance activity was determined as per the guideline of ASTM STP663, 1997 [26].

In rapid chloride permeability test (RCPT), the as prepared geopolymer mortar cylinders of individual category (100 mm diameter x 200 mm height) were cured first for 28 days at their respective curing condition. Each cylinder was then cut into small cylinders (100 mm diameter x 50 mm height), epoxy coated along their edges and put under water for 24 h. The RCPT of the samples were tested as per ASTM C1202, 2000 [27].

2.4 Microstructure analysis

Fragmented geo-polymeric mortars of all curing categories were individually crushed into fine powder and sieved to make the particles size lesser than 5 μm. The field emission scanning electron microscope (FE-SEM; HITACHI S-4800, JAPAN)) equipped with energy dispersive X-ray analyzer (EDAX; Philips XL30) was used for microstructure observations of the samples. X-ray diffraction (XRD; Bruker AXS Inc, Model D8, WI, USA) of the samples were also done with a scanning speed of 0.5 s / step at 40 kV (2θ = 10 to 80^o) for structural information. The diffraction spectra were analyzed by JCPDS data files.

2.5 Toxicity study of the bacterial cells

Twelve adult male (body weights: 140-160 gm) and twelve adult female (body weights: 130-150 gm) albino rats of Wister strain were procured from the animal housing facility of Jadavpur University. They were maintained according to the guidelines of Instructional Animal Ethics Committee of Jadavpur University, Kolkata (Ref. No.: AEC/PHARM/1502/14/2015, Dated: 30/07/2015). The animals, maintaining with normal protein diet (18% casein, 70% carbohydrate, 7% fat, 4% salt mixture and 1% vitamin mixture), were divided into four groups (e.g., Group 1 - Control, Group 2 - 10^2 cells/ml bacteria treated, Group 3 - 10^4 cells/ml bacteria treated, and Group 4 - 10^6 cells/ml bacteria treated) and each group was further divided into two subgroups (male and female) having three animals in each.

Animal in Group 1 were injected subcutaneously with 0.1 ml of normal saline [0.9 % (w/v) NaCl solution], and in Groups 2, 3 and 4 were injected subcutaneously with 0.1 ml suspensions of transformed *Bacillus* cells at doses of 10^2, 10^4 and10^6 cells/ml in normal saline respectively for every alternate days (3 days in a week). After 28 days

of treatment, the animals keeping fasting overnight were sacrificed on the following morning. Blood was collected in sterilized tubes and serum was separated by centrifugation followed by storage at - 20 ºC. biochemical analysis was done by using the standard kit of MERCK as per the manufacturer protocols.

The transformed bacterial cells were also used to treat on two human cell lines (WI38 and HaCaT cells). The cells were seeded on 24 well cell culture plates and treated them with transformed *Bacillus* cells in concentrations of 10^1,10^2,10^3, 10^4, 10^5 cells/ml. The survivability assay was carried out by using MTT assay.

2.6 Statistical analysis

All categories of samples were prepared by the standard procedures. Each experiment was repeated for at least two times and the data of each experiment were presented as averaged over 10 samples (5 samples in each set) with ± S.D.

3. Results

The compressive, tensile and flexural strengths were seen to increase in all categories of bacteria-incorporated geo-polymeric mortars compared to their respective controls. The maximum strengths were observed at ambient temperature air curing (Supplementary Table 1). The compressive strengths of the self-healed geo-polymeric mortars were considerably increased with respect to their corresponding controls at all curing conditions (Table 2).

Table 2. Compressive Strength (MPa) of Self-healing of bacteria incorporated geopolymer mortar samples

Samples	3 days	7 days	14 days	28 days
1C	15.0 ± 1.0	16.0 ± 2.0	17.0 ± 1.8	17.8 ± 1.9
1S	19.0 ± 1.0	21.4 ± 1.9	24.1 ± 0.9	28.3 ± 1.0
	(26.66↑)	(34.75↑)	(41.76↑)	(58.98↑)
2C	14.5 ± 0.9	15.0 ± 1.0	16.0 ± 2.0	17.4 ± 2.0
2S	17.1 ± 1.2	18.6 ± 2.2	21.1 ± 1.0	25.7 ± 1.4
	(17.93↑)	(24.00↑)	(31.87↑)	(47.70↑)
3C	16.0 ± 2.0	17.0 ± 1.8	17.9 ± 1.9	18.0 ± 1.0
3S	19.5 ± 1.8	22.2 ± 1.0	24.8 ± 0.5	27.9 ± 1.0
	(21.87↑)	(30.58↑)	(38.54↑)	(55.00↑)
4C	16.0 ± 1.8	17.0 ± 2.0	18.0 ± 1.2	18.9 ± 2.0
4S	19.0 ± 2.0	21.4 ± 1.4	24.3 ± 1.9	28.3 ± 0.9
	(18.75↑)	(25.88↑)	(35.00↑)	(49.73↑)
5C	14.0 ± 1.0	14.5 ± 0.9	15.5 ± 2.0	16.4 ± 1.8
5S	15.8 ± 1.8	17.5 ± 0.5	19.9 ± 1.2	23.1 ± 2.0
	(12.85↑)	(20.68↑)	(28.38↑)	(40.85↑)
6C	13.2 ± 0.8	14.0 ± 1.0	14.9 ± 1.9	15.5 ± 2.0
6S	14.9 ± 2.0	16.8 ± 0.9	18.9 ± 1.0	21.5 ± 0.8
	(12.87↑)	(20.00↑)	(26.84↑)	(38.70↑)
7C	17.0 ± 1.9	18.0 ± 2.0	18.5 ± 1.8	19.2 ± 1.9
7S	20.5 ± 0.9	23.4 ± 1.2	25.4 ± 0.5	29.5 ± 0.9
	(20.58↑)	(30.00↑)	(37.29↑)	(53.64↑)
8C	14.7 ± 1.9	15.0 ± 1.9	16.2 ± 1.0	17.0 ± 2.0
8S	14.4 ± 0.9	15.6 ± 0.8	18.2 ± 0.9	21.3 ± 1.9
	(0↑)	(4.00↑)	(12.34↑)	(25.29↑)

* Data are presented mean ± S.D. (n = 10). The increased percentage of data for experimental samples (S) was calculated with respect to the corresponding control and was shown within the parenthesis.

Almost, 60% compressive strength was increased with respect to their control at 28 days ambient temperature air curing. Higher temperatures air curing (50 or 90 ºC) or steam curing also showed effective compressive strength increments (Table 2).

Bacteria incorporated geo-polymeric mortars showed remarkably increased flexural strength and split tensile strength compared to their control samples irrespective of curing conditions in self-healing studies (Fig. 1A and 1B respectively).

The maximum flexural strength (142.8%) the maximum split tensile strength (100%) were seen to increase in bacteria incorporated mortars at ambient temperature air curing. Similar strength increments (63.48% of compressive, 140.9% of flexural and 93% of tensile) of geo-polymeric mortars were noticed in crack-repairing study (Table 3; Figs. 1C and 1D) at ambient temperature air curing. The UPV of the self-healed (Table 4) and the

crack-repaired (Table 5) samples were increased in bacteria incorporated mortars at all ages compared to their corresponding controls. At ambient temperature air curing, it was 41.37% increment for self-healing and 45.86% increment for crack repairing samples.

The minimum water ingress was noted in self-healing samples (1.5%; Table 6) and crack-repairing samples (1.09%; Table 7) when cured at ambient temperature air curing condition.

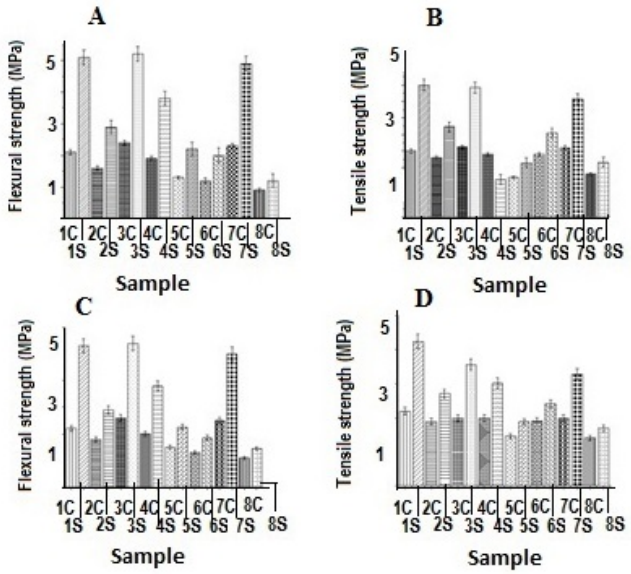

Figure 1. Mechanical strength analysis of bacterial incorporated geopolymer cured at different curing conditions. (A) Flexural strengths of self-healed samples, (B) Split Tensile strengths of self-healed samples, (C) Flexural strengths of crack-repaired samples, (D) Split Tensile strengths of crack-repaired samples.

Table 3. Compressive Strength (MPa) of Crack repairing of bacteria incorporated geopolymer mortar samples

Samples	3 days	7 days	14 days	28 days
1C	16.0 ± 0.9	16.5 ± 0.8	17 ± 0.9	17.8 ± 0.6
1S	20.6 ± 1.0 (28.75↑)	22.4 ± 0.8 (35.75↑)	25.1 ± 1.2 (47.64↑)	29.1 ± 0.9 (63.48↑)
2C	15.0 ± 0.8	16.0 ± 0.8	16.5 ± 0.6	17.0 ± 0.9
2S	18.0 ± 1.0 (20.00↑)	20.3 ± 1.4 (26.87↑)	22.7 ± 1.4 (37.57↑)	25.3 ± 0.5 (48.82↑)
3C	17.0 ± 0.6	18.0 ± 0.8	18.5 ± 0.9	19.0 ± 0.7
3S	21.4 ± 1.0 (25.88↑)	23.7 ± 1.2 (31.66↑)	26.6 ± 1.0 (43.78↑)	30.2 ± 1.2 (58.94↑)
4C	16.0 ± 1.6	16.5 ± 1.2	17.0 ± 1.0	18.0 ± 1.0
4S	19.3 ± 0.9 (20.62↑)	21.1 ± 0.5 (27.87↑)	23.8 ± 0.9 (40.00↑)	27.3 ± 1.2 (51.66↑)
5C	13.0 ± 1.0	14.0 ± 1.2	14.0 ± 1.0	14.5 ± 1.0
5S	15.0 ± 0.8 (15.38↑)	17.0 ± 1.4 (21.42↑)	18.7 ± 0.7 (33.57↑)	20.7 ± 1.4 (42.75↑)
6C	14.0 ± 1.0	14.5 ± 0.9	15.0 ± 0.9	15.5 ± 0.8
6S	16.1 ± 1.5 (15.00↑)	17.4 ± 1.0 (20.00↑)	19.8 ± 1.2 (32.00↑)	22.1 ± 1.4 (42.58↑)
7C	17.0 ± 1.1	18.0 ± 1.0	18.0 ± 0.9	19.5 ± 1.5
7S	21.2 ± 1.2 (24.70↑)	23.6 ± 0.4 (31.11↑)	25.3 ± 0.9 (40.55↑)	30.6 ± 0.4 (56.92↑)
8C	15.0 ± 1.0	15.0 ± 0.9	16.0 ± 1.2	16.5 ± 1.0
8S	14.5 ± 0.9 (0↑)	16.5 ± 1.0 (10.0↑)	18.5 ± 1.0 (15.62↑)	22.4 ± 0.8 (35.75↑)

* Data are presented mean ± S.D. (n = 10). The increased percentage was calculated with respect to the corresponding control and shown within the parenthesis.

Table 4. Ultrasonic pulse velocity (Km/s) of self-healed geopolymer mortars

Samples	3 days	7 days	14 days	28 days
1C	2.8 ± 0.9	2.8 ± 1.0	2.9 ± 0.9	2.9 ± 0.8
1S	3.1 ± 0.8	3.5 ± 0.6	4.0 ± 1.0	4.1 ± 0.6
	(10.71↑)	(25.00↑)	(37.93↑)	(41.37↑)
2C	2.7 ± 1.0	2.7 ± 0.8	2.8 ± 0.9	2.8 ± 0.7
2S	2.8 ± 0.9	3.1 ± 0.4	3.5 ± 0.6	3.6 ± 0.4
	(3.70↑)	(14.81↑)	(25.00↑)	(28.57↑)
3C	2.9 ± 0.7	3.0 ± 0.8	3.0 ± 0.6	3.1 ± 0.9
3S	3.1 ± 0.8	3.6 ± 1.0	3.9 ± 0.8	4.3 ± 0.5
	(6.89↑)	(20.00↑)	(30.00↑)	(38.70↑)
4C	2.8 ± 0.9	2.9 ± 0.7	2.9 ± 0.8	3.0 ± 0.6
4S	2.9 ± 0.6	3.4 ± 0.4	3.7 ± 0.6	3.9 ± 0.9
	(3.5↑7)	(17.24↑)	(27.58↑)	(30.00↑)
5C	1.9 ± 1.0	2.0 ± 0.8	2.0 ± 0.9	2.1 ± 0.8
5S	1.96 ± 0.8	2.2 ± 0.6	2.4 ± 0.5	2.6 ± 0.6
	(3.15↑)	(10.00↑)	(20.00↑)	(23.80↑)
6C	2.2 ± 0.8	2.3 ± 0.9	2.3 ± 0.8	2.4 ± 0.7
6S	2.27 ± 0.9	2.5 ± 0.5	2.7 ± 0.9	2.9 ± 0.6
	(3.18↑)	(8.69↑)	(17.39↑)	(20.83↑)
7C	2.5 ± 0.8	3.0 ± 0.9	3.0 ± 0.8	3.1 ± 1.0
7S	2.6 ± 0.9	3.6 ± 0.5	3.9 ± 1.0	4.2 ± 0.8
	(4.00↑)	(20.00↑)	(30.00↑)	(35.48↑)
8C	2.25 ± 0.9	2.3 ± 0.8	2.3 ± 0.7	2.3 ± 0.6
8S	2.25 ± 0.4	2.4 ± 1.0	2.7 ± 0.9	2.71 ± 0.9
	(0↑)	(4.34↑)	(17.39↑)	(17.82↑)

* Data are presented mean ± S.D. (n = 10). The increased percentage was calculated with respect to the corresponding control and shown within the parenthesis.

Table 5. Ultrasonic pulse velocity (Km/s) of crack-repaired geopolymer mortars

Samples	3 days	7 days	14 days	28 days
1C	2.4 ± 0.1	2.7 ± 0.6	2.8 ± 0.9	2.9 ± 0.8
1S	2.8 ± 0.9	3.51 ± 0.8	3.86 ± 0.4	4.23 ± 1.0
	(16.66↑)	(30.00↑)	(37.85↑)	(45.86↑)
2C	2.3 ± 0.9	2.4 ± 0.8	2.5 ± 0.8	2.8 ± 0.9
2S	2.53 ± 0.4	2.92 ± 0.7	3.27 ± 0.8	3.83 ± 0.6
	(10.00↑)	(21.66↑)	(30.80↑)	(36.78↑)
3C	2.6 ± 0.6	2.8 ± 0.5	2.9 ± 1.0	3.0 ± 0.6
3S	2.99 ± 0.4	3.58 ± 0.9	3.98 ± 0.9	4.26 ± 1.0
	(15.00↑)	(27.85↑)	(37.24↑)	(42.00↑)
4C	2.4 ± 0.8	2.7 ± 0.9	2.8 ± 0.6	2.9 ± 0.4
4S	2.75 ± 0.7	3.36 ± 0.8	3.78 ± 0.9	4.0 ± 0.7
	(14.58↑)	(24.44↑)	(35.00↑)	(37.93↑)
5C	1.9 ± 0.9	2.0 ± 0.9	2.0 ± 0.6	2.0 ± 0.8
5S	2.07 ± 0.8	2.38 ± 0.6	2.52 ± 0.8	2.60 ± 0.9
	(8.94↑)	(19.00↑)	(26.00↑)	(30.00↑)
6C	2.0 ± 0.8	2.0 ± 1.0	2.1 ± 0.8	2.1 ± 0.9
6S	2.14 ± 0.5	2.34 ± 0.9	2.69 ± 0.6	2.7± 0.9
	(7.00↑)	(17.00↑)	(28.09↑)	(28.57↑)
7C	2.46 ± 0.9	2.51 ± 0.8	2.52 ± 0.6	2.6 ± 0.7
7S	2.8 ± 1.0	3.31 ± 0.9	3.4 ± 0.8	3.66 ± 0.9
	(13.82↑)	(31.87↑)	(34.92↑)	(40.76↑)
8C	2.0 ± 0.4	2.1 ± 0.3	2.14 ± 0.4	2.2 ± 0.8
8S	1.99 ± 0.8	2.37 ± 0.4	2.58 ± 0.7	2.81 ± 0.5
	(0↑)	(12.85↑)	(20.56↑)	(27.72↑)

* Data are presented mean ± S.D. (n = 10). The increased percentage was calculated with respect to the corresponding control and shown within the parenthesis.

Table 6. Water Absorption of self-healed geopolymer mortars

Samples	Initial mass (g)	Mass after 30 mins (g)	Percent increase	Mass after 24 hrs.	Percent increase
1C	724 ± 3.0	737.75 ± 3.0	1.89	742.10 ± 5.0	2.5
1S	712 ± 2.5	716.98 ± 3.0	0.69	722.68 ± 2.4	1.5
2C	726.4 ± 5.0	743.83 ± 3.2	2.39	751.09 ± 1.9	3.39
2S	718 ± 4.2	729.48 ± 4.0	1.59	734.51 ± 4.0	2.29
3C	725 ± 6.0	739.50 ± 1.9	2.0	745.30 ± 2.5	2.8
3S	716 ± 5.4	722.44 ± 3.0	0.89	729.60 ± 1.4	1.89
4C	729.4 ± 4.0	746.17 ± 2.8	2.29	752.74 ± 1.9	3.19
4S	720 ± 3.0	729.36 ± 4.0	1.30	735.84 ± 3.4	2.20
5C	730.5 ± 2.5	749.49 ± 1.9	2.59	756.79 ± 1.8	3.59
5S	721 ± 3.0	733.97 ± 2.5	1.79	739.74 ± 4.0	2.59
6C	731 ± 4.0	750 ± 4.0	2.59	758.41 ± 1.5	3.74
6S	724.2 ± 5.0	738.68 ± 3.4	1.99	743.75 ± 2.0	2.69
7C	724 ± 4.5	739.20 ± 4.0	2.09	746.44 ± 4.2	3.09
7S	718 ± 2.0	726.61 ± 3.5	1.19	733.07 ± 4.0	2.09
8C	729 ± 3.0	749.41 ± 2.5	2.79	757.43 ± 1.5	3.89
8S	722 ± 4.2	738.60 ± 1.9	2.29	743.66 ± 2.0	3.00

* Data are presented mean ± S.D. (n = 10). The increased percentage was calculated with respect to the corresponding control and shown within the parenthesis.

Table 7. Water Absorption of crack-repaired geopolymer mortars

Samples	Initial mass (g)	Mass after 30 mins (g)	Percent increase	Mass after 24 hrs.	Percent increase
1C	730 ± 1.9	744.6 ± 2.5	2.0	747.52 ± 1.8	2.40
1S	715 ± 2.5	717.86 ± 3.0	0.4	722.86 ± 2.4	1.09
2C	732 ± 4.0	752.49 ± 4.1	2.79	754.69 ± 3.2	3.09
2S	716 ± 2.2	723.16 ± 1.9	1.00	731.75 ± 2.2	2.19
3C	733 ± 3.5	748.39 ± 3.9	2.09	752.05 ± 2.0	2.59
3S	719 ± 4.0	724.03 ± 2.0	0.69	729.06 ± 3.0	1.39
4C	731 ± 6.2	751.46 ± 3.0	2.79	752.93 ± 1.9	3.00
4S	722 ± 3.4	728.49 ± 4.0	0.89	735.71 ± 2.2	1.89
5C	732 ± 2.9	754.69 ± 1.4	3.09	756.88 ± 1.9	3.39
5S	723 ± 1.8	732.39 ± 2.5	1.29	740.35 ± 2.9	2.39
6C	733 ± 6.0	757.18 ± 1.9	3.29	759.38 ± 3.2	3.59
6S	725 ± 1.9	735.15 ± 2.5	1.40	742.40 ± 4.0	2.40
7C	734 ± 4.2	751.61 ± 2.0	2.39	754.55 ± 3.0	2.79
7S	721 ± 3.4	726.04 ± 3.2	0.69	732.53 ± 1.9	1.59
8C	735 ± 1.9	761.46 ± 2.9	3.60	763.66 ± 2.4	3.89
8S	729 ± 2.8	742.85 ± 3.0	1.89	750.14 ± 1.4	2.89

* Data are presented mean ± S.D. (n = 10). The increased percentage was calculated with respect to the corresponding control and shown within the parenthesis.

The sulfate resistance test showed that the minimum increment of weight in bacterial assimilated self-healed (1.3%) and crack repaired (1.0%) geo-polymeric at ambient temperature air curing (Table 8).

The chloride ions permeability was decreased in bacterial amended self-healed as well as crack repaired samples with respect to their controls cured at different curing conditions (Figs. 2A and 2B), which was maximized at ambient temperature air curing.

The bacteria incorporated self-healing of geo-polymeric material is shown in Fig. 3.

The FE-SEM and EDAX analyses of the microbial amended geopolymer powdered samples acquired from the self-healed portions of geopolymer mortar showed that there were formation of various phases in the developing repaired portion of the samples. Rod like structures (approx. 80 nm; Fig. 4B) were appeared inside the matrix of the microbial-amended samples, which was absent in the control samples (Fig. 4A).

The XRD analysis of the healing material confirmed the formation of various new phases, e.g., mullite ($3Al_2O_3$, $2SiO_2$), sodium metasilicate ($Na_2Si_2O_3$), ferric oxide (Fe_2O_3) along with the enhanced formation of sodium aluminium-silicate ($NaAlSi_3O_8$), calcium-silicate (Ca_3SiO_5), calcium-carbonate ($CaCO_3$) and silica (SiO_2) as shown in Fig. 5.

Table 8. Sulfate Resistance Activity of self-healed and crack-repaired geopolymer mortar samples

Samples	SELF-HEALING			CRACK-REPAIRING		
	Initial mass (g)	Mass after 90 days (g)	% of Increment	Initial mass (g)	Mass after 90 days (g)	% of Increment
1C	724.0 ± 3.0	739.20 ± 2.4	2.1	730.0 ± 1.9	743.14 ± 2.9	1.8
1S	712.0 ± 2.5	721.25 ± 3.2	1.3	715.0± 2.5	722.15 ± 4.2	1.0
2C	726.4 ± 5.0	749.64 ± 1.8	3.2	732.0 ± 4.0	753.22 ± 4.0	2.9
2S	718.0 ± 4.2	733.79 ± 4.0	2.2	716.0 ± 2.2	731.03 ± 3.0	2.1
3C	725.0 ± 6.0	742.40 ± 2.5	2.4	733.0 ± 3.5	749.12 ± 4.6	2.2
3S	716.0 ± 5.4	726.74 ± 3.2	1.5	719.0 ± 4.0	728.34± 4.0	1.3
4C	729.4± 4.0	751.28 ± 1.2	3.0	731.0 ± 6.2	750.00 ± 1.8	2.6
4S	720.0 ± 3.0	735.12 ± 1.4	2.1	722.0 ± 3.4	734.27 ± 2.9	1.7
5C	730.5 ± 2.5	756.06 ± 2.6	3.5	732.0 ± 2.1	754.69 ± 3.0	3.1
5S	721.0 ± 3.0	738.30 ± 4.0	2.4	723.0 ± 1.8	740.35 ± 1.9	2.4
6C	731.0 ± 4.0	757.31 ± 1.9	3.6	733.0 ± 6.0	757.92 ± 2.5	3.4
6S	724.2 ± 5.0	743.02 ± 2.9	2.6	725.0 ± 1.9	744.57 ± 3.4	2.7
7C	724.0 ± 4.5	744.27 ± 3.0	2.8	734.0 ± 4.2	752.35 ± 1.6	2.5
7S	718.0 ± 2.0	731.64 ± 2.2	1.9	721.0 ± 3.4	731.09 ± 3.4	1.4
8C	729.0 ± 3.0	757.43 ± 1.2	3.9	735.0 ± 1.9	762.93 ± 3.9	3.8
8S	722.0 ± 4.2	743.66 ± 2.4	3.0	729.0 ± 2.8	750.87 ± 4.2	3.0

* Data are presented mean ± S.D. (n = 10). The increased percentage was calculated with respect to the corresponding control samples and shown within the parenthesis.

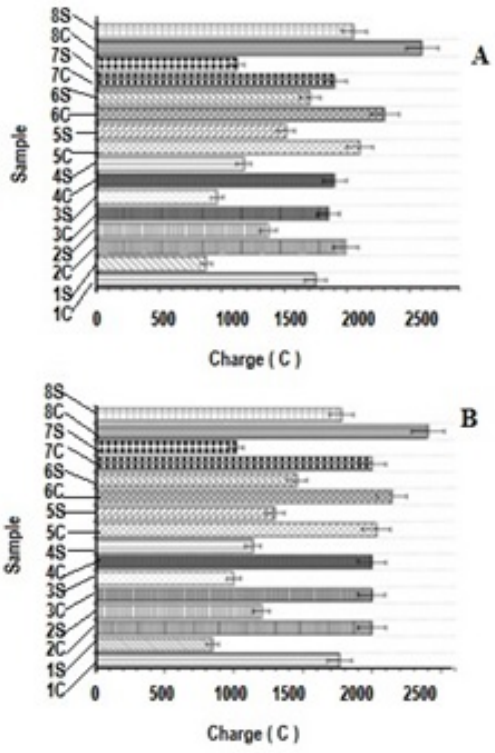

Figure 2. Rapid chloride permeability test results of bacterial incorporated geopolymer cured at different curing conditions: (A) Self-healed geopolymer mortar samples, (B) Crack-repaired geopolymer mortar samples.

The toxic effect of the transformed *Bacillus* cells on human cell lines was done by MTT assay, which did not exhibit any marked cell death (Table 9). The results of toxicity study of bacterial cells on rats were shown in Table 10. Only, with the higher concentration of bacterial treatment (10^6 cells/ml), the total cholesterol, HDL, LDL and triglyceride levels were increased slightly. Whereas, all the other parameters were well within the reference range (Table 9).

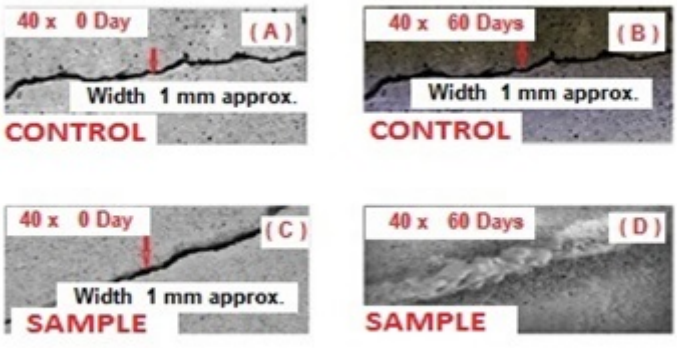

Figure 3. Crackscopic image of self-healed bacterial incorporated geopolymer mortar ample.

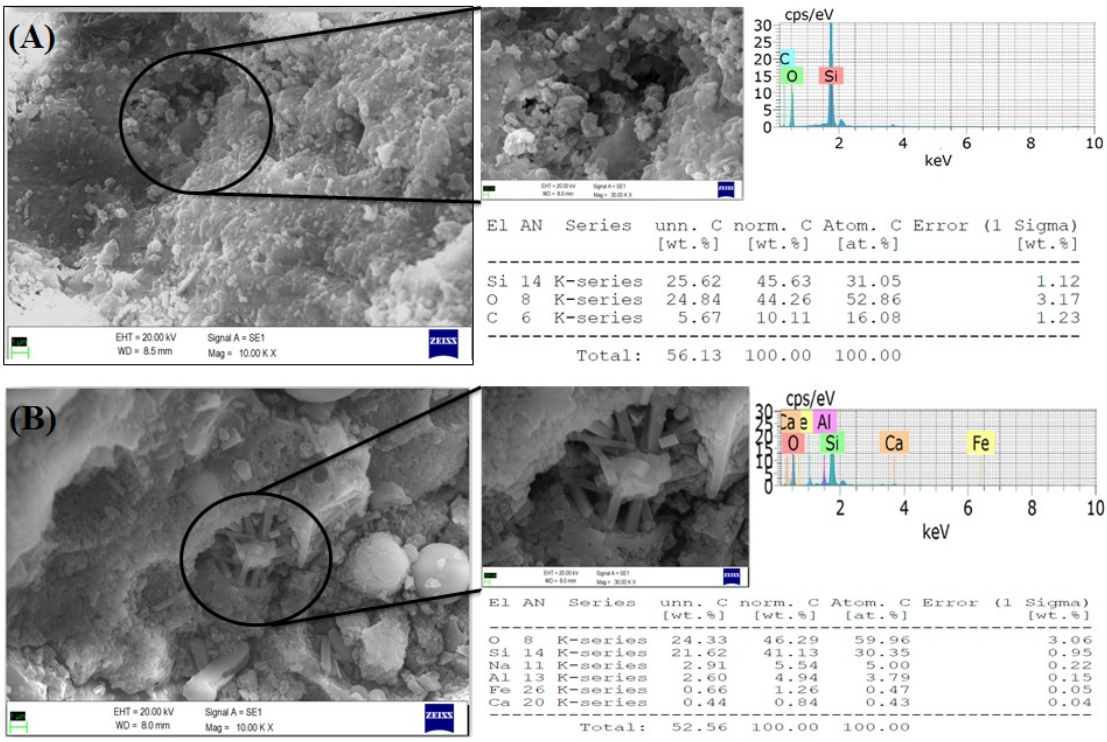

Figure 4. Microstructure analysis of geopolymer samples cured at ambient temperature air curing: (A) FE-SEM image and EDAX analysis of control geopolymer mortar, (B) FE-SEM image and EDAX analysis of bacterial cells amended geopolymer mortar.

Table 9. Survival data of human cell lines against Bacteria treatment

Cell concentration	Cell survivability percentage	
	WI38 cell line	HaCaT cell line
Control	100	100
10^1 cells/ml	97.9	92.1
10^2 cells/ml	95.0	91.4
10^3 cells/ml	94.6	92.3
10^4 cells/ml	90.4	89.1
10^5 cells/ml	83.7	84.8

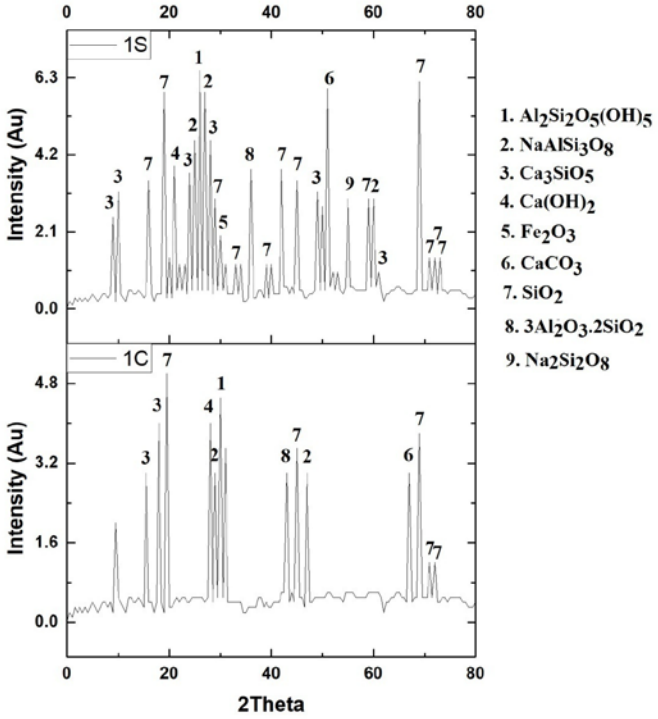

Figure 5. X-ray Diffraction study of geopolymer mortar cured in ambient temperature air curing: (1C) XRD analysis of control mortar sample, (1S) XRD analysis of bacterial cells amended mortar sample.

Table 10. Biochemical parameters of bacterial treated rats at different levels

Biochemical Parameter		Group 1	Group 2	Group 3	Group 4	Ref. Range
TG	Male	39.75	51.95	66.66	69.55	32.94 – 70.79
(mg/dL)	Female	31.78	48.30	60.36	66.16	25.88 – 65.88
TC	Male	60.07	67.10	89.66	119.05	60.00 – 100.00
(mg/dL)	Female	65.05	73.82	82.72	112.81	62.00 – 104.00
HDL-C	Male	46.86	49.52	67.49	75.56	39.02 – 72.20
(mg/dL)	Female	46.81	49.20	53.14	72.59	39.02 – 78.05
LDL-C	Male	7.16	7.19	8.84	29.61	2.39 – 27.34
(mg/dL)	Female	11.88	14.96	17.50	26.98	7.81 – 20.86
ALP	Male	5.00	7.85	9.00	9.94	2.20 – 9.20
(KA/100mL)	Female	4.80	6.20	7.92	9.40	2.20 – 9.20
Creatin-ine	Male	0.60	0.71	0.86	0.98	0.40 – 0.80
(mg/dL)	Female	0.48	0.56	0.69	0.80	0.40 – 0.80
Urea	Male	18.20	20.89	22.64	25.96	14.00 – 23.00
(mg/dL)	Female	16.57	19.58	21.63	23.22	14.00 – 23.00
SGPT	Male	20.23	22.89	26.71	29.16	17.50 – 30.20
(U/L)	Female	18.67	20.93	24.22	27.87	17.50 –30.20
SGOT	Male	52.94	66.84	75.89	80.65	45.70 – 80.80
(U/L)	Female	49.12	56.22	68.97	73.71	45.70 – 80.80

4. Discussions

Cement industries play a significant role to green-house effect, for which people are looking for suitable alternative(s) where, various industrial-waste materials are used in constructions for reduction of cement consumption and minimize of ecological problems created by cement [1, 14, 28]. Scientist and technologist are facing challenges in reducing CO_2 emissions while producing enough cement to meet demand of construction industries. Several research works are going on these directions, which include improving energy efficiency, switching to lower-carbon fuels, promoting material effectiveness and advancing new near zero emission manufacturing processes [16 -19, 28, 29]. Development of bacterial amended fly ash-based geo-polymeric material

is one of such attempt in this context. To make the process energy efficient, the energy consumption for geo-polymerization process has been modified by heating the mixture of alkali-activated solution mixed-fly ash only at 65 ºC for 45 mins, rather heating the whole mixture of alkali-activated fly ash and sand at 65 to 80 ºC for 48 h [3, 14].

Though highly alkaline environment resists the microbial growth inside the geo-polymeric matrix, the alkilophilic and thermophilic transformed *Bacillus subtilis* bacterium is chosen in this work because this bacterial species has ability to survive for quite long time inside the concrete matrices due to it spore forming attribute. The long term survival of the *Bacillus* cells within geopolymer is understood by the bio-silicification activity of the bacterial cells, which are isolated from geo-polymeric material of different ages (Supplementary Table 2).

Curing condition has a significant role in the development of strength and durability of concretes [29, 30]. Proper curing of concrete is a necessary because it promotes cement hydration resulting more hydration products, which is useful for the development of long-term strength. In addition, proper curing regimes enhances the development of concrete microstructures, which is favourable for durability improvement of the concrete [29, 30]. Furthermore, improper curing affects on the strength gains and creates several defects viz. micro cracks and poor surfaces, which greatly reduces the safety and durability of concrete structures [31]. The bacteria impregnated geo-polymeric mortars are thus cured under various conditions in order to achieve the best possible result for developing mechanical strength and self-healing efficacy. Weak tensile or flexural strength of the cementitious materials compels to steel reinforcement to protect the structure. Unfortunately, corrosion of steel reinforcement reduces the service life of the structure and thus creates added problem. Our results suggest that ambient temperature air curing is the most suitable curing condition for achieving the highest mechanical strengths (compressive, tensile and flexural) and extended longevity of the bacteria incorporated geo-polymeric mortars. Rabie et al. [32] have investigated the feasibility of producing sustainable cement-free composites and its environmental impact, which corroborates with our experimental results. High temperature air curing (90 ºC heat curing for 72 h) also showed good performance on mechanical strengths and durability of the bacterium incorporated geo-polymeric materials (Table 2). The spore forming ability of the bacterium may help the bacterium to remain active at high temperature.

It is known that the presence of sulphate salts in cement paste causes increased formation of Ettrengite at high rates that negatively affects the hardened cement paste due to a large volume increase in the hardened cement paste [33]. Similarly saline water also affects the workability, strength and durability of cementitious structures [34]. There are reports, which demonstrate that alkali-activated mortars possess better chemical stability, which provides resistance to acid attack [35] and salts such as chlorides [36] and sulfates [37]. Compare to controls, it is observed that the bacteria amended mortars cured in 5% sulphuric acid or 5% saline water environments have achieved higher mechanical strength, which clearly establishes the fact that the developed geopolymer material possesses good acid resistant and salt resistant attributes. This is again in agreement with the previously published results as demonstrated earlier.

As, low temperature hampers the bacterial growth and activity, this could be explained the poor mechanical strength developed in the bacteria-amended samples at 8 ºC for 72 h curing.

It is demonstrated earlier that anaerobic hot spring bacteria execute higher mechanical strength and enhanced durability due to the formation of gehlenite phase when incorporated in the material [19, 32]. The short lifespan of the bacterium in concrete opposes the bacterium to act as a true self-healing activator for a prolonged period. Bacillus can produce the self-healing calcite minerals in concrete [38]. The gene of Bioremediase-like protein from BKH2 bacterium has been transferred to spore forming *Bacillus subtilis*, for which the transformed *Bacillus subtilis* bacterium is able to form calcite and gehlenite both providing synergistic self-healing effect to the incorporated cementitious material. In fly ash geopolymeric composite, the transformed *Bacillus subtilis* bacterium leads to increased formation of various thermo-stable phases like Mullite ($3Al_2O_3$. $2SiO_2$), Albite ($NaAlSi_3O_8$) and Alite (Ca_3SiO_5) etc. in the matrices, which are primarily responsible for the increased mechanical strengths and longevity of the material [14, 19]. Our results also describe that the transformed *Bacillus subtilis* have an amicable crack repairing abilities when incorporated in the geo-polymeric material. The crack repaired mortars show remarkable improvement of compressive strength (63.5%; Table 3), split tensile strength (93%; Fig. 1C) and flexural strength (140.9%; Fig. 1D) respectively compared to controls after ambient temperature air curing (28 days). The highest increment of UPV was also noted in such case (Table 5). The bacterium was seen to fill the cracks of 1 mm width completely in ambient temperature water curing (60 days; Fig. 3). This implies the significant self-healing efficacy of the bacterium in geo-polymeric material, which arises from the fact that the transformed Bacillus bacterium produces various crack-sealing materials as mentioned above.

The results of water absorption test for self-healing study (Table 6) and crack-repairing study (Table 7), sulfate resistant study (Table 8) and rapid chloride ions permeability test (Figs. 2A and 2B) suggest the increased longevity of the bacteria amended geo-polymeric material. The transformed *Bacillus subtilis* possesses both the urease gene and bioremediase like-protein gene. Urease gene is responsible for calcite production and bioremediase like-protein gene is responsible for gehlenite and different themostable novel phases (Mullite, Albite, Alite etc.)

production inside the geo-polymeric material [14]. These phases fill the micro-pores and cracks, thus inhibit the water molecules or various ions (sulfate ions, chloride ions etc.) to ingress inside the matrix of geo-polymeric samples and act as self-healing agents [39, 40, 14]. The extended stability of the bacterium incorporated geo-polymeric material is therefore basically due to the self-healing attribute of the incorporated bacterial cells.

The transformed Bacillus bacterial cells neither produced any toxic effects on animals (Table 9) nor on human cell lines (Table 10). The toxicity study of the transformed *Bacillus subtilis* cells in rat models do not produce any harm to the animals, even when they are used directly by injection at high cell concentrations (Table 9). Similarly, MTT assay did not exhibit any marked cell death on two different human cell lines. The bacterium incorporated material will be thus eco-friendly and safe towards human populations.

5. Conclusions

Our study shows that, the genetically enriched alkilophilic *Bacillus subtilis* bacteria efficiently repair and heal the cracks in totally (100%) fly ash-based geo-polymeric materials cured in different conditions. The bacterial cells remain viable for longer time at adverse curing conditions inside the geopolymer material. The formation of various thermo-stable phases by the transformed *Bacillus* cells makes the geopolymer material eco-efficient and more durable. The ambient temperature air curing is the most suitable energy-efficient curing condition for achieving the higher mechanical strengths and increased durability of the bacterium incorporated geo-polymeric material. This geo-polymeric material may be used for an alternative of cement in construction industries.

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