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

A Thesis Submitted for the Degree of Philosophy in Science at Jadavpur University

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Certificate from the Supervisors

This is to certify that the thesis entitled "**Modified Bacteria Incorporated Geopolymer- A Qualitative Approach for an Eco-friendly, Energy- efficient and Self-healing Construction Material**" submitted by **Avishek Chatterjee** who got his name registered on 16/08/2016 for the award of Ph.D. (Science) degree of Jadavpur University, is absolutely based upon his own work under the supervision of **Prof. Brajadulal Chattopadhyay, Department of Physics, Jadavpur University and Prof.Saroj Mandal, Department of Civil Engineering, Jadavpur University, Kolkata** and that neither this thesis nor any part of it has been submitted for either any degree/diploma or any other academic award anywhere before.

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DECLARATION

I do hereby declare that the work embodied in this thesis entitled "**Modified Bacteria Incorporated Geopolymer- A Qualitative Approach for an Eco-friendly, Energy- efficient and Self-healing Construction Material**" which is being submitted for the degree of Doctor of Philosophy (Science) has been carried out by me in the Concrete and Structural laboratory, Department of Civil Engineering, and Bio-Physics laboratory, Departmnt of Physics, Jadavpur University, Kolkata, India. Neither this thesis nor any part thereof has been presented/submitted anywhere for either any degree/diploma or any other academic award before.

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May 2024, KOLKATA **Avishek Chatterjee**

This thesis is dedicated to my Mother

CONTENTS

ABBREBIATIONS

- MPa - Mega Pascal
- kN - Kilo Newton
- GPa - Giga Pascal
- CM - Control Cement Mortar
- GPC - Geopolymer Concrete
- FESEM - Field Emission Scanning Electron Microscope
- µL - Micro-litre
- µg - Micro-gram
- RCPT - Rapid Chloride PenetrationTest
- XRD - X-Ray Diffraction
- EDS - Energy Dispersive Spectroscopy
- EDTA - Ethylene Diamine Tetra-Acetate
- S.D. - Standard Deviation

INTRODUCTION

❖ General view

Concrete industry contributes at least 5 - 8% of the global carbon dioxide emissions [1]. The decomposition of lime stone emits a substantial amount of CO_2 in the atmosphere and reduce the lime stone resources during the manufacturing of cement. During the last few decades, there has been a rapid increase in production of coal ash, which is annually estimated to be around 780 million in the world due to increased amount of energy being generated by coal-fired power plants [2 – 4]. The utilization of fly ash is about 35% in landfills, embankments, production of blended cement etc. and remaining as an industrial waste.

Therefore, there is a need to develop an alternative material, which will not only reduce the demand for Portland cement, but also decrease the CO_2 emissions and increase the utilization of fly ash along with the cement alternative must possess mechanical and structural properties comparable to the Portland cement, but it should emit CO_2 at a much lower rate. A possible solution to this problem might be the use of fly ash based geopolymer mortar.

Geopolymers are a novel class of materials that are formed by the polymerization of silicon, aluminium and oxygen species from an amorphous three dimensional structures [5-8]. Geopolymers are mainly composed of the source material, whih are rich in silica and aluminium and alkaline activator liquid. The source materials could be obtained from natural minerals such as kaolinite, clays, etc. and the by-product materials such as silica fume, slag, red mud and fly ash. The combination of sodium hydroxide or potassium hydroxide and sodium silicate or potassium silicate used as an activator liquid for geopolymerisation[9]. It is being reported that alkali-activated geopolymer material has excellent mechanical properties, does not dissolve in acidic solutions, and does not generate any deleterious alkali-aggregate reaction even in high alkaline conditions [10].

Alkali- activated Kaolinite materials have been used as an alumino-silicate oxide source to synthesize geopolymer products which sems to be greener alternative to OPC [11, 12]. Alkali activation of metakaoline based geopolymer concrete showed the enhanced mechanical strength and durability with an increase of NaOH concentration [13].

One of the most common industrial by-product used as binder materials is ground granulated blast furnace slag (GGBS). GGBS had been widely used as a cement replacement material due to its latent hydraulic properties. GGBS shows an important role in

the development of the compressive strength of alkali activated geopolymer concrete. Moreover in the presence of high concentration of alkaline solution, this green cement alternative shows results higher compressive strength when heat cured at 60°C from 6 to 24 hours [14-16].

Fly ash ,another industrial by-product, which is most common sources for geopolymer mortar because for its easy availability [17]. The fly ash-based geopolymer matrix binds the loose coarse aggregates, fine aggregates and other un-reacted materials together to form the geopolymer concrete. There are several factors on which the strength and durability of fly ash based geopolymer concrete, depends are the concentration of alkali activator, mix proportion, curing temperature , mode of curing and curing time etc. It has been generally seen that higher concentrations of NaOH used as an alkali activator appeared to provide higher compressive strength at early age [18, 19]. The sodium hydroxide leaches the silicon and aluminium in the amorphous phase of fly ash and the sodium silicate acts as a binder. Also, the mechanical strength of geopolymer mortar depends on the concentration as well as ratio of sodium hydroxide and sodium silicate [20-23].In general, heat activation is needed for the development of geopolymer mortar in the presence of alkali activator. Several researchers have reported that the mechanical strength and durability of geopolymer concrete depends on heat curing period along with its respective curing temperature [24-27]. The Compressive strength of such geopolymer mortar is more at 60ºC comparable to 80ºC for a given molar concentration [22-27]. In previous studies, it has been found that the indirect tensile strength of fly ash-based geopolymer concrete when heat cured is greater than the values recommends by the draft Australian Standard AS3600 (2005) and Neville (2000) for Portland cement concrete [28]. It was also reported that the flexural strength of heat cured geopolymer mortar/concrete is about 1 – 1.4 times higher than that of OPC cement concrete [29].The high tensile and flexural strength of the geopolymer concrete help to decrease the rate and extent of cracking in response to corrosion of steel reinforcements [30-31]. Geopolymer product does not have stoichiometries composition and comprise mixtures of amorphous to semi-crystalline structure and crystalline Al-Si particles [32]. In spite of the complexities in their molecular structures, they can be used extensively in real world applications. The microstructural analysis of heat cured geopolymer mortar/concrete using Scanning Electron Microscopy (SEM) and Energy Dispersive X-ray (EDS), X-ray diffraction (XRD), Fourier Transform Infrared Spectroscopy (FTIR), helps us to understand the underlying mechanisms of reactions and morphology of the system [27,33-37].

However, geopolymer mortar/concrete provides poor strength at ambient temperature (about 27 ± 2°C) curing due to slow polymerization process. Thus the scope of geopolymer concrete to be used as a cement alternative is less preferred for the requirement of heat activation after casting.

Previous studies have reported that the compressive strength and durability of concrete can be increased substantially (> 30%) by using some specific hot spring bacteria (BKH1 and BKH2) or their extra-cellular protein (e.g. bioremediase, M.W. ~ 28 kDa) [38-40] . Those bacteria possess silica leaching (biosilicification) activity which can be used to develop new phase (Gehlenite) inside the mortar matrices for getting higher strength and more durability in concrete structures [41-42]. The use of *Bacillus pasteurii* bacteria in concrete is associated with mineral precipitation (calcium carbonate) that helps to fill micropores and cracks, thus reducing its permeability and increasing its strength and durability [43]. However, the highly alkaline pH environment within the concrete matrices restricts the growth of the bacteria [44]. To overcome this problem, different authors have suggested the use of different immobilization solutions (clay capsules, silica gel or polyurethane encapsulation). De Beile et al. (2018) have shown the first in situ applications of encapsulated bacterial spores which have the ability to self-heal cracks in concrete [45]. Inagaki et al. (2003) have shown that thermophilic or hyper-thermophilic microorganisms living in geothermal environments are involved in the formation of biogenic siliceous deposits (siliceous sinter, geyserite and silica scale) [46]. The slow growth rate of the hot spring bacteria may restrict them to use in the concrete industry. The problem has been overcome by transferring the bioremediase gene into *E. coli* JM 107 [40] and *Bacillus subtilis* bacterial strains and used those transformed bacteria in concrete/mortar mix to increase the strength and durability of the cementitious material in short time period [47]. *Bacillus subtilis* is a spore forming bacterial strain which can remain in dormant form within the concrete/mortar matrices for quite long time and becomes active when water ingresses within the concrete [48]. 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 [49]. 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 [49-50]. It occurs due to formation of calcite (calcium carbonate) [51] or gehlenite (calcium-aluminium silicate) crystals inside the matrices by the action of some specific incorporated-bacteria [38]. 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. Therefore, the study deals with the development of genetically modified bacterium amended geopolymer mortar without heat activation after casting and assessment of mechanical properties of such modified geopolymer mortar along with their crack repairing ability and its microstructural studies.

❖ Background

There are limited literatures available on geopolymer to eliminate the shortcomings of ambient temperature curing [52-53]. It has been found that mechanically activated fly ash based geopolymer paste cured at ambient temperature showed about 80% more compressive strength compared to raw fly ash based geopolymer paste [54]. It has been reported that the compressive strength and microstructure of the ground fly ash based geopolymer pastes cured at ambient temperatures depends on NaOH concentration [55]. The incorporation of 2% nano silica by mass of cementitious materials increased compressive strengths of high-volume fly ash concrete cured at ambient temperature has been reported in previous studies [56]. Researchers have found that the mechanical strength of high calcium fly ash based geopolymer paste had been increased with the addition of nano-SiO_2 and nano-Al_2O_3 due to formation of Calcium Silicate Hydrate (CSH or CASH) and Sodium Alumino-Silicate Hydrate (NASH) gels in the matrix [57-58]. However there is almost no systematic study on mechanical strength and durability performance of bacterium amended low calcium fly ash based geopolymer concrete cured at ambient temperature and their crack repairing ability when cured at various curing conditions. Based on the above background the following section discusses the goals of the present study.

❖ Goals

The present research is aimed at making a significant contribution towards the development bacterium amended alkali-activated fly ash based geopolymer mortar cured at ambient temperature and also to promote its use for practical purposes. The use of this

bacterium incorporated modified geopolymer mortar for various purposes will certainly reduce CO_2 emission to certain extent. Heat activation, which is an essential requirement to accelerate the polymerization process for the development of physical and mechanical properties of geopolymer concrete. Thus, the scope of geopolymer concrete is limited due to requirement of heat activation after casting. The main goal of present study focuses on developing bacterium incorporated modified geopolymer cured at ambient temperature and their self-healing behavioural study, survivability of the bacteria inside the geopolymer matrices for an year and the toxicity study of the bacteria in rat model as well as human cell line.

❖ Research Objectives:

The objectives of the research are:

- To develop bacterium amended geopolymer mortar cured at ambient temperature at specific bacterial cell concentration.
- Mechanical strength, durability study, acid and thermal resistance of bacterium incorporated geopolymer mortar (cured at ambient temperature) and compare with geopolymer mortar (without bacteria) and cement mortar (with and without transformed bacteria *Bacillus subtilis*).
- Microstructural study of Genetically enriched bacterium amended geopolymer mortar by Field Emission Scanning Electron Microscopy with Energy Dispersive X-Ray and X-Ray Diffraction analysis.
- Study on structural behaviours such as compressive strength, flexural strength, split tensile strength and bond strength of self-healed and crack repaired bacterium amended geopolymer mortar cured at different curing conditions and to compare with control geopolymer mortar without bacteria.
- X-Ray Diffraction (XRD) for qualitative identification of the chemical composition of the self healed bio-geopolymer mortar.
- Field Emission Scanning Electron Microscopy (FESEM) and Energy Dispersive X-Ray Spectroscopy (EDS) for observing the morphology of bacterium amended self-healed geopolymer mortar.

- Toxicity study of the genetically modified bacteria in rat model as well as human cell line.

The next chapter presents a detailed literature review on geopolymer mortar.

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REVIEW OF LITERATURE

❖ General view

This chapter presents the background on the development of fly ash based geopolymer in brief. This chapter also discusses the current research work on the development of bacterial concrete and their crack repairing abilities , including its mechanical behaviour and durability performance.

❖ History of Geopolymer:

'Geopolymer' term was being used by *Prof. Joseph Davidovits*, to describe a family of mineral binders which is having a chemical composition similar to zeolite but with an amorphous structure [1]. Geopolymers are gnerally member of the family of inorganic polymers,having chain structures formed on a backbone of Al and Si ions in presence of highly alkaline condition, which forms a three dimensional polymeric chain and ring structure consists of Si-O-Al-O [2]. The three known chemical units in the geopolymer structure are: (i) Si-O-Al-O, or polysialate group, (ii) Si-O-Al-O-Si-O, or poly (sialate - siloxo) group, and (iii) Si-O-Al-O-Si-O –Si-O, or poly (sialate – disiloxo) group [3]. This polymeric chain binds the inert aggregate to form geopolymer concrete.

❖ Source Materials:

Geopolymers are developed by alkali activation of any materials that contains Silicon (Si) and Aluminium (Al) in amorphous form to form cement alternative [4]. Metakaolin or Calcined Kaolin, ASTM Class F fly ash, GGBFS, combination of calcined mineral and non calcined materials, combination of fly ash and metakaolin, and combination of granulated blast furnace slag and metakaolin are generally used as source materials [5 - 10]. The research works which relate to other source materials used in cement alternatives are now discussed.

Besides these materials, geopolymer composite is also synthesized from industrial wastes, red mud (RM) and rice husk ash (RHA), at various mixing ratios of raw materials and the mechanical strength of this geopolymer composite also showed better performance at higher rice husk ash to red mud ratios [11].

An innovative geopolymer developed by *Hajjaji et al.* (2013) by alkali activation of metakaolin along with iron oxide and red mud mixtures which showed better mechanical

strength of such geopolymer [12]. *R. H. Kupaei et al.* (2013) developed the light weight geopolymer concrete, synthesised from fly ash (FA) and oil palm shell (OPS) which showed improved compressive strength at higher molar concentration of alkali activator[13]. Geopolymer concrete developed by *Yang et al.* (2008, 2009) by using recycled aggregates as partial replacement for the fresh ones and mixture of waste concrete powder and metakaolin along with silica fume as the source materials for the geopolymeric binder showed increament in compressive strength at high alkaline concentrations[14,15].

❖ Activator solution:

The major role in the development of strength and durability of gopolymer composites is because of alkali activator solution which is mainly Sodium hydroxide (NaOH) and sodium silicate (Na_2SiO_3) solution [2]. Although potassium hydroxide (KOH) and potassium silicate (K_2SiO_3) are also have been used by many researchers as alkaline activator but Sodium hydroxide (NaOH) and sodium silicate (Na_2SiO_3) solution shows improved mechanical strength when used in fly-ash based geopolymer[16, 17].

It have been also seen that geopolymeric reactions occur at a high rate when the alkaline activator contains soluble silicate, either sodium or potassium silicate, compared to the use of only alkaline hydroxides [7].

Fly ash activated with higher Molar concentrations (8-12 M) of NaOH solution, cured at 85°C for 24 hours produced the geopolymer composite with a compressive strength between 35 and 40 MPa, this strength increses to nearly 90 MPa with the addition of water glass to the NaOH (SiO_2 / Na_2O = 1.23) [18]. *Hardjito et al.* (2005) , *G. S. Ryu et* al. (2013) and *Chindaprasirt et al.* (2007) also reported that compressive strength is increased and developed earlier at higher molar concentrations of NaOH and higher ratio of Na_2SiO_3/NaOH [19,20,21].

G. Görhan and *G. Kürklü* (2014) reported that due to increase in the coagulation of silica, geopolymer concrete activated with 6M NaOH and cured at 65°C showed better compressive strength than geopolymer concrete activated with 9M NaOH solution and cured at 85°C [22].

❖ Curing Conditions:

Heat activation is a much desirable activity for geopolymerization for the development of strength of geopolymer concrete. Most of the research works on fly ash based geopolymer are on the mix proportion and strength variation of geopolymer concrete cured at different temperatures. The compressive strength of heat cured geopolymer concrete is generally higher than normal temperature geopolymer concrete. At early age within 7 days, heat cured geopolymer concrete achieved maximum compressive strength, whereas the compressive strength of normal temperature cured geopolymer concrete increased after 7 days to 28 days [33, 34].

Chindaprasirt et al. (2013) established that microwave radiation effectively enhanced the geopolymerizationof fly ash based geopolymer mortar. Early-stage of microwave radiation promoted the dissolution of Si and Al species and enhanced the gel formation of geopolymer and stimulated the breaking of hydrogen bonds in water molecules. The microwave radiation (98W) for 5 min followed by 6.0 hour at 60°C resulted in the densification of the matrices and increase in the bulk density and compressive strength of the samples.The micro wave radiation followed by conventional heat curing reduced the heat curing time and energy [35].

Bakharev (2005) reported the influence of elevated temperature curing types of activator on phase composition, microstructure and strength development in geopolymer materials using Class F fly ash and sodium silicate and sodium hydroxide solutions [36]. Long pre-curing of geopolymer concrete / mortar at room temperature before application of heat was beneficial for strength development than 1 month of curing at elevated temperature. The importance of elevated temperature curing particularly for the samples exposed to 2 to 5 hours curing, where a significant increase in strength was observed at 85°C as compared to 65°C. Also, this study investigated of the effect of different curing regimes and types of activator on the strength development and hydration products of fly ash activated by alkali activated solution.

Hardijito and *Rangan* (2004) observed that heat curing at higher temperature increased the compressive strength of geopolymer concrete up to 60°C, beyond this temperature did not increase the compressive strength. It was also reported that heat

activation of geopolymer concrete at 60°C for 24 hours showed optimum strength development [37].Similarly, *Škvára et al.* (2005) reported alkali activated geopolymer concrete achieved the maximum of strength after 6 – 12 hours of heat activation at a temperature of 60 - 80°C [38].

J.C. Swanepoel et al. (2002) conveyed that the compressive strength of geopolymer concrete after 28 days of curing (Fig. 2.1) indicated that the geopolymerization reaction occurred in the samples heated to 60 and 70°C for 48 hours, as the sample remained at the same strength after heating for longer time periods of 72 hours. Also, to the samples heated at the lower temperatures, 40°C and 50°C showed a decrease in strength for longer heating times. The changes that occurred in the samples heated at 40 and 50°C seems to be the result of mainly physical changes, while changes in the samples heated at 60 and 70°C seem to be more of a permanent chemical nature [8].

Mishra et al. (2008) reported that compressive strength of geopolymer concrete was increased with the increase of curing time 24 to 48 hours at 60°C. It was also reported that when curing time further increased from 48 hours to 72 hours, no significant variation in compressive strength was observed (Fig 2.2) [39].

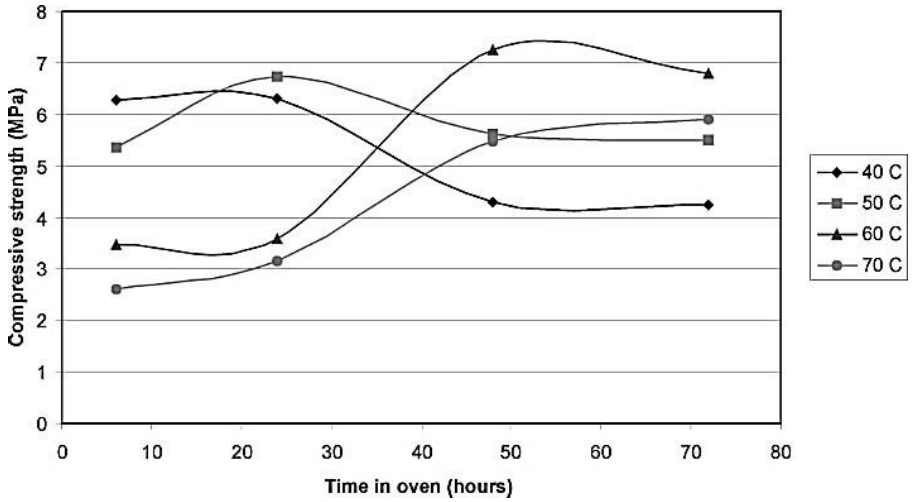

Figure 1 – : Compressive strength of geopolymer concrete after 28day at different temperature (2002).

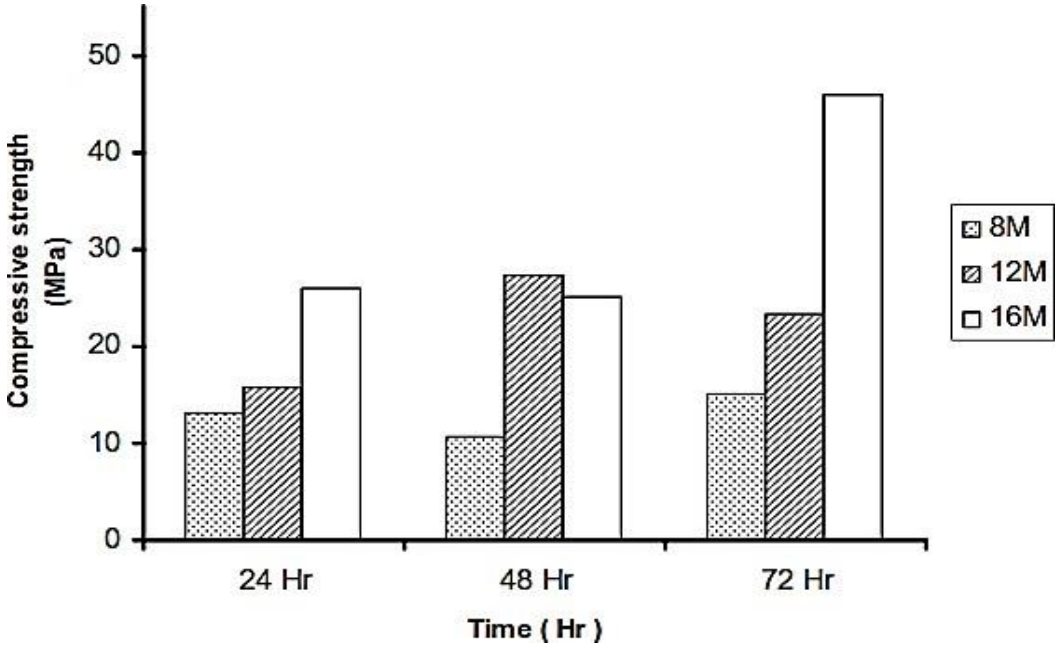

Figure 2 – : Variation of compressive strength with curing time (2008)

❖ Compressive strength:

The compressive strength of conventional Portland cement based concrete is the most important parameter in the mix design of concrete. The target compressive strength of conventional concrete depends upon the cement content and water / cement ratio of the mix. Similarly, compressive strength of geopolymer mortar/concrete depends upon several factors such as fluid / fly ash ratio, molar concentration of NaOH, NaOH / Na_2SiO_3 ratio, heat activation time and temperatures, mix proportion etc.

Higher concentration (in terms of molar) of sodium hydroxide solution results in higher compressive strength of geopolymer concrete. *Chindaprasirt et al.* (2009) showed that heat cured geopolymer concrete at low NaOH concentration of 5M reached 24 MPa compressive strength, whereas higher compressive strength of 35 and 33 MPa obtained with the use of 10M and 15M NaOH solution respectively (Fig 2.7). It was also reported that at higher molar concentration of NaOH fly ash based geopolymer concrete showed maximum compressive strength than that of bottom ash geopolymer concrete [40].

Palomo et al. (1999) reported that higher molar concentration (up to 12M) of alkali activator solution increased the geopolymerisation process and subsequently increased compressive strength [23]. Similarly, *Alvarez-Ayuso et al.* (2008) reported higher

compressive strength values were attained at curing time of 48 h, having curing temperature of 80°C and 12M NaOH solutions as activation media (Fig 2.8) [41].

The higher the ratio of sodium silicate to sodium hydroxide solution and solid to liquid ratio of activated fluid by mass, the higher the compressive strength of geopolymer concrete [42].

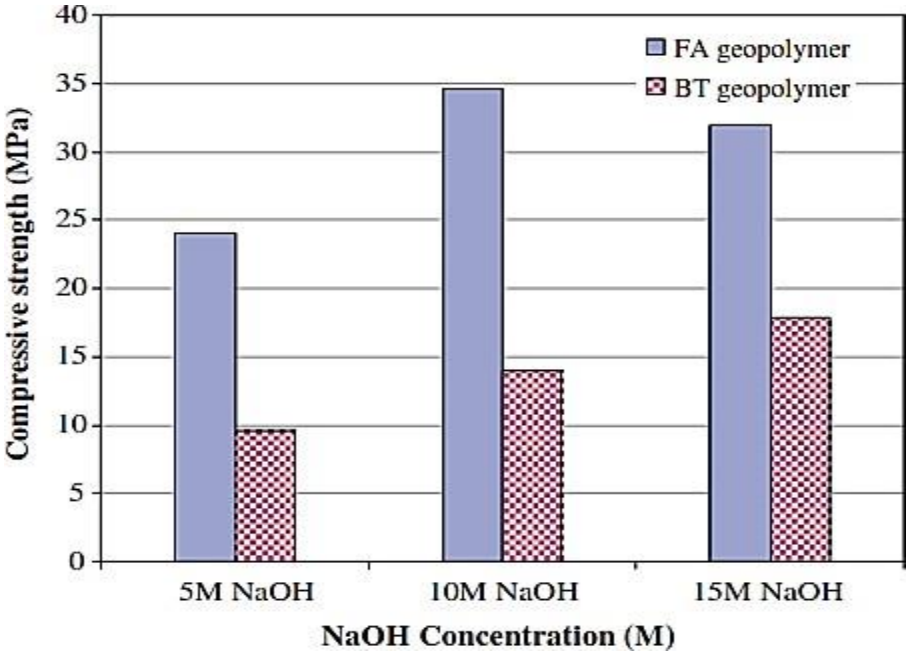

Figure 3 – :Compressive strength of fly ash and bottom ash based geopolymer at different molar concentration (2009).

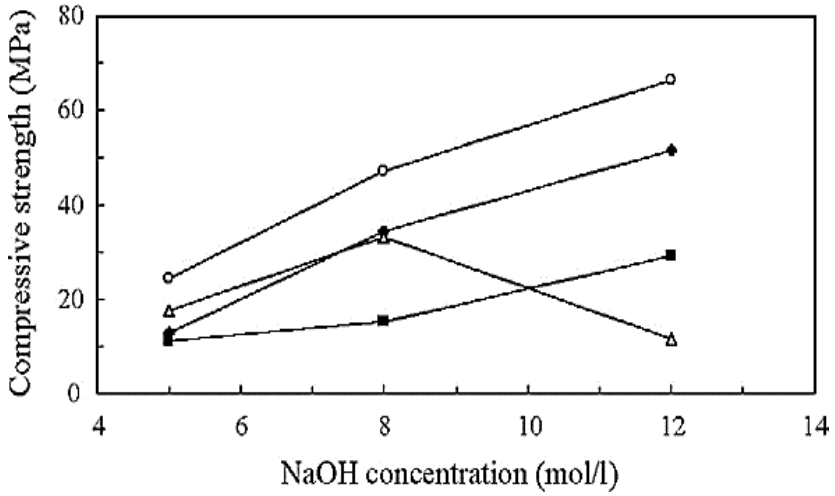

Figure 4– : Compressive strength of based geopolymer at different molar concentration (2008).

Chindaprasirt et al. (2007) reported that compressive strength of geopolymer mortar with sodium silicate to sodium hydroxide ratio of 0.67 and 1.00 were significantly higher than Na_2SiO_3 / NaOH ratio of 1.5 and 3.0. The variation in the ratio of sodium silicate to sodium hydroxide ratio affects the pH conditions and affectes on the strength development of the geopolymer mortar [26] (Fig 2.9).

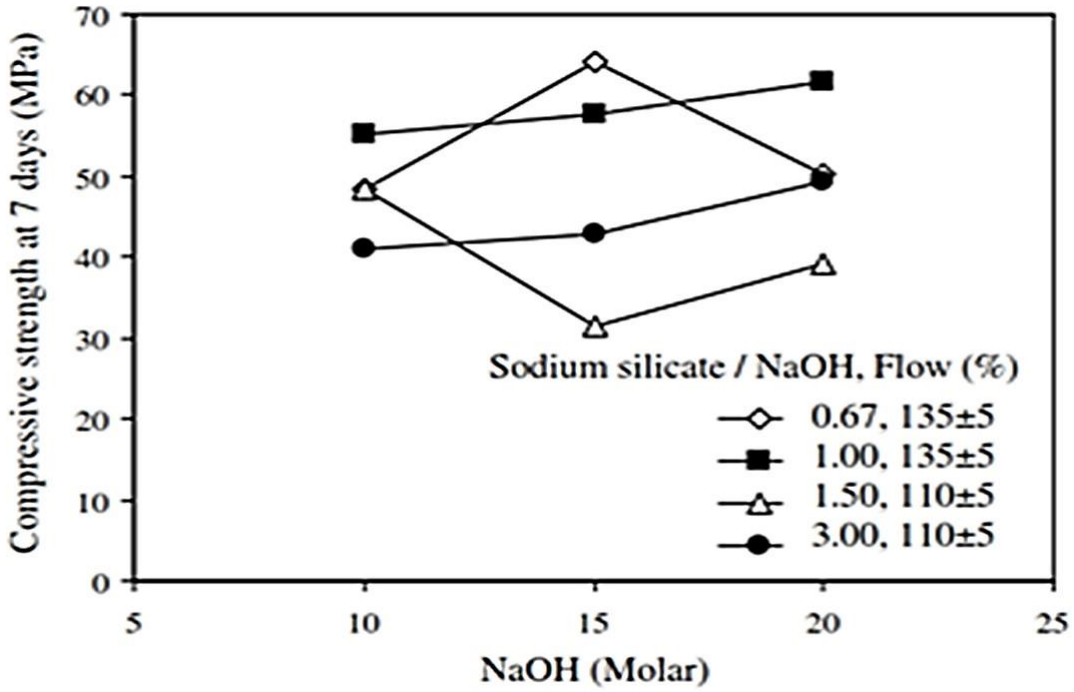

Figure 5 – : Compressive strength of geopolymer concrete at different molar concentration (2007).

Salih et al. (2014) showed the relation between compressive strength and solid to liquid ratio of geopolymeric mixes. The compressive strength was increased by increasing the sodium silicate (Na_2SiO_3) to sodium hydroxide (NaOH) ratio. The increment in the Na_2SiO_3/NaOH ratio increased the amount of sodium silicate in the activator solution and increased geopolymerization (Fig 2.10). The maximum compressive strength observed at sodium silicate to sodium hydroxide ratio of 2.50 [43].

The compressive strength of geopolymer concrete also depends on H_2O to Na_2O molar ratio. *Barbosa et al.* (1999) reported that the compressive strength of geopolymer concrete was decreased with the increase of H_2O to Na_2O molar ratio [44, 45].

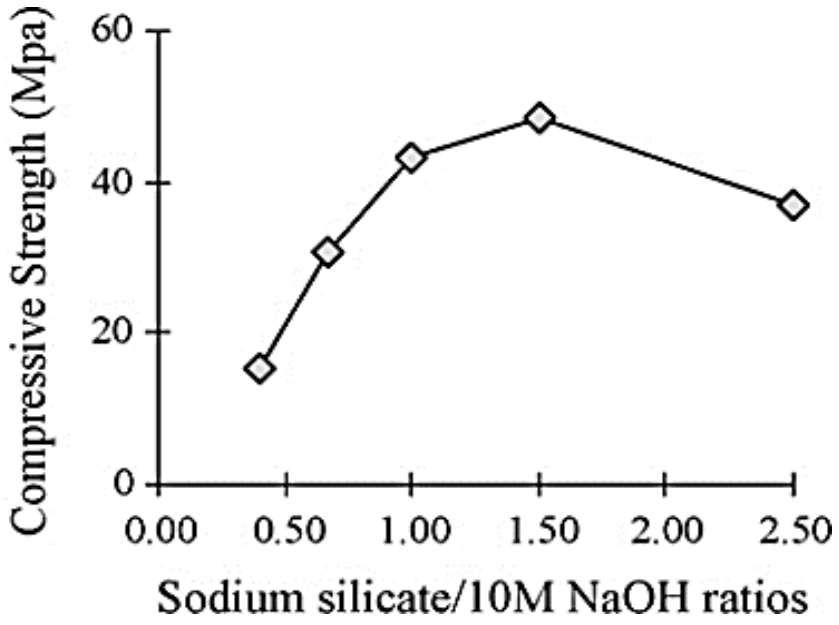

Figure 6 – : Compressive strength of bottom ash geopolymer mortar (2009).

Longer curing time and higher curing temperature increases the compressive strength of geopolymer concrete. *Palomo et al.* (1999) reported compressive strength of geopolymer cured at 85°C was higher than the samples cured at 65°C. Longer curing time of the samples showed maximum average compressive strength [23]. Geopolymer concrete cured at 40°C disclosed very low compressive strength due to slow polymerisation process. Curing time played a positive role on the compressive strength performance of geopolymer concrete. *Rovnaník* (2012) reported that compressive strength of geopolymer concrete was the function of curing temperature and curing time. Geopolymer concrete cured at 60°C and 80°C showed better compressive strength than geopolymer concrete cured at 40°C for 4h [46].

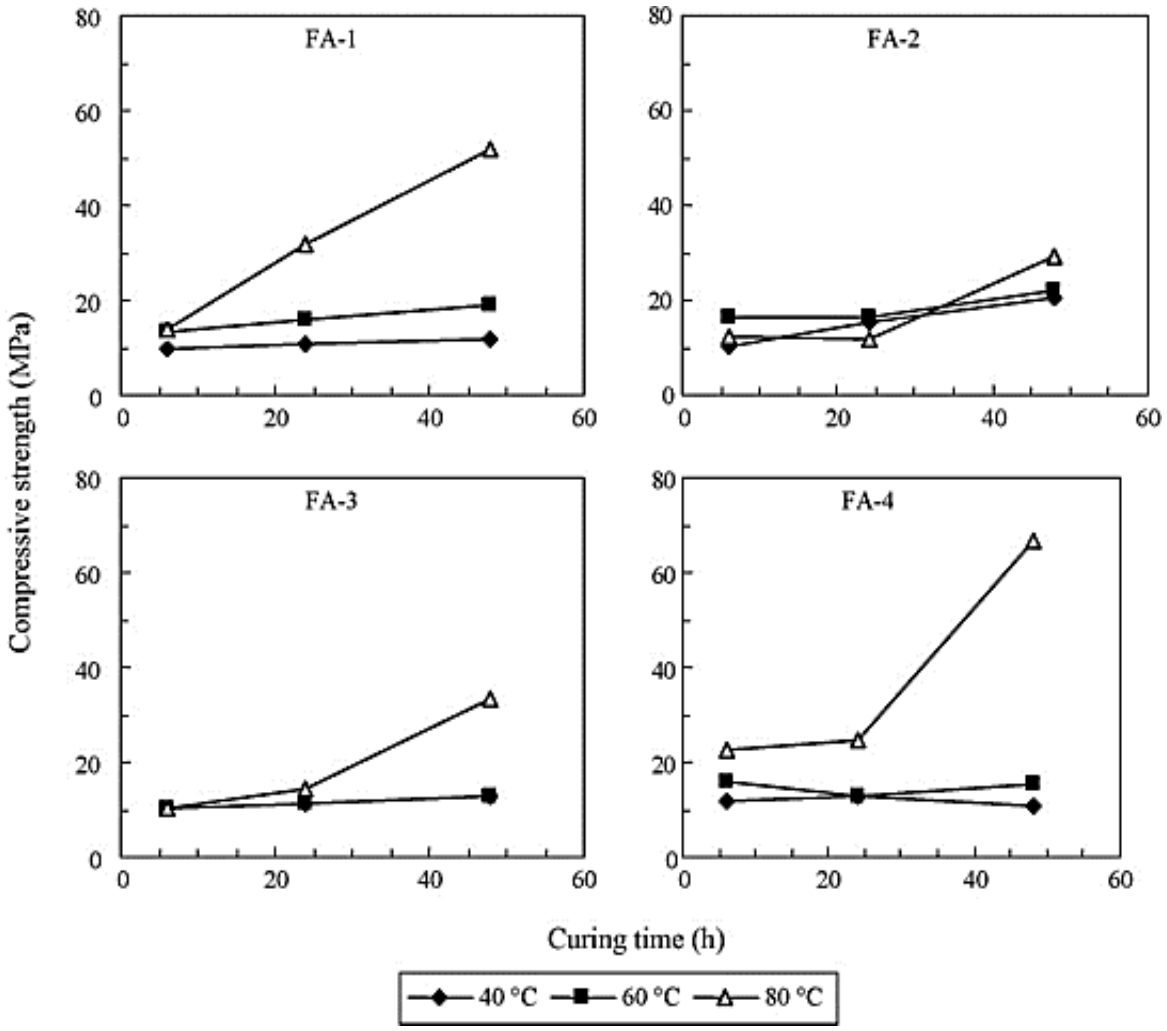

Figure 7 – : Compressive strength of geopolymer at different curing temperature and curing time (2012).

❖ Tensile strength:

The tensile strength is one of the basic and important properties of the concrete. The concrete is not usually expected to resist the direct tension because of its low tensile strength and brittle nature. However, the determination of tensile strength of concrete is necessary to determine the load at which the concrete members may crack. The cracking is a form of tension failure.

The tensile splitting strength (indirect tensile strength) of geopolymer concrete is only a fraction of the compressive strength, as in the case of Portland cement concrete [24]. It was also reported that indirect tensile strength of fly ash-based geopolymer concrete is larger than the values recommended by the draft Australian Standard AS3600 (2005) and Neville (2000) for Portland cement concrete.

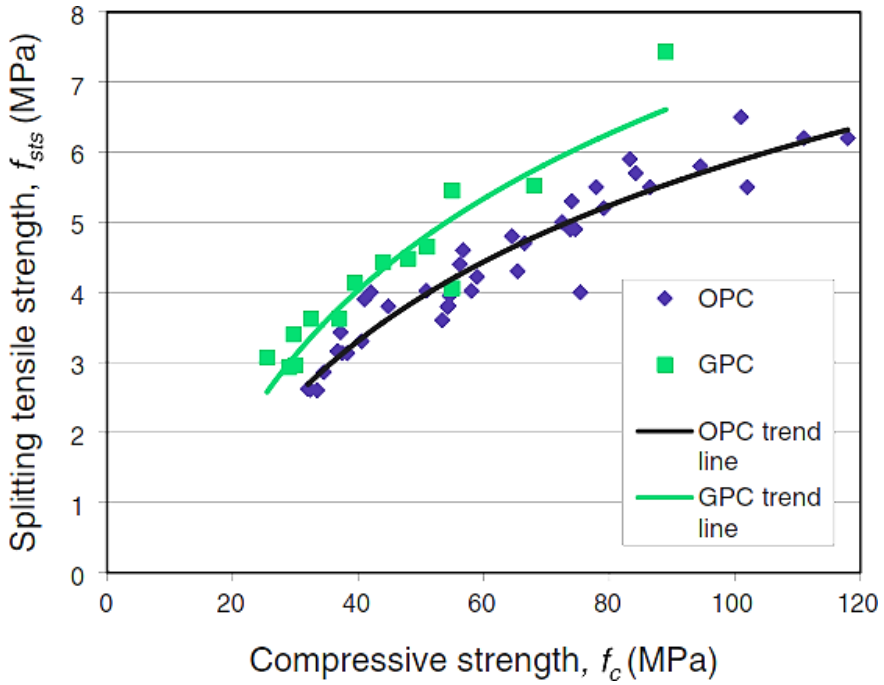

Figure 8– : Split tensile strength with compressive strength of GPC (2011).

Ryu et al. (2013) reported a model to describe the relationship between compressive strength and split tensile strength of geopolymer concrete with the mix of 9 M NaOH and sodium silicate at mass ratio of 1:1 and cured at 60°C for 24h (Fig 2.17 & Eq. 8) [29]. *Yellaiah et al.* (2014) conveyed that direct tensile strength of geopolymer mortar cured at 30°C and 60°C was 0.11 and 0.14 times of compressive strength at activator liquid to fly ash ratio of 0.30. It was also reported that the tensile strength of geopolymer mortar was more at lower curing temperature and was less for higher curing temperature for lower activator fly ash ratio due to insufficient alkaline liquid for complete polymerization [47].

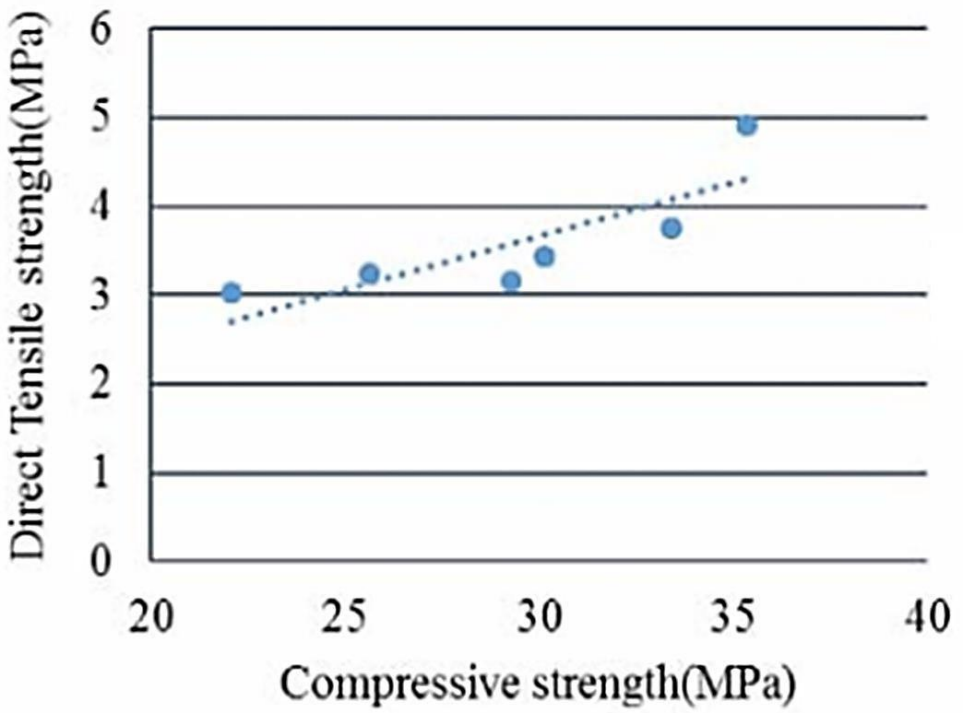

Figure 9 – :Variation of direct tensile strength with compressive strength of geopolymer (2014).

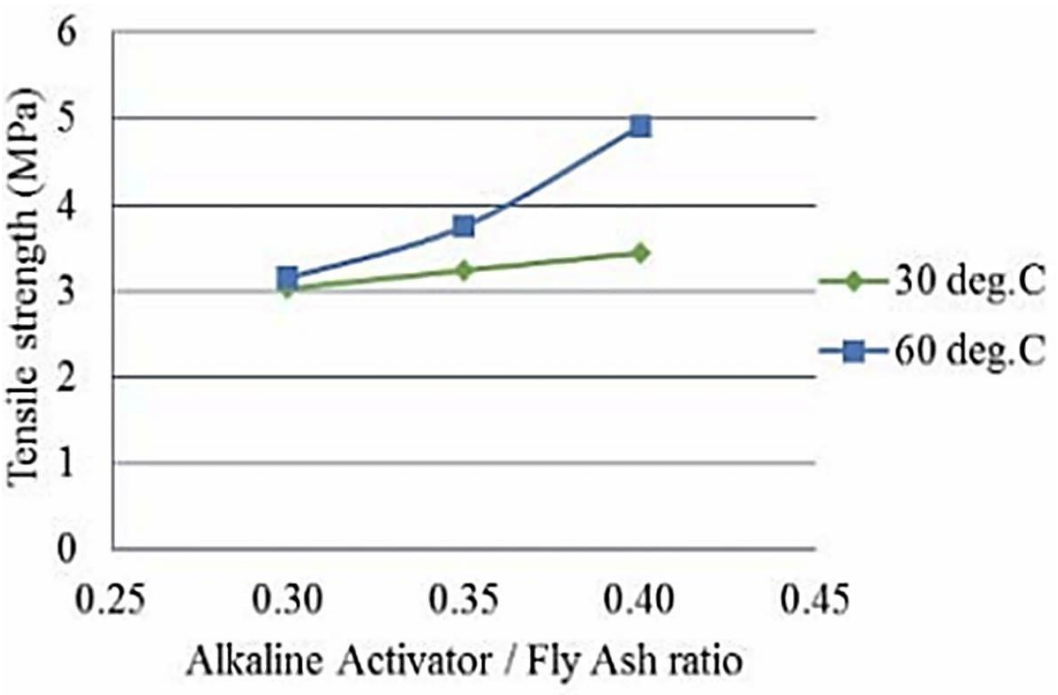

Figure 10 – : Variation of tensile strength with alkali activator and fly ash ratio (2014).

❖ Flexural strength:

Flexural strength is one measure of the tensile strength of concrete. It is a measure of an unreinforced concrete beam or slab to resist failure in bending. *Sofi et al*. (2007) reported that flexural strength of 100×100×300 mm steam cured prisms at 30°C – 35°C for 24h was more than OPC concrete prism. It was also informed that the difference between splitting tensile and flexural strength of geopolymer concrete mixes had been found to be approximately 2.0 MPa [48].

Yellaiah et al. (2014) reported that flexural strength of 70.6mm×70.6mm×141.2mm prism moulds cured at 30°C and 60°C for 24h increased with the increase of alkaline activator to fly ash ratio. The maximum flexural strength of fly ash based geopolymer was achieved at 60°C for 24h curing [47].

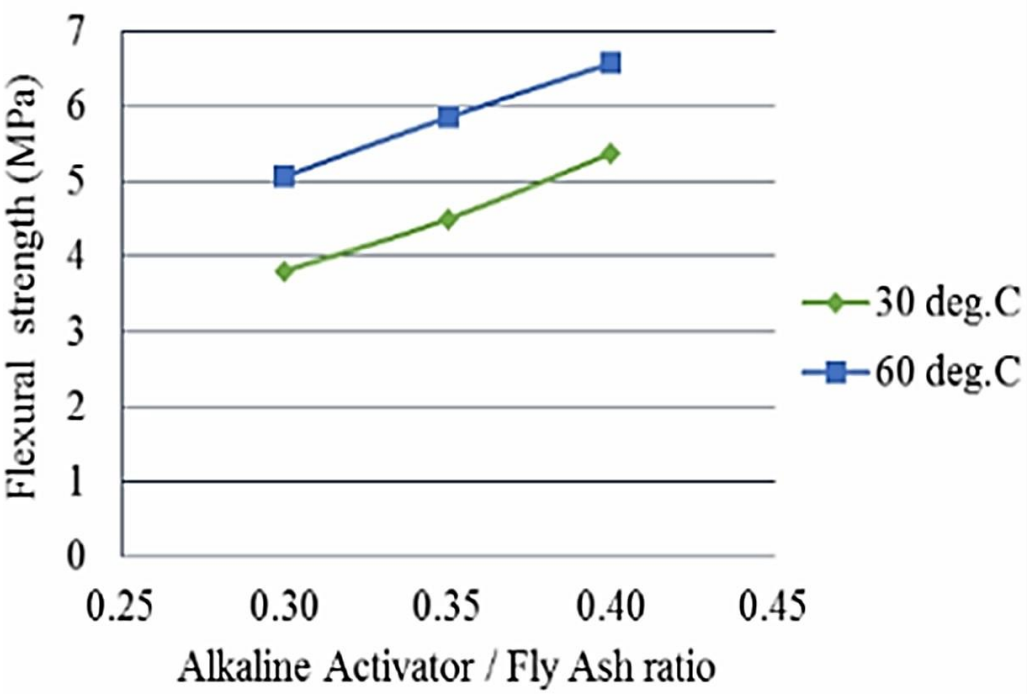

Figure 11–: Variation of modulus of rupture with alkaline activator / fly ash ratio for different curing temperature (2014).

❖ Microstructure analysis of geopolymer concrete

A brief outline about the microstructures of geopolymer matrix relevant to the present researchis presented in this section.

❖ X-ray Diffraction analysis:

XRD analysis is based on constructive interference of monochromatic X-rays and crystalline sample. The X-rays are generated by a cathode ray tube, filtered to produce monochromatic radiation, collimated to concentrate, and directed towards the sample. The interaction of incident rays with the sample produces constructive interferences. The constructive interference of X-rays diffracted from the planes of atoms within the solid give rise to a characteristic diffraction peak or a sequence of peaks that are unique to a particular material. Thus, XRD may be a useful tool for the present study even though the amount of information which can be obtained is limited due to the substantial amorphous nature of geopolymers.

XRD analysis confirmed that in the presence of alkali activated solution and heat activation of amorphous compounds present in fly ash transform into semi crystalline and semi crystalline compound. The peaks of hematite, quartz, and mullet have been distinctly observed and the hump denotes presence of amorphous silica in the geopolymer matrix as shown in diffractrogram [68, 69].

T. Bakharev (2005) had reported that the phases of geopolymer matrix were amorphous, and only in the case of materials prepared with sodium hydroxide solution were semi crystalline zeolitic phases. The peaks were observed in XRD analysis of heat activated geopolymer matrix due to quartz, mullite and hematite of the crystalline component of the fly ash (Fig 2.29). The broad peak in the region 20 – 32° 2θ arising from the glassy phase of the fly ash and broad peaks in the region 6 – 10° and 12 – 16° 2θ arising from alumino-silicate gel [36].

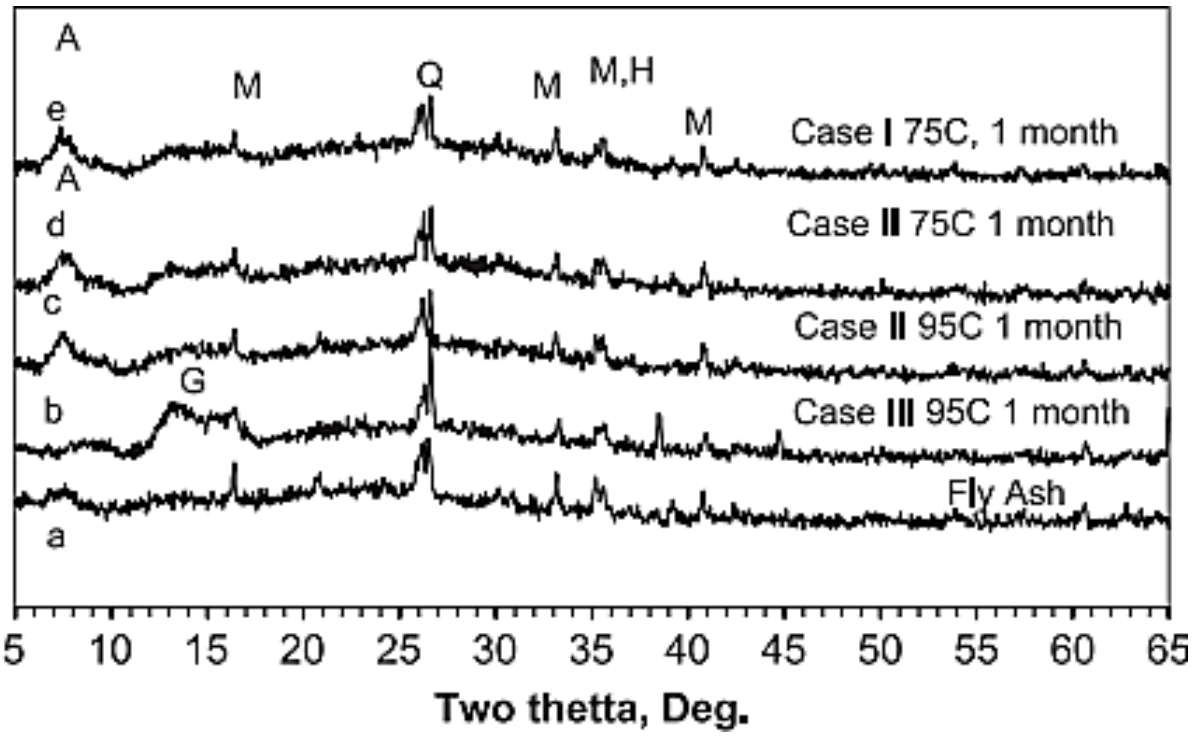

Figure – 12 :XRD analysis fly ash based geopolymer concrete, A=poorly crystalline, G=poorly crystalline alumino-silicate gel, Q=quartz, M=mullite, H=hematite (2005).

XRD analysis results of fly ash based geopolymer concrete/mortar depends on molar concentration and heat activation. The alumina-silicate substances with amorphous structure are the main products generated by geopolymerization through the alkali-activation process. Finding the patterns of the products in amorphous phase by XRD is very difficult. There were numerous peaks of mullite ($3Al_2O_3$, SiO_2) and quartz (SiO_2) in the crystalline phase was observed [29].

Chindaprasirt et al. (2013) reported that picks of crystalline quartz (SiO_2), calcium sulfate ($CaSO_4$) and calcium oxide (CaO) were observed in the XRD analysis of high calcium fly ash based geopolymer paste under microwave radiation followed by heat curing for 3h, 6h and 12h at 65°C. The broad hump at 22 – 38° observed in XRD analysis of geopolymer matrix, indicated the presence semi crystalline phase with high amount of amorphous gel. It was also reported that the calcium silicate compounds from the reaction between high calcium fly ash, silica and silicate solution. Microwave radiation cured geopolymer paste exhibited the sharp peaks of crystalline phases with high degree of amorphous phase of the semi-crystalline geopolymer [35].

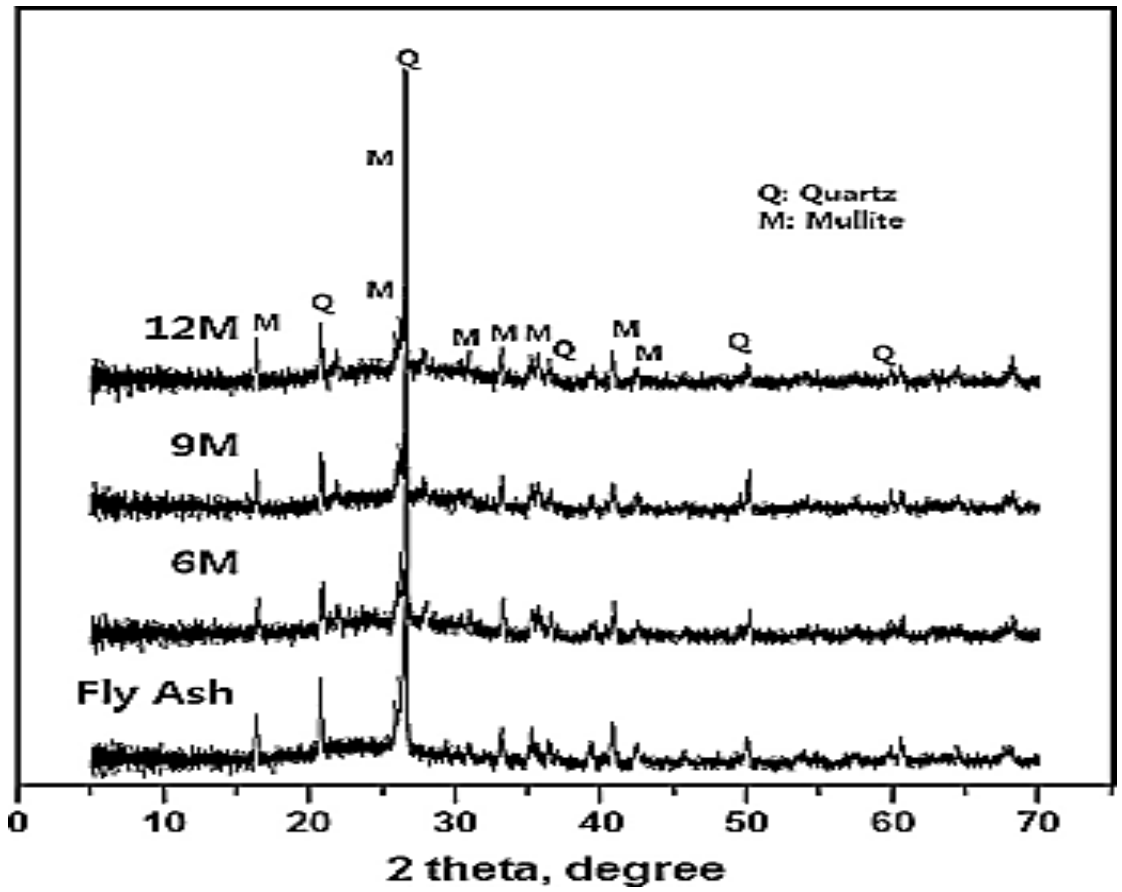

Figure – 13 :XRD analysis of fly ash and geopolymer matrix (2013).

❖ Scanning Electron Microscopy (SEM) and Energy Dispersive X-ray (EDX):

The scanning electron microscope (SEM) uses a focused beam of high energy electrons to generate variety of signals at the surface of solid sample. The high energy electron carry a significant amount of kinetic energy and this energy dissipated as a variety of signals produced by electron-sample interaction. These signals include secondary electron, backscattered electron and X-rays provide the information about SEM. The secondary product is readily interpretable images of the surface to determine sample morphology. The emitted X-ray has an energy characterization of the parent element. The detection and measurement of energy permits elemental analysis.

Ryu et al. (2013) observed that fly ash particles polymerized in presence of alkali activator produced amorphous, semi crystalline and crystalline product.The un-reacted fly

particle is also observed in spherical shape. The un-reacted fly Ash particle in the matrix do not act as filler, but instead increase the strength of the matrix with age through the bonding strength provided by the complex reaction between the surfaces of the particles (Fig 13A & B) [29] .

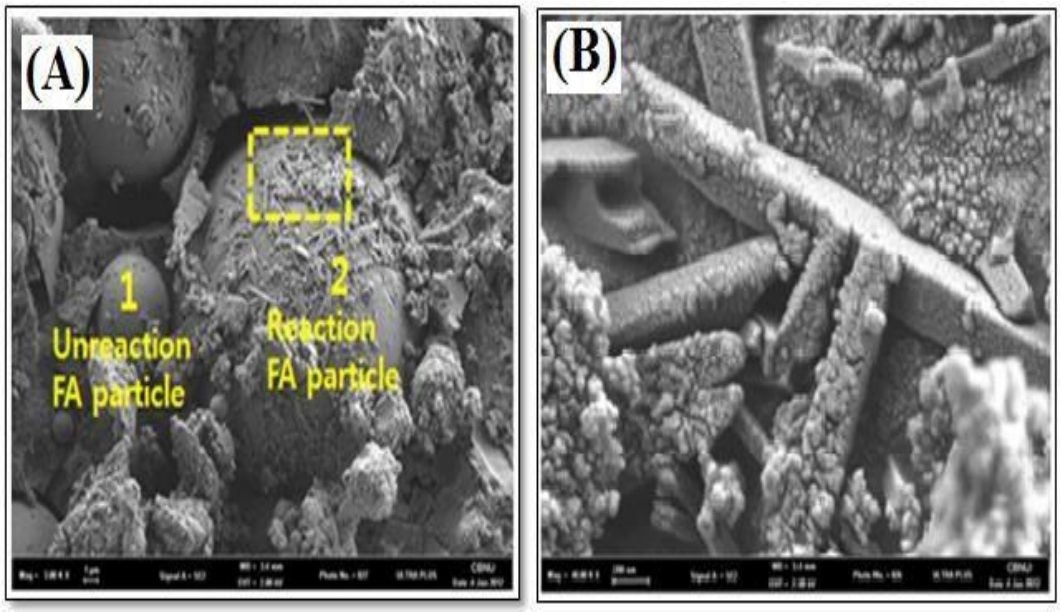

Figure – 14:(a) Unreacted fly ash and (b) reaction product after polymerization (2013).

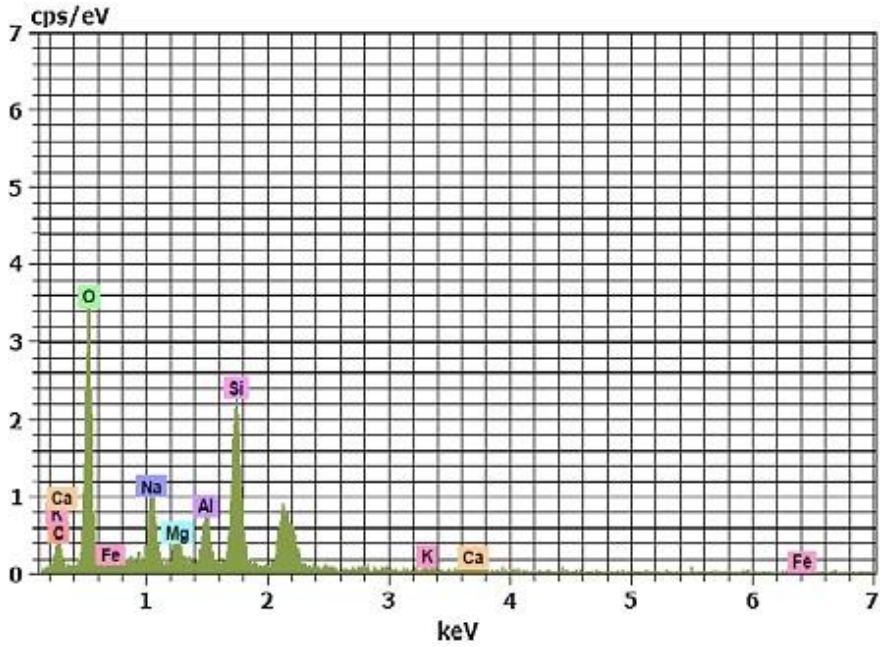

Figure – 15: EDX analysis of the geopolymer matrix (2013).

Also EDX results are presented in Figure 2.33 of alkali activated fly ash geopolymer matrix. It is clearly observed that most of the reactant is composed of silica and alumina. The

sodium is observed due to presence of alkali activator (NaOH + Na_2SiO_3) in geopolymerisation process. These reactants in turn combine with the Na ions dissociated from the external NaOH, causing the reaction products to agglomerate and enabling the strength to develop through combinations of fly ash particles [29].

Chindaprasirt et al. (2013) reported the SEM images of un-reacted spherical fly ash, partially reacted grains of fly ash particles of microwave cured geopolymer mortar (Fig 2.33). Gel formation on fly ash particles showed the dissolution of glassy phase in the alkaline solution as showed in Figure 2.34A & 2.34C. A large number of gels were formed on the fly ash particles owing to the promoted dissolution of Si and Al species from fly ash with microwave radiation. It was found in FESM study that the Interfacial Transition Zone (ITZ) between aggregates and concrete matrix in heat cured geopolymer matrix stronger than that in cement concrete. The stronger ITZ contributed to the higher splitting tensile strength and bond strength of geopolymer concrete [35].

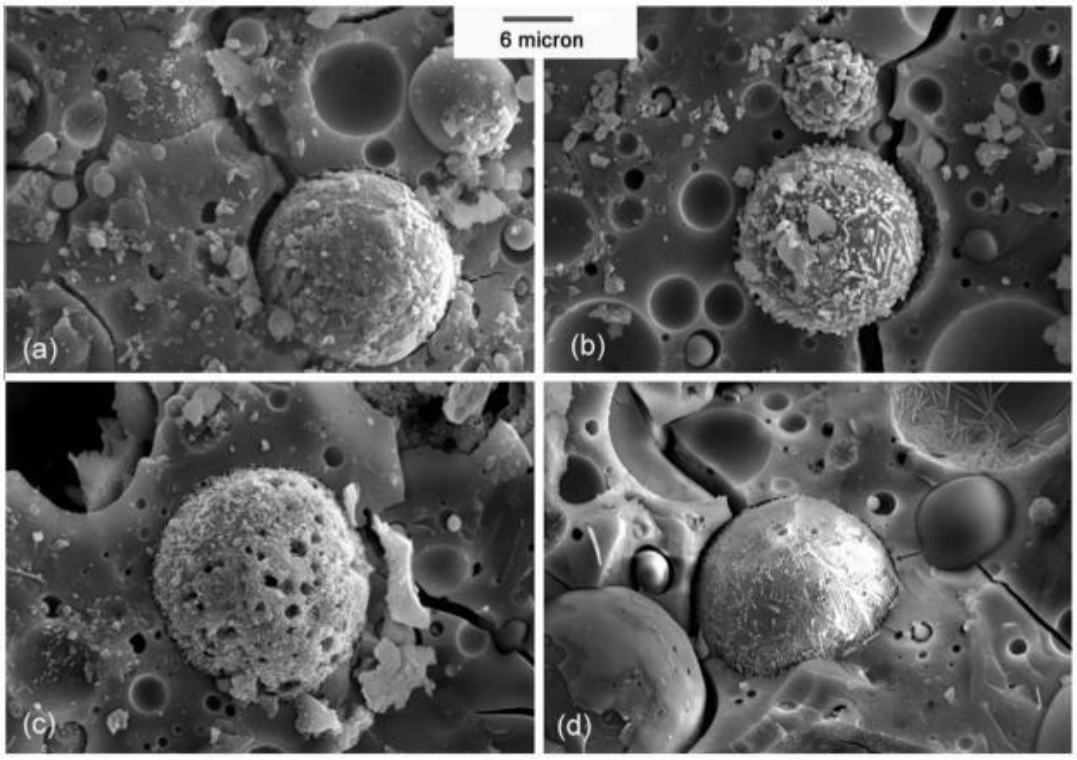

Figure – 16 :Microstructure of geopolymer pastes with microwave radiation and additional 65°C heat curing; (a) 3h, (b) 6h, (c) 12h, and (d) Control (2013).

Chindaprasirt et al. (2009) compared in SEM images of fly ash and bottom ash based geopolymer matrix. Both SEM images showed a continuous mass of alumino-silicate with

un-reacted, partially reacted ash particles. The ratio of Si/Al for of bottom ash geopolymer matrix was higher than that of fly ash based geopolymer as observed in EDX analysis. The higher ratio of Si/Al results in geopolymers with lower strength and higher elasticity.

EDX analysis of geopolymeric gel showed that the gel was mostly consists of the phases containing Na-Si-Al in the bulk region suggesting the formation of silicate-activated gel by polymerization throughout the inter particles volume. Sodium, silica and alumina and a small amount of iron, calcium, potassium and magnesium were observed in the gel by Energy Dispersive X-Ray Analysis (EDX). These elements (Fe, Ca, K, Mg) was obviously represented the fly ash phases, which for various reasons, did not dissolve during alkali activation [70].

❖ Applications of Geopolymer mortar:

Most applications of geopolymer mortar/concrete to date have been in the precast industry using accelerated curing. However, geopolymers have various other areas of applications from civil engineering field to automobile and aerospace industries. *Rangan et al.*(2005) have identified various economic benefits of using fly ash based geopolymer concrete, by taking into account that the cost of purchasing fly ash (excluding transportation) is relatively low. Therefore, after taking into account the cost of activator liquids, it was estimated that the production of fly ash-based geopolymer concrete may be 10 – 30% cheaper than that of Portland cement concrete. This is not the case though in practice, as the large cement companies usually control the supply of raw materials, including fly ash, which is often locked up by long term agreements [71].

Since geopolymers are considered as two-component systems (reactive solid components alkaline activation solution) they can be used as suitable binders in precast industry for the manufacture of reinforced products such as large-diameter pipes and roofing tiles. Immobilization techniques are used for the treatment of large amounts of heavy metals and radioactive wastes, thus geopolymerisation has received over the years significant attention due to its low cost, flexibility and increased durability versus time [1, 2].

Table – 1: Application of geopolymer mortar:

Si:Al ratio	Applications
1	- Bricks - Ceramics - Fire protection
2	- Low CO_2 cements and concretes - Radioactive and toxic waste encapsulation
3	- Fire protection fibre glass composite - Foundry equipment - Heat resistant composites, 200°C to 1000°C - Tooling for aeronautic titanium processing
>3	- Sealants for industry, 200°C to 600°C - Tooling for aeronautics SPF aluminium
20 - 35	- Fire resistant and heat resistant fibre composites

❖ Modified Geopolymer concrete without heat activation:

The scope of geopolymer concrete is limited to the precast member due to requirement of heat activation after casting. Most of the research works on fly ash based geopolymer are on the mix proportion and strength variation of geopolymer concrete cured at different temperature range of 45 ºC to 80 ºC for about 2 -3 hours [35]. It is well-known that the geopolymer mortar provides poor strength at ambient temperature of about 27 ± 2 °C curing due to slow polymerization process. There are limited literatures on available on geopolymer to eliminate the shortcomings of ambient temperature curing. The literate available on these areas are now presented. There were several technique has been adopted for the development of strength and durability of ambient temperature cured geopolymer concrete. *Temuujin et al.* (2009) reported the mechanical activation of the fly ash results in particle size and morphology changes with concomitant increase in reactivity with alkaline liquid. Mechanical activation of fly ash in a vibration mill with milling media to powder ratio of 10:1 leads to a reduction of particle size and change in particle shape but little change in mineralogical composition. Mechanically activated ash based geopolymer paste cured at ambient temperature showed 80 % increase in compressive strength than raw fly ash based geopolymer paste. The main contribution to increased compressive strength of the geopolymer is attributed to reduction of particle size and change in morphology allowing a higher dissolution rate of the fly ash particles [68].

Wongpa et al. (2010) reported that an ambient temperature cured inorganic polymer concretes (IPCs) can be produced from rice husk–bark ash (RHBA) combined with fly ash

(FA). The compressive strength of RHBA and FA modified geopolymer concrete cured at ambient temperature were influenced by the ratios between the paste content and the aggregate content and the weight ratio between the solution content (S) and the ash content (A). The solution to ash ratio was the most important factor that controlled the rate of reduction in compressive strength of IPCs while paste to aggregate ratio had the less influence. The higher the S/A ratio, the lower the compressive strength. On the other hand, for the same solid to ash, the mixtures containing higher paste to aggregate ratio produced lower compressive strength than that with lower paste to aggregate ratio . It was stated that the geopolymer mix of paste to aggregate ratio 0.34 and solid to ash content ratio 0.63 showed the highest compressive strength at ambient temperature curing [69].

Somna et al. (2011) reported that ground fly ash synthesised with hydroxide for 5 minutes cured at room temperature produced more strength than ordinary fly ash based geopolymer paste. The ground fly ash had the higher surface area than ordinary fly ash, resulted in a significant improvement compressive strength at ambient temperature. XRD results of NaOH activated ground fly ash showed the presence of more amorphous and crystalline phase of quartz (SiO_2) and hematite (Fe_2O_3) than ordinary fly ash geopolymer matrix [70].

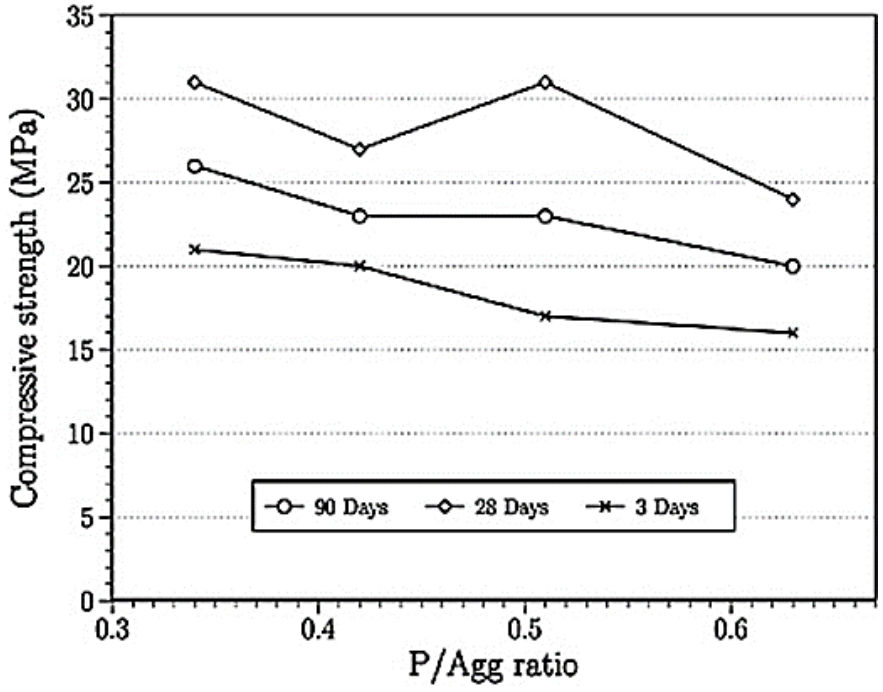

Figure – 17: Relation between compressive strength and paste to aggregate ratio of IPCs (2010).

Nath et al. (2012) reported that the inclusion of slag up to 30 % of total binder in the fly ash based geopolymer mixture decreased the setting time and increased the compressive strength up to 55MPa at 28days of normal temperature cured geopolymer mortar. With the increase of alkaline activator solution in the mix from 35% to 45% of total binder, the setting time increased and compressive strength decreased (Fig 2.46). The improvement of strength of fly ash and slag blended geopolymer concrete was due to the increase of calcium bearing compound in the dissolute binder which produced reaction product from both alkali activated fly ash and slag. The higher Si/Al ratio of slag incorporated mixes also contributed to harden fast and develop strength [71].

❖ Bio-concrete

Bakreshwar is in the Birbhum district of West Bengal, India, located at 23.88 °N 87.37 °E. It has an average elevation of 84 metres (276 feet). It is a place of geological interest with many hot springs. These hot springs due to their unique chemical richness harbor different types of thermophilic and hyper-thermophilic microbial community [72]. Moreover, these heat-stable enzymes of thermophiles proved to be very important to the field of biotechnology. Bacterial mineralization or biomineralization is a common and widespread phenomenon occurring in various geothermal systems. It is the process by which living forms influence the precipitation of materials [73]. Bacteria are very small (1.5 μm3) but have the largest surface to volume ratio of any life form. Biomineralization is classified either as extracellular or as intracellular. Extracellular biomineralization involves inorganic, often crystalline materials forming on the outer wall of the cell, within the cell wall or in the immediate surrounding tissue areas. In nature, gigatons of silica– SiO_2, an oxide form of silicon – are produced yearly from silicon, the second most abundant element on earth, by a diverse group of eukaryotes [74]. The study of the hydrolysis and condensation reactions during biosilicification is complicated owing to the sensitivity of silica, silicates and silicic acid to pH concentration and temperature [75]. As silicic acid precursors, tetra-alkoxy-silanes are easily hydrolyzed and the resulting silanols are condensed during the formation of particulate silica. The demand for new materials and products is still growing and the interest in naturally formed biopolymers and bio-minerals, such as chitin, calcium precipitates and silica is increasing. Different microorganisms and photosynthesizing micro algae of the family Bacillariophyceae (diatoms), produce calcite, silica exoskeletons etc. with a potential

to be used in specific industrial or technological processes [76]. Promising results of an innovative biotechnology based on microbial carbonate precipitation have laid to researchconcerning the use of bacteria in or on concrete, called Bioconcrete, an inherent self-repairing biomaterial that can remediate the cracks and fissures in concrete . A novel approach to rejuvenate or remediate fractures and fissures in concrete structures employing *Bacillus* species mediated biomineralization of calcium carbonate has been explored.Their study exhibited that production of "microbial concrete" by Bacillus sp. on constructed facilities increased the durability of building materials [77].

A variety of additives or replacements of cement can be applied in order to improve the durability of the final concrete products but not all these are easy and good and also sometimes cause environmental pollution. Other commonly applied additives that improve or change certain concrete characteristics needed for specific applications where high strength is required, affect the durability and result in the unwanted early deterioration of concrete structures. One major cause that initiates various mechanisms of concrete deterioration is the process of cracking what dramatically increases the permeability of concrete. As cracking links both isolated and connected pore systems, this result in a substantially increased permeability which plays a major role in deterioration of concrete and the life of the concrete structures become shorter. Also this causes costly repair though sometimes it fails to protect the structures. Therefore cracking of concrete should be minimized and that a potential healing mechanism should ideally result in the sealing or plugging of newly formed cracks in order to minimize increases in matrix permeability [77].

In natural environments, mineral precipitation processes constantly occur at a slow rate over geologic time, plugging or selectively cementing permeable porous media. Microbial metabolic activities often contribute to selective cementation by producing environment for a long time even after cell death [78].

Though the study on the mortar and concrete incorporating favorable microorganism is very limited, it has been shown that under favorable conditions for instance Bacillus pasteruii, a common aerobic soil bacterium and *Pseudomonas aeruginosa*(aerobic microorganism) can continuously precipitate a new highly impermeable calcite layer over the surface of an already existing concrete layer [79]. This new additional calcite layer is highly insoluble and increases the impermeability of the specimen. Thus it resists the penetration of

harmful solutions into the concrete (alkali, sulfate etc) and thereby decreases the deleterious effects they may cause. As a result, it would eventually increase the compressive strength (5%-10%) and overall durability performance of the concrete .

Cracks and fissures are a common problem in building structures, pavements and historic monuments. Reinforcement corrosion and crack formation are the major causes of limiting the durability or lifetime of concrete structures. A variety of additives or replacements of cement can be applied in order to improve the durability of the concrete. Cracking causes early deterioration of concrete that can be minimized by microbial-induced sealing or plugging of the cracks. Methods that were used previously for crack remediation often use synthetic polymer that need to be applied repeatedly. But later a novel technique was introduced in fixing cracks with environmentally friendly biological processes that is continuous self-remediating process. Based on this bio-mineralogy concept, an attempt has been made to develop an eco-friendly, bacteria incorporated geopolymer material with more durability and mechanical strength [80].

2.1 Aim of the study:

Based on the review of literature on the geopolymer mortar/concrete, a comprehensive experimental programme has been taken up on fly ash based geopolymer mortar. The basic aim is to develop genetically modified bacterium amended fly ash based geopolymer mortar using low calcium fly ash (abundantly available in India) without heat curing after casting. The details of the study are as follows:

- ✓ To develop bacterium amended geopolymer mortar cure at ambient temperature .
- ✓ To determine the mechanical strength and durability performance in terms of RCPT and water absorption of genetically modified bacterium incorporatedgeopolymer mortar (cured at ambient temperature) and compare to the geopolymer mortar without bacteria and cement mortar (with and without bacteria *Bacillus subtilis*).
- ✓ To access the thermal resistance property of bacteria incorporated geopolymer mortar cured at ambient temperature.
- ✓ To study the self healing attributes of bacterium amended geopolymer along with crack repairing.

- ✓ To study mechanical strength and durability study bacterium incorporated geopolymer mortar after application of 50% load and cured at different curing conditions.
- ✓ To determine the microstructural analysis of modified geopolymer mortar and compare with conventional geopolymer mortar without bacteria.

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MATERIALS & METHODS

❖ Materials and Chemicals

Standard Ennore sand [1] and low calcium Class-F fly ash (specific gravity: 2.05; grain size: 150–300 µM (6.02%), 90–150 µM (33.32%), 45–90 µM (53.40%), < 45 µM (6.21%) obtained from National Thermal Power Corporation Ltd; Farakka Plant) were used for geopolymer [2]. 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 [3]. Transformed *Bacillus subtilis* cells (Laboratory stock culture), grow in a specific mineral media (pH 10.0), were used as bacterial agent [3].

❖ Mix proportion and curing for comparative analysis of OPC with bacteria amended Geopolymer

There were 4 categories samples (1—OPC mortar; 2—OPC mortar + bacteria; 3—Fly ash geopolymer and 4—fly ash geopolymer + bacteria) prepared for the study. Mixing proportions of 4 categories are shown in Table 1. The NaOH solution (12 molar) was mixed with commercially available Na_2SiO_3 solution in the proportion of 1:1.75 (v/v) to make alkali activator fluid. The effective bacterial cells concentration (10^5 cells/ml) was prepared by diluting the bacterial cells with water or activator fluid for preparing bacteria-incorporated cementitious mortar or fly ash geopolymer samples, respectively. A 2% LB medium (v/v) (pH 10.0) as was added during bacteria incorporated mortar specimens' preparation. Ordinary Portland cement of 43 grade mixed with sand properly and was used for making mortar samples of categories 1 and 2. After 24 h of casting, all the specimens were removed from moulds and cured under water for several days until testing. For the preparation of category 3 and 4 samples, the fly ash- and bacteria cells-containing activator fluid was mixed properly for 2 min and the mixture was heat-cured at 60 °C for 45 min before casting. After 24 h of casting, all the specimens were removed from moulds and cured at ambient temperature for several days until testing [2].

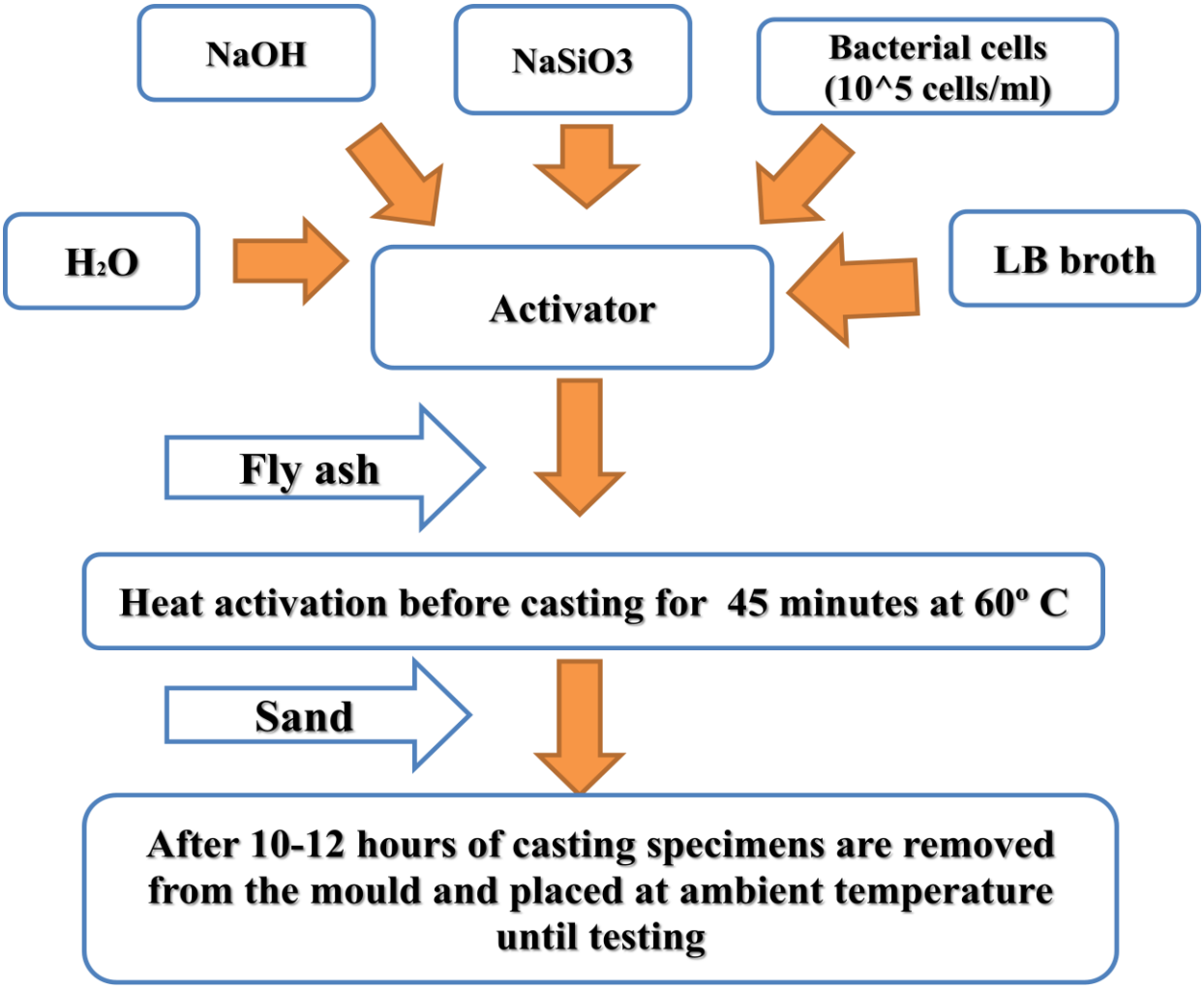

Figure 1: Process of development of bacterium amended geopolymer

Table 1: **Mixing ratio of different category samples**

Sample Category	Fly ash /Cement : Sand	Activator Fluid : Fly ash	Water : Cement	Bacterial cells/ml	Curing conditions
OPC	1:3	Nil	0.4	Nil	Water curing
OPC + bacteria	1:3	Nil	0.4	10^5	Water curing
Fly ash geopolymer	1:3	0.4	Nil	Nil	Ambient temperature curing .
Fly ash geopolymer + Bacteria	1:3	0.4	Nil	10^5	Ambient temperature curing

❖ Sample preparation for Self healing and Crack repairing ability under various curing conditions

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 [2]. 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 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. The prepared samples for all categories were initially cured for 28 days in 8 different curing conditions as shown in Table 2.

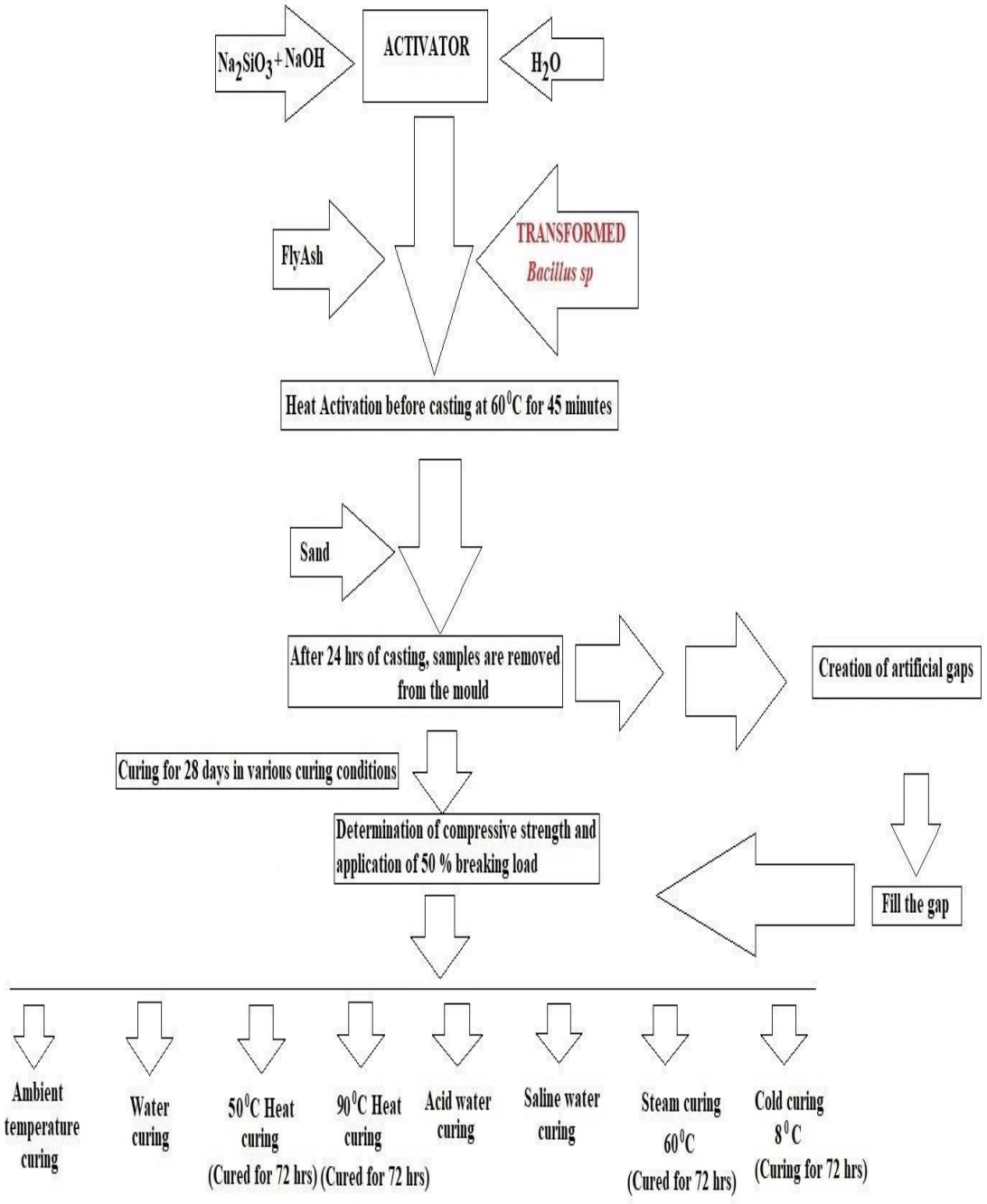

Figure 2- Schematic representation of the development of bacterium amended geopolymer mortar and its self-healing in various curing conditions.

Table 2: 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

❖ Compressive strength and Ultrasonic pulse velocity dtermination of bacterium amended geopolymer

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 [4]. 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 . 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.

❖ Study of Self- Healing of Micro- cracks

Samples used for self-healing event, were employed to 50% average corresponding breaking load to the respective category to make artificial microcracks . The samples were then kept for further curing for different days (3, 7, 14 and 28 days respectively) at their respective curing conditions. The images of the cracks were captured and the width of the cracks were determined by Crackscope. 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.

❖ Study of Crack-Repairing ability of geopolymer mortar

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.

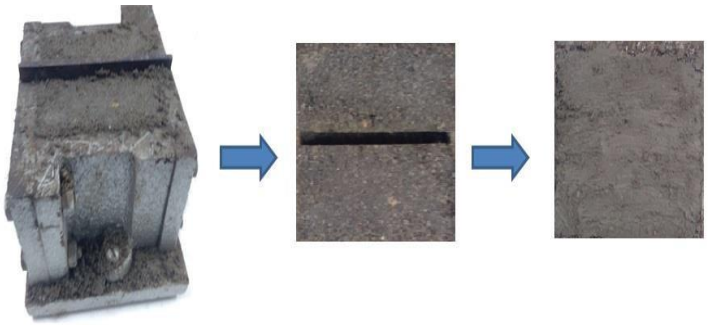

Figure 3: Crack-repairing image of geopolymer samples.

❖ Split Tensile and flexural strength test of mortar

Mortar cylinder specimens (5 in each) of size 100 mm diameter × 200 mm height were cast for 4 different category samples to determine the split tensile strength. After respective curing, the cylinder of each category was placed horizontally between the loading surfaces of compression testing machine and the load is applied perpendicularly to the axis of the cylinder. The maximum breaking load applied to the specimen was recorded to calculate the split tensile strength of the specimen as per IS: 5816 (1999) [5].

The flexural strength testing was carried out on 50 mm × 50 mm × 200 mm geopolymer mortar bar for all 4 categories samples. The beam specimens have been tested in flexural testing machine under a uniform rate of loading after 28 days of curing. The centre point loading method was adopted for determination of flexural strength (AASHTOT-67 2005) [6]

Figure 4 : Flexural strength test setup for reinforced concrete beam.

❖ Rapid Chloride Penetration Test (RCPT) of mortar

Chloride permeability test—For rapid chloride ion permeability test (RCPT), mortar cylinders (3 in each) of each category (100 mm diameter × 200 mm height) were prepared. After 28 days of respective curing, each cylinder was cut into three small cylinders (100 mm diameter × 50 mm height) and epoxy-coated along with theirs edges and left under water for 24 h before measuring the chloride ion permeability by Rapid Chloride Ion Penetration Cell. The test was done as per ASTM C1202 (2000) [7].

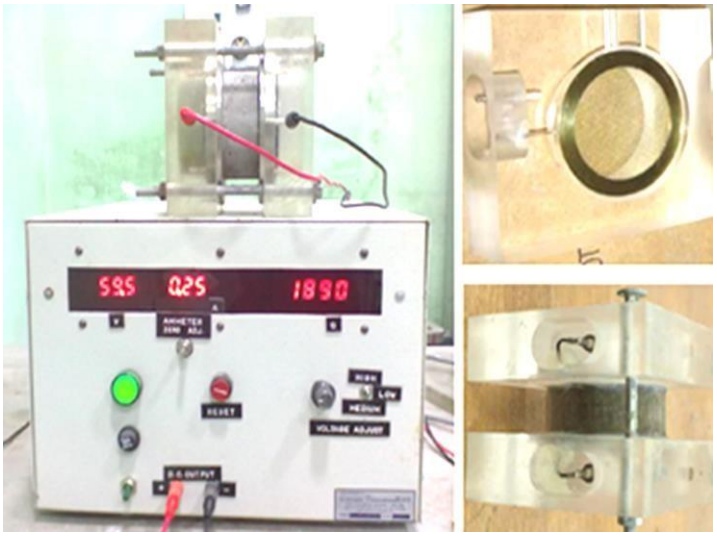

Figure 5 : RCPT arrangement.

❖ Water absorption test on mortar

After 28 days of respective curing, all the OPC and geopolymer mortar specimens (5 for each category) along with self-heal and crack-repair both (5 for each category) were air-dried for 24 h at room temperature and their initial masses were determined. The samples were then kept under deionized water for 30 min. The samples were then removed from water and cleaned with tissue paper, and their wet masses were registered again immediately. Then, samples were kept under water again for another 24 h, and after that, the samples were removed from water and cleaned with tissue paper, and their final wet masses were measured. Water absorption capacity was determined as per Neville's method (Neville 1996) [8].

❖ Sulfate resistance and Acid resistance test on Geopolymer mortar

After 28 days of respective curing, the initial masses of the mortars (5 numbers for each category i.e OPC, OPC + Bacteria, Fly ash geopolymer, Flyash geopolymer + Bacteria) , as well as self-healed and crack –repaired specimens cured in different curing conditions (5 for each category of curing condition) were determined. The samples were then immerged in sulphate solution (5% MgSO4, pH 7.0 in deionized water)-containing tank. The samples were kept under sulphate solution for 90 days.

After curing, the samples were removed from the tank and air-dried, and their masses were again determined. The test was carried out as per the guideline of ASTM STP663 (1997) [9].

For Acid resistance test, mortar samples (20 samples for each category) were prepared. After respective curing for 28 days, 5 samples from each category were taken and their average compressive strengths were determined. The rest of the 15 samples of each category were then immerged in 10% sulphuric acid solution for different days of curing (4, 8 and 12 weeks, respectively). After each curing period, 5 samples of each category were taken and their average compressive strengths were determined..

❖ Thermal resistance Test on Geopolymer mortar

After respective curing for 28 days, the samples from 4 different categories were kept in the oven for heating at different temperatures (100–400 °C, respectively) for 4 h each. After cooling, the compressive strength of the heat-treated mortar samples was measured to observe the thermal tolerance of respective samples.

Figure 6 : Thermal resistance test of geopolymer mortar samples

❖ Microstructural analysis of mortar

❖ X-Ray Diffraction analysis

XRD analysis of the powder mortar sample was done by X-ray diffractometer (Bruker AXS Inc, Model D8, WI, USA). The experiment was conducted with a scan speed 0.5 s/step at 40 kV. The XRD spectrum was analysed in the range 2Θ = 10–80 degree, and the peak positions were marked and analysed by using JCPDS data file.

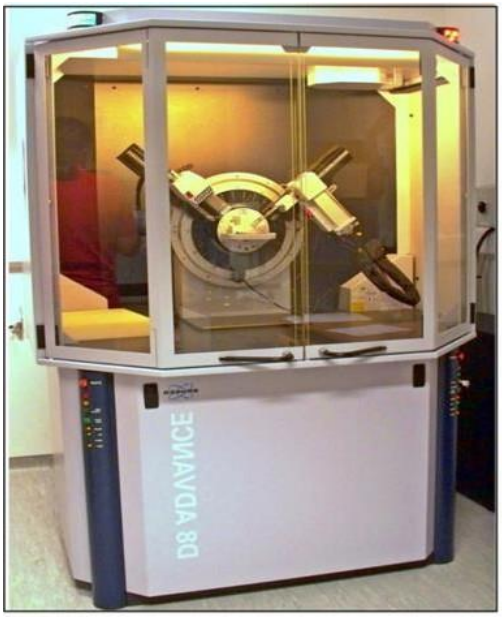

Figure 7 : XRD test arrangement.

❖ Field Emission Scanning Electron Microscopy and Energy-dispersive X-ray Spectroscopy (EDS) analysis

The scanning electron microscopy is a versatile, non-destructive technique that reveals detailed information about the morphology and the composition of natural and manufactured materials. In a scanning electron microscope, the specimen is exposed to a narrow electron beam from an electron gun, which rapidly moves over or scans the surface of the specimen. The electrons interact with the atoms that make up the sample producing signals that contain information about the sample's surface topography,

composition and electrical conductivity. The types of signals produced by theFESEM include secondary electrons, back-scattered electrons (BSE), characteristic X-rays, light (cathodoluminescence), specimen current and transmitted electrons. Secondary electron detectors are standard equipment in all FESEMs, but it is rare that a single machine would have detectors for all possible signals. The signals result from interactions of the electron beam with atoms at or near the surface of the sample. In the most common or standard detection mode, secondary electron imaging or SEI, can produce very high-resolution images of a sample surface, revealing details less than 1 nm in size. Due to the very narrow electron beam, SEM micrographs have a large depth of field yielding a characteristic three-dimensional appearance useful for understanding the surface structure of a sample.

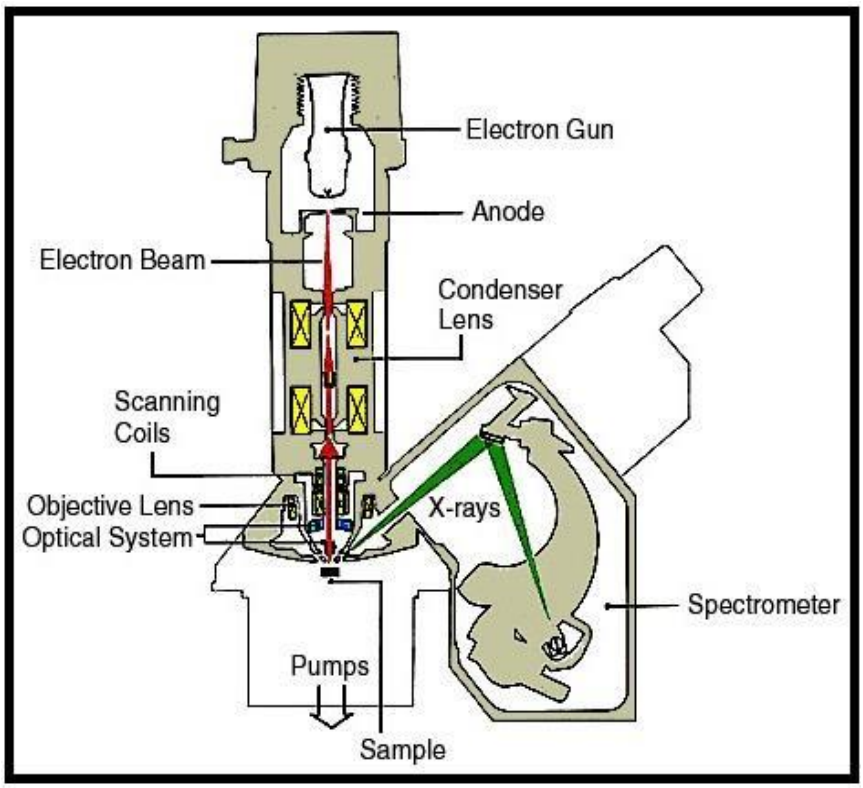

Figure 8 : Graphical presentation of FESEM setup.

After measuring the compressive strength, the fragmented mortar sample of each category was ground into fine powder and sieved to make the size less than 5 micrometres for analysis. For FESEM and EDX analyses, the fine powder was

dispersed with ethanol (99.9%) to make a film on carbon tape and then kept under vacuum desiccators for evaporation. Finally, the dried samples were gold-coated for field emission scanning electron microscope FESEM (HITACHI S-4800, JAPAN) and EDX (EDX-equipped Philips XL30) analysis.

❖ Mercury Intrusion Porosimetry (MIP) Test

Mercury intrusion porosimetry (MIP) analysis was done to observe the modification of pore size distribution on 4 different categories samples. Liquid nitrogen-frozen fragmented samples (collected after measuring the compressive strength) were cryovacuum-dried for several days prior to analysis.

❖ Bacterial survivability inside the geopolymer samples

Transformed *Bacillus subtilis* bacterium-incorporated mortar samples of different ages (3, 7, 14, 28, 60, 120, 240, 360 days) were made to powder form by pestle-mortar and a pinch of each powdered bacteria-incorporated mortar sample (approximately 10 mg) was added in 5 ml of sterile liquid LB medium and kept some time for precipitation of the heavier dust particles. One millilitre supernatant of each was inoculated to the 5 ml sterile LB medium (pH 10.0) and kept for 24 h at 37 °C in shaker-incubator followed by observation for growth. The growth of the bacterium was detected by measuring the optical density of the growth medium at 620 nm with respect to control growth medium.

❖ Biosilicification assay

It was done to confirm the presence of bioremediase-like protein leached by the active bacterial cells as described by Biswas et al. 2010 [10] .To study the activity of the protein / enzyme, biosilicification assay was designed which confirmed the silica leaching features of the protein. In the reaction mixture, 100 µL of protein(s) solution, 20 µL of Tris-HCl buffer (20 mM, pH 8) and 50 µL of silica rich substrate tetra-ethyl-orthosilicate (TEOS) were added and final volume was made up to 1 mL with deionized sterile water. The reaction was performed at 65 ºC for 180 min and then terminated by centrifugation at 11000 x g for 20 min. The precipitate thus obtained was washed twice with absolute ethanol and air-dried. The precipitate was then dissolved in 1 mL 1(M) NaOH and the released silicic acid was determined by molybdate assay). At first 200 µL of 5 (N) HNO_3 and 500 µL of ammonium molybdate (Laboratory standard) were added to 1 mL of 1(M) NaOH containing the precipitate and then 100 µL of 10% metol solution (metol: oxalic acid 1: 1 v/v) was added to the reaction mixture. Optical densities of all reaction samples were measured at 405 nm against control sample (without protein).

❖ Toxicity study of the bacterial cells

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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 ^{o}C. 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 101,102,103, 104, 105 cells/ml. The survivability assay was carried out by using MTT assay.

❖ Statistical analysis

Since the cementitious mortars or geopolymer mortars are heterogeneous substances, the experimental data may vary from sample to sample. For our experimental purposes, all category samples (5 samples in each) as described in earlier were prepared. Each experimental data were presented as average ± SD. Each set of experiment was repeated at least three times.

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RESULTS
&
DISCUSSIONS

❖ General view

At first, the experimental results on the bacterium amended geopolymer mortar have been presented respectively and critically discussed. Further, the results of self-healing bacterium amended geopolymer cured under various curing conditions and crack-repairing ability of it is been discussed.

❖ Bacterium amended fly ash based Geopolymer mortar

The aim of the study is to eliminate the much needed heat activation for the preparation of bacterium amended flyash based geopolymer mortar with appropriate mechanical strength and durability. Figure 1 summarises the compressive strength of bacterium amended geopolymer mortar cured at ambient temperature and its comparative analysis with geopolymer mortar without bacteria, cement mortar with and without bacteria at 3, 7, 14 and 28days of curing.

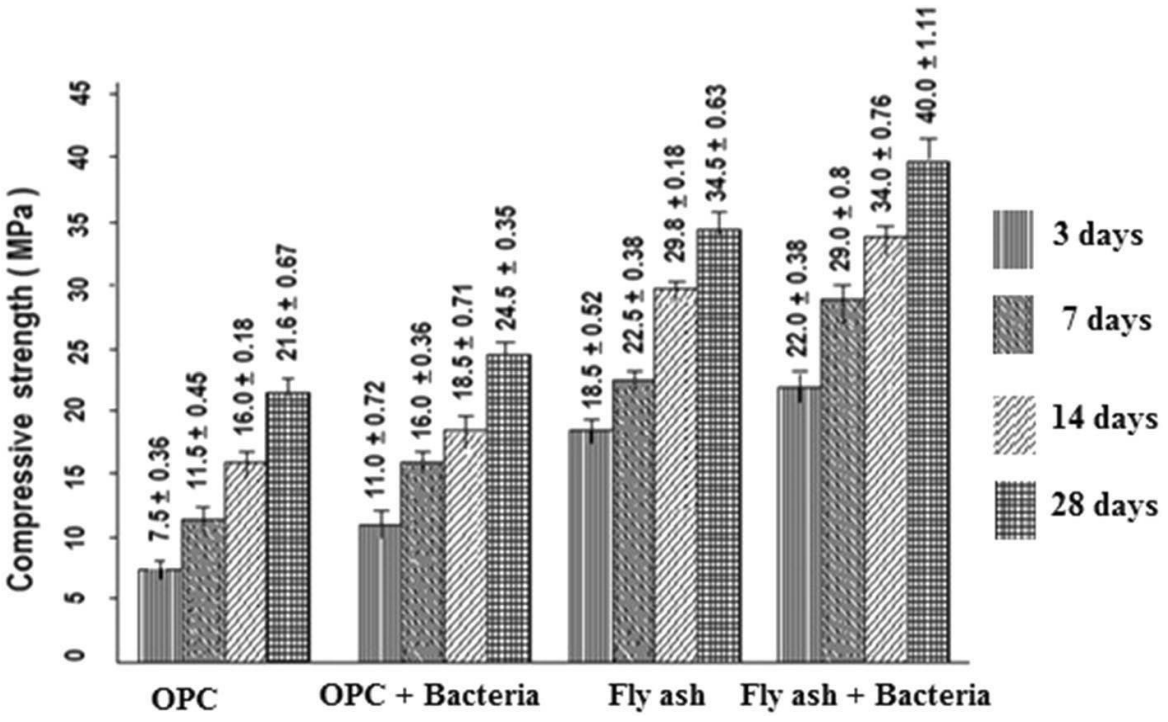

Figure 1: Compressive strength of different category mortar samples

The 100% fly ash geopolymer mortars possessed higher compressive strength than that of cement-based mortars (Fig. 1). The incorporation of bacteria increased the compressive

strength of both cementitious (OPC + bacteria) and geopolymer (Fly ash geopolymer +bacteria) samples at all ages of curing as seen from Fig. 1.

Table 1- Effect of concentration of *T. Bacillus* cells on compressive strength of fly ash geopolymer mortar

Days of curing	Fly ash Control	Fly ash incorporated with *T. Bacillus* in the concentration				
		10^2 cells /ml	10^3 cells/ml	10^4 cells/ml	10^5 cells/ml	10^6 cells/ml
3	18.5 ± 0.52	19.0 ± 0.40 (2.70)	19.5 ± 0.50 (5.40)	20.0 ± 1.00 (8.10)	22.0 ± 0.38 (18.91)	20.0 ± 0.50 (8.10)
7	22.5 ± 0.38	23.0 ± 0.40 (2.22)	24.0 ± 0.60 (6.66)	25.0 ± 0.80 (11.11)	29.0 ± 0.80 (28.88)	26.0 ± 0.40 (15.55)
28	34.5 ± 0.63	35.0 ± 0.45 (1.44)	36.2 ± 0.30 (4.90)	37.4 ± 0.60 (8.40)	40.0 ± 1.11 (15.94)	37.5 ± 0.80 (8.69)

Data are presented mean ± SD. The value within parenthesis indicates the % increment with respect to its control

The compressive strength increment is maximized at a particular bacterial cells' concentration (10^5 cell/ml alkaliactivated fluid used) when incorporated to the fly ash-based geopolymer Table 1. It is already established that genetically improved *Bacillus* bacterial strain (transformed *Bacillus subtilis*) has the ability to increase the compressive strength and durability of the cementitious mortar samples when incorporated within the samples [1]. The bacterium *(Bacillus subtilis*) possesses urease gene which is responsible for the formation of calcite ($CaCO_3$) in the matrix of cementitious mortars [2]. On the other hand, the bioremediase-like gene is responsible for the leaching of nano-silica from various silicate phases present within the concrete environment due to its biosilicification activity [1,3,4]. The leached nano-silica forms different phases (e.g. gehlenite or calcium aluminium silicate) by reacting with the different oxides inside the mortar matrix [1]. The transformed *Bacillus subtilis* bacterium thus becomes useful for the development of high-performance geopolymer because calcite and gehlenite synergistically fill the micropores of the biopolymer mortars thereby increasing the compressive strength.

Table 2-Effect of bacterium on Flexural and Tensile strength

Sample	Flexural strength (MPa)	Tensile strength (MPa)
OPC	**4.3 ± 0.89**	**2.0 ± 0.32**
OPC + Bacteria	**5.4 ± 0.69 (25.6↑)**	**3.0 ± 0.41 (50↑)**
Fly ash geopolymer	**2.0 ± 0.97**	**1.59 ± 0.20**
Fly ash geopolymer + Bacteria	**5.3 ± 0.68 (165↑)**	**3.86 ± 0.20 (142↑)**

Data are presented mean ± S.D. (n = 9). The value within parenthesis indicates the % of increment (↑) with respect to its control

It was noted that the transformed *Bacillus subtilis* also increased the flexural strengths (25.6% for OPC + bacteria and 165% for fly ash geopolymer + bacteria) and tensile strengths (50% for OPC + bacteria and 142% for Fly ash geopolymer + bacteria) both with respect to their control samples as shown in Table 2.

The increments of flexural strength (25.6% for OPC + bacteria and 165% for fly ash geopolymer + bacteria) and tensile strengths (50% for OPC + bacteria and 142% for fly ash geopolymer + bacteria) of the transformed bacterium incorporated specimens also can be explained similarly that various crystalline phases like calcite and gehlenite formed by the bacterium synergistically fill the micropores of the biopolymer mortars.

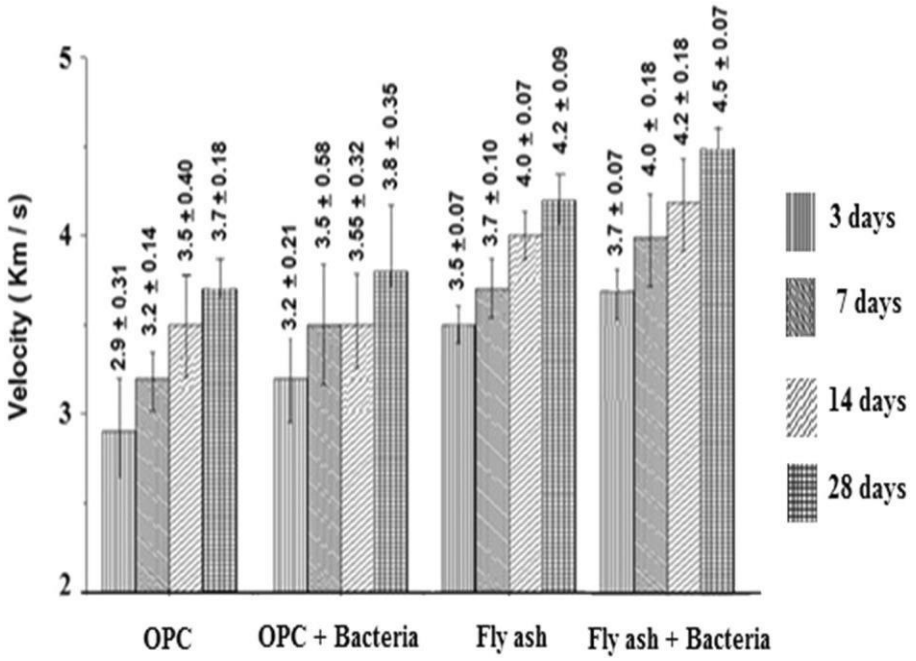

Figure 2 : Ultrasonic pulse velocity of different category mortar samples

A significant increase in ultrasonic pulse velocities was observed in OPC + bacteria and fly ash geopolymer + bacteria mortar at all ages of curing in comparison with their respective controls (Fig. 2) which clearly signifies the less number of void space available within the cementitious as well as bacterium amended geopolymer matrices due to the leaching of silica and formation of various thermostable phases which can be also observed in the following experiments.

The water absorption results showed that the percentage increments of mass of the OPC + bacteria samples were less compared to OPC control samples (Table 3). Minimum increment of mass (2.1%) was noted in the case of bacterium mended fly ash geopolymer samples.

Table 3: Water absorption test of bacteria treated samples

Sample	Mass (g)				
	Initial	After 30 min	% Increase	After 24 h	% Increase
OPC	730 ± 3.5	750 ± 9.4	2.7	760 ± 7.0	4.1
OPC + bacteria	725 ± 11.1	740 ± 7.90	2.0	749 ± 10.5	3.3
Fly ash geopolymer	730 ± 7.9	745 ± 5.00	2.0	753 ± 8.3	3.1
Fly ash geopolymer + bacteria	715 ± 7.9	720 ± 3.53	0.7	730 ± 7.0	2.1

Data are presented mean ± SD. (n = 15). The increased percentage was calculated with respect to the corresponding control.

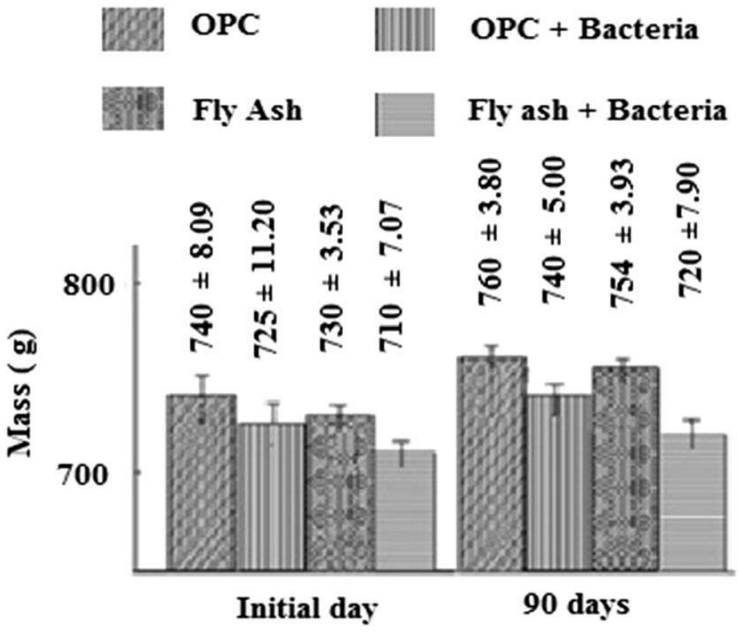

Figure. 3: Sulphate resistance activity of different category mortar samples

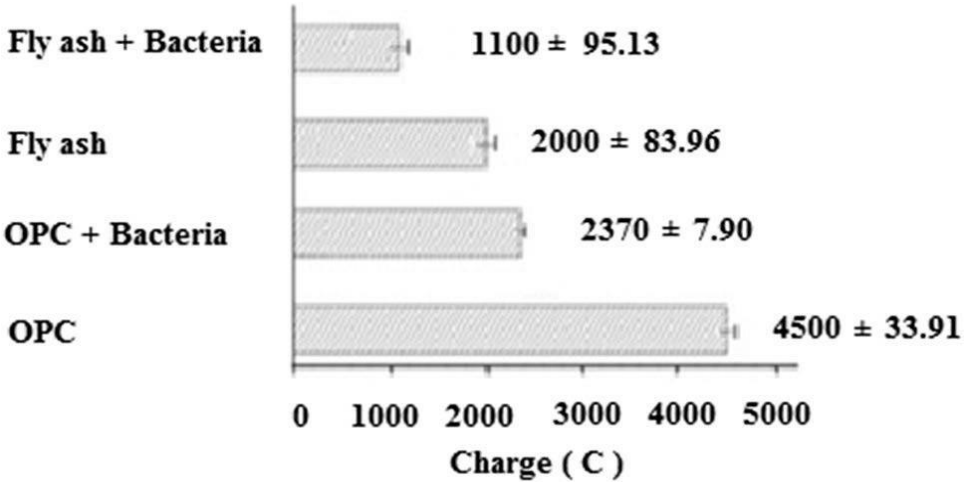

Figure 4 :Rapid chloride permeability of different category mortar samples

The water absorption results showed that the percentage increments of mass of the OPC + bacteria samples were less compared to OPC samples (Table 3). Minimum increment of mass (2.1%) was noted in the case of fly ash geopolymer + bacteria samples. It was observed that the bacterial cells-amended fly ash geopolymer mortar samples had minimum increment of weight (1.40%) compared to the fly ash geopolymer mortar samples (3.29%) during sulphate absorption test (Fig. 3). The results of chloride permeability test also showed that bacterial cells increased the chloride resistance of the samples (Fig. 4). The chloride ions permeability was decreased 47.3% in OPC + bacteria samples compared to OPC samples and 45% in fly ash geopolymer + bacteria samples compared to fly ash geopolymer samples as seen from Fig. 4. The overall increased strength and ultrasonic pulse velocity of the bacterium-incorporated cementitious mortar or geopolymer samples are the clear indication of less porosity present within matrix of the samples. This makes the specimens to allow less water, sulphate or chloride ions whatever may be to ingress within the samples. This effect is more prominent in the case of bacterium-incorporated geopolymer samples. The results thus suggest that bacterium-incorporated 100% fly ash geopolymer mortars are comparatively more durable material than the cementitious mortar samples.

Table 4: Acid resistance of mortar samples in 10 % sulphuric acid

Samples	Compressive strength (MPa)			
	Initial	4 weeks	8 weeks	12 weeks
OPC	21.60 ± 0.67	12.96 ± 0.90 (40.0)	9.20 ± 1.20 (57.4)	6.30 ± 1.20 (70.8)
OPC + Bacteria	24.50 ± 0.35	16.21 ± 0.75 (33.8)	13.50 ± 1.00 (44.8)	10.90 ± 1.35 (55.5)
Fly ash geopolymer	34.50 ± 0.63	26.00 ± 1.20 (24.6)	20.70 ± 1.00 (40.0)	18.00 ± 0.90 (47.8)
Fly ash geopolymer + Bacteria	40.00 ± 1.11	36.10 ± 1.40 (9.7)	34.00 ± 1.90 (15.0)	31.00 ± 1.60 (22.5)

Data are presented mean ± SD (n = 15). The value within parenthesis indicates the % decrement with respect to its control.

Acid resistance activity of the mortar samples showed that transformed *Bacillus subtilis* also protected the bacteria-incorporated samples significantly (Table 4). It was noted that fly ash geopolymer + bacteria mortar samples exhibited comparatively much better acid resistant activity than the other geopolymer and cementitious mortar samples (Table 4).

The compressive strength of the OPC mortars was decreased by 44.4% with increasing the curing temperatures (100–400 °C) as shown in Table 5, whereas the compressive strength of the OPC + bacteria samples was decreased only by 23.1% compared to OPC mortar samples. The fly ash geopolymer + bacteria samples showed a significant increase in compressive strength (15.9%) at elevated curing temperature as seen from Table 5. It is noted that the cementitious mortar samples fail to maintain its structural integrity at very high temperature due to which the compressive strength of the samples is decreased by 44.4% with increasing the curing temperatures from 100 to 400 °C (Table 5). The pore water that remained within the concrete/mortar specimen expands at elevated temperature and causes volume expansion

of the samples resulting in the generation of cracks. The cracks lead to reduction of compressive strength. Also the compressive strength deterioration is attributed to the $Ca(OH)_2$ decomposition that occurs at about 400 °C [5]. Whereas, incorporation of bacterial cells to the cementitious mortars, the thermal tolerance of the samples is increased to some extent as revealed by the compressive strength (23.1% reduction only) of the OPC + bacteria samples. Chattopadhyay et al. (2010) have demonstrated that bioremediase protein helps the formation of more calcium silicate-hydrate (CSH) gel within the matrix by enhancing the hydration of the unused cement particles in the bioremediase protein-amended cement-paste samples [6]. Due to this, less pore water is available within the matrix and thus causes less volume expansion at elevated temperature. This may be one of the causes for high-temperature tolerance of the OPC + bacterial mortar samples.

Table 5: Effect of temperature on Compressive strength

Temp. (°C)	OPC	OPC + Bacteria	Fly ash	Fly ash + Bacteria
100	21.6 ± 0.6	26.0 ± 0.9 (↑20.00)	34.5 ± 0.4	41.0 ± 1.6 (↑18.80)
150	22.0 ± 0.4	26.5 ± 0.6 (↑20.00)	36.0 ± 0.3	42.0 ± 0.9 (↑16.60)
200	20.0 ± 0.8	25.0 ± 0.5 (↓25.00)	37.5 ± 0.5	42.5 ± 1.0 (↑13.30)
250	20.0 ± 0.6	24.0 ± 0.9 (↓20.00)	38.0 ± 0.8	44.0 ± 0.9 (↑15.70)
300	16.0 ± 0.9	23.0 ± 0.4 (↓43.75)	36.0 ± 0.6	45.0 ± 1.2 (↑25.00)
350	14.0 ± 0.8	21.0 ± 0.3 (↓50.00)	35.0 ± 0.8	46.0 ± 0.5 (↑31.40)
400	12.0 ± 0.6	20.0 ± 0.6 (↓66.60)	33.0 ± 0.6	47.5 ± 0.8 (↑43.90)

Data are presented mean ± SD (n = 5). The value within parenthesis with ↑ symbol indicates the % of increment and with ↓ symbol indicates the % of decrement with respect to its control.

Higher compressive strength and greater ultrasonic pulse velocity of the bacteria-incorporated fly ash geopolymer samples indicate more compactness and lesser micropores of the samples in comparison with its control or cementitious mortar samples. It is known that water released during the formation of geopolymers was expelled from the geopolymer matrix during heat curing, which causes discontinuous nano-pores within the matrix resulting in improvement of strength of geopolymers [7]. In this study, the strength of geopolymer mortar specimens is found to increase with the increase in the temperature, attaining peak strength at 250 °C. Subsequently, this strength is observed to reduce gradually to some extent for the remainder of the heating regime. This result supports the finding of early studies [7]. On the other hand, the compressive strength of the bacterial cells-incorporated fly ash geopolymer specimen after curing at ambient temperature for 28 days shows significant increment (15.9%) of compressive strength at elevated temperature (400 °C). This may be explained due to the increased formation of various high-temperature stable phases (e.g. mullite or Al_2O_3–SiO_2, hematite or Fe_2O_3, etc.) inside the bacteria incorporated geopolymer matrices during the curing for several days.

Microstructures analysis by environmental scanning electron microscope equipped with EDX showed that formation of various phases inside the pores of the fly ash geopolymer + bacteria mortar samples. A significant increased concentration of silicon was observed within the phases of micropores of fly ash geopolymer + bacterial samples (Fig. 5). Increased formation of silica (SiO_2) and calcium aluminium silicate ($Ca_2Al_2SiO_7$) was observed in OPC + bacteria mortar samples.

The XRD analysis similarly suggested that new phases (e.g. $3Al_2O_3$, $2SiO_2$, $Na_2Si_2O_3$) along with the increased concentration of sodium aluminium silicate ($NaAlS_{i3}O_8$), calcium silicate (Ca_3SiO_5), calcium carbonate ($CaCO_3$) and silica (SiO_2) were appeared in the bacterium-incorporated OPC and fly ash geopolymer samples (Fig. 6). From the results of MIP test, it was noticed that specific density within micropore region (pore diameter < 50 μm) of the bacteria-incorporated mortar samples was less in both OPC + bacteria and fly ash geopolymer + bacteria samples compared to their respective controls (Fig. 7).

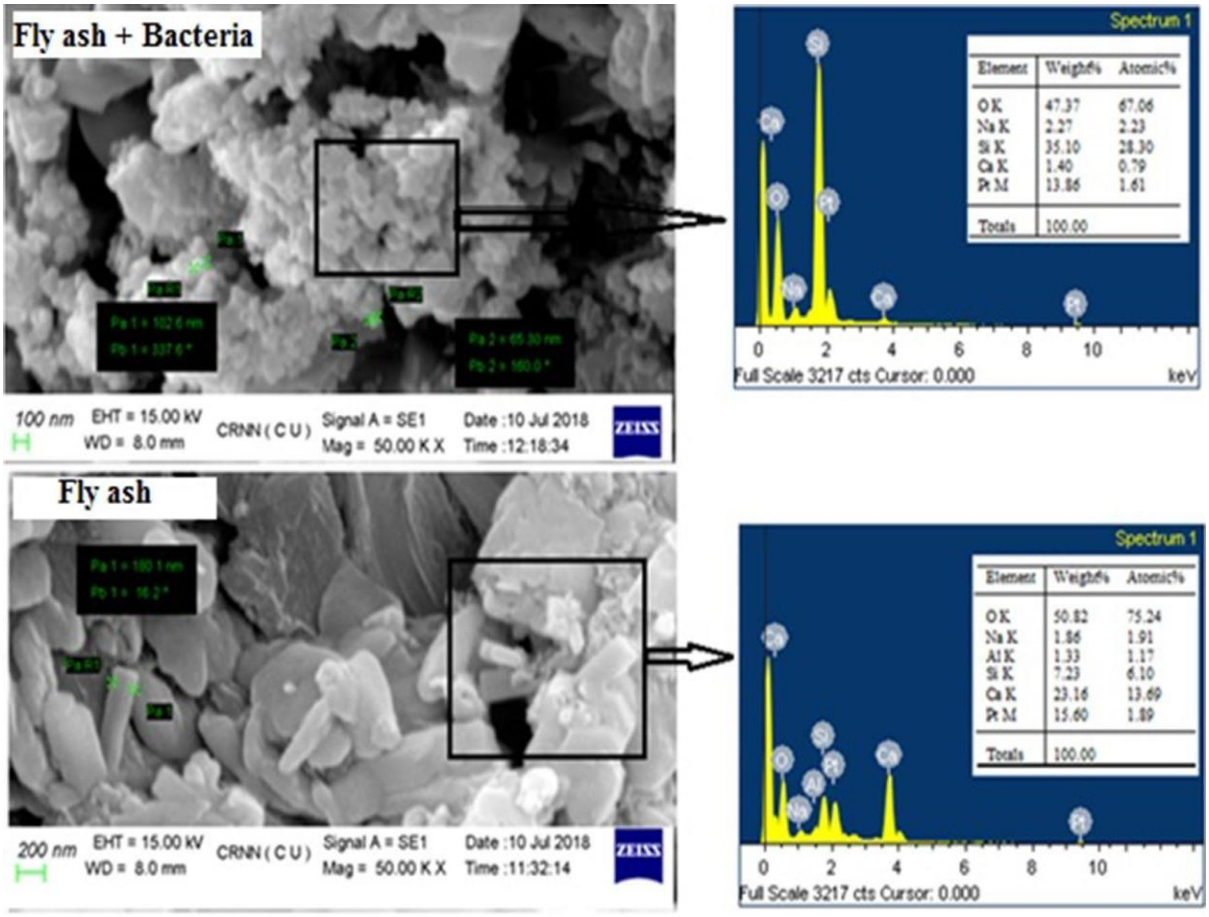

Figure. 5 : SEM image with EDX analysis of fly ash geopolymer and fly ash geopolymer + bacteria-incorporated mortar sample

Microstructure analysis of fly ash geopolymer mortar and bacteria cells-incorporated fly ash geopolymer mortar samples by environmental scanning electron microscope showed that there is a significant difference between the elementary compositions in the mortar matrices (Fig. 5). Abundance of silicon atoms (almost 5 times greater as compared to its control) is seen within the pores of the bacterium-amended fly ash geopolymer. The powder crystal XRD spectra exhibit some new peaks as well as some higher-intensity minor peaks in the bacterium-incorporated mortar samples compared to the their respective control samples (Fig. 6). An intricate investigation of all those peaks from JCPDS data file clearly confirms that those peaks are due to the newly formation of silica (SiO_2) and gehlenite by the action of bioremediase-like protein and calcite ($CaCO_3$) by the action of urease protein within the OPC + bacteria samples. Similarly, the new peaks that appeared in the fly ash geopolymer + bacteria are also due to enhanced formation of silica , mullite albite and alite , etc., by the

action of bioremediase-like protein and calcite ($CaCO_3$) by the action of urease protein within the fly ash geopolymer + bacteria samples. These newly formed phases are responsible for higher strength more thermostable as explained earlier. These newly developed phases also reduce the porosity of bacteria-amended samples as revealed by the MIP test results (Fig. 7). The MIP test results clearly indicate that the specific densities of the bacteria treated mortars are significantly lesser in both bacteria-amended OPC and fly ash specimens as compared to their respective controls. The MIP test results thus corroborate with the findings of UPV test and durability test.

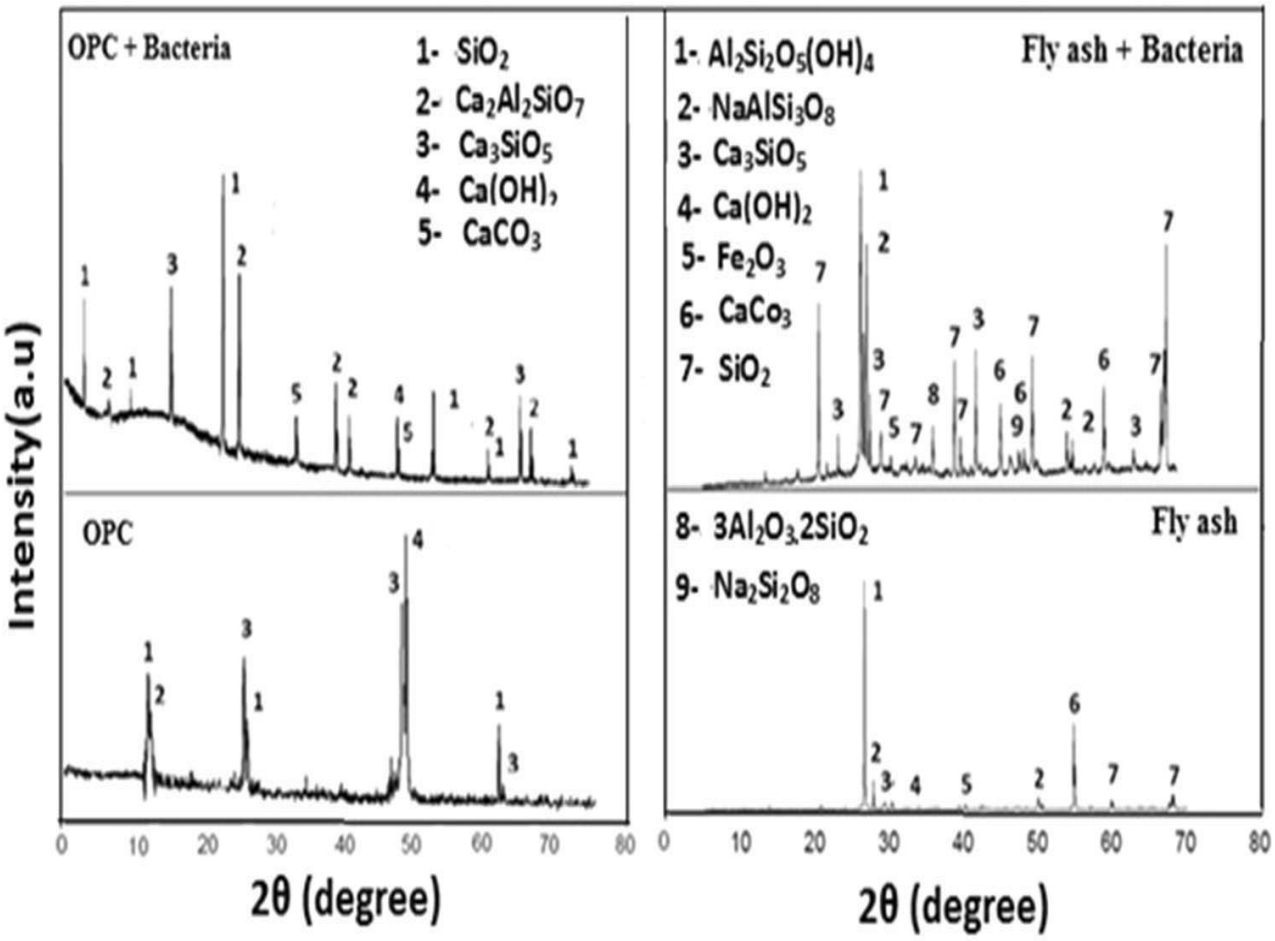

Figure 6: XRD analyses of different category mortar samples

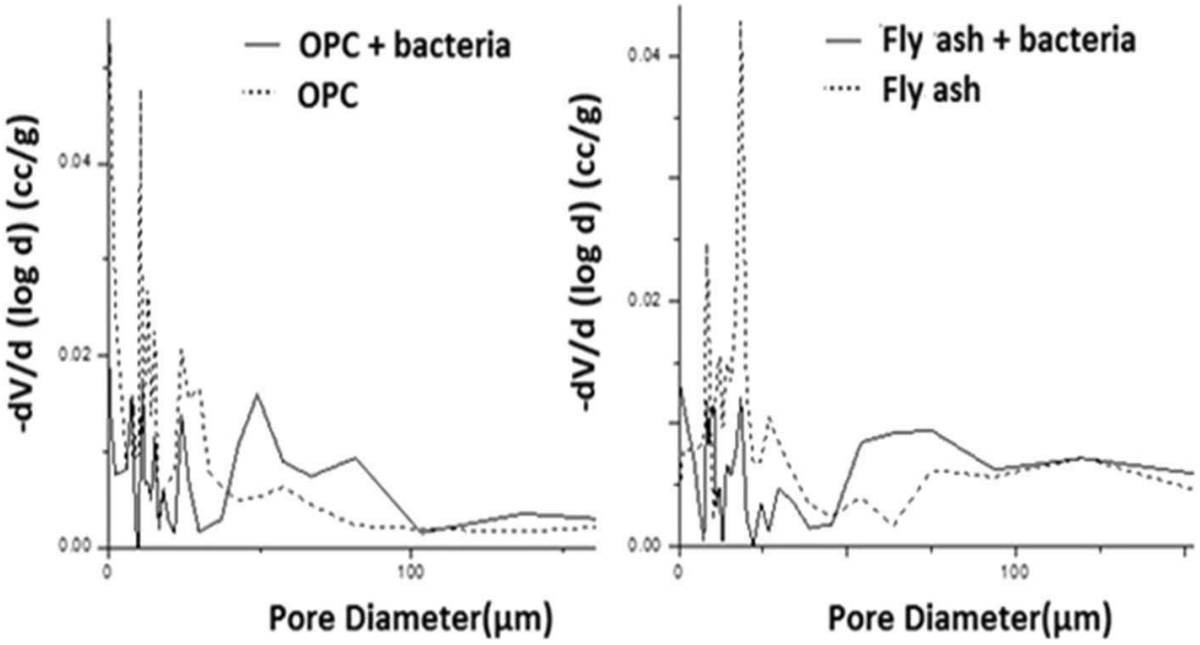

Figure 7: MIP analysis of different category mortar samples

The bacterial survivability and biosilicification results showed that the incorporated bacterial cells remained active for long time (1 year) within the cementitious and geopolymer mortar samples.

Table 6 :Bio-silicification assay from fly ash samples incorporated with *T. Bacillus* of different ages

Fly ash samples incorporated with *T. Bacillus* of different ages	Absorbance at 405 nm
7 days	0.27
28 days	0.30
60 days	0.31
120 days	0.28
365 days	0.25

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❖ Self Healing and Crack repairing study of Bacterium amended fly ash based Geopolymer mortar cured at various curing conditions

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 (Table 7).

The compressive strengths of the self-healed geo-polymeric mortars were considerably increased with respect to their corresponding controls at all curing conditions (Table 8).

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 ^{o}C) or steam curing also showed effective compressive strength increments (Table 8).

Curing condition has a significant role in the development of strength and durability of concretes [8, 9]. 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 [8,9]. 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 [10]. The bacteria impregnated geopolymeric 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. The 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. [11] have investigated the feasibility of producing sustainable cement-free composites and its environmental impact, which corroborates with the experimental results. High temperature air curing (90 ^{o}C heat curing for 72 h) also showed good performance on mechanical strengths and durability of the bacterium incorporated geo-polymeric materials (Table 8). 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 [12]. Similarly saline water also affects the workability, strength and durability of cementitious structures [13]. There are reports, which demonstrate that alkali-activated mortars possess better chemical stability, which provides resistance to acid attack [14] and salts such as chlorides [15] and sulfates [16]. 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 ^{o} C 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 . 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 [17]. 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 . The 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 9), split tensile strength (93%; Fig. 8) and flexural strength (140.9%; Fig. 8) respectively compared to controls after ambient temperature air curing (28 days). The highest increment of UPV was also noted in such case (Table 11 and 12). The bacterium was seen to fill the cracks of 1 mm width completely in ambient temperature water curing (60 days; Fig. 12). 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.

Table 7 :Compressive strength of bacteria incorporated geopolymer mortar samples

	Compressive Strength (MPa)
Samples	**28 days**
1C	32.1 ± 2.8
1S	39.0 ± 1.9 (21.4↑)
2C	29.5 ± 2.0
2S	33.92 ± 2.5 (15.0↑)
3C	33.3 ± 3.2
3S	39.8 ± 2.8 (19.5↑)
4C	29.2 ± 1.8
4S	33.20 ± 3.4 (13.7↑)
5C	27.6 ± 1.6
5S	30.8 ± 2.2 (12.9↑)
6C	26.2 ± 1.9
6S	29.0 ± 2.8 (10.9↑)
7C	32.5 ± 1.9
7S	38.02 ± 2.5 (16.98↑)
8C	16.9 ± 3.2
8S	18.7 ± 1.2 (10.8↑)

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

Table 8: Self-healing of bacteria incorporated geopolymer mortar samples

	Compressive Strength (MPa) at different curing periods			
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.

Table 9: Crack repairing of bacteria incorporated geopolymer mortar samples

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

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

Table 10: Ultrasonic pulse velocity of self-healed geopolymer mortars

	Ultrasonic pulse velocity (Km/s)			
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 controland shown within the parenthesis.

Table 11: Ultrasonic pulse velocity of crack-repaired geopolymer mortars

	Ultrasonic pulse velocity (Km/s)			
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 controland shown within the parenthesis.

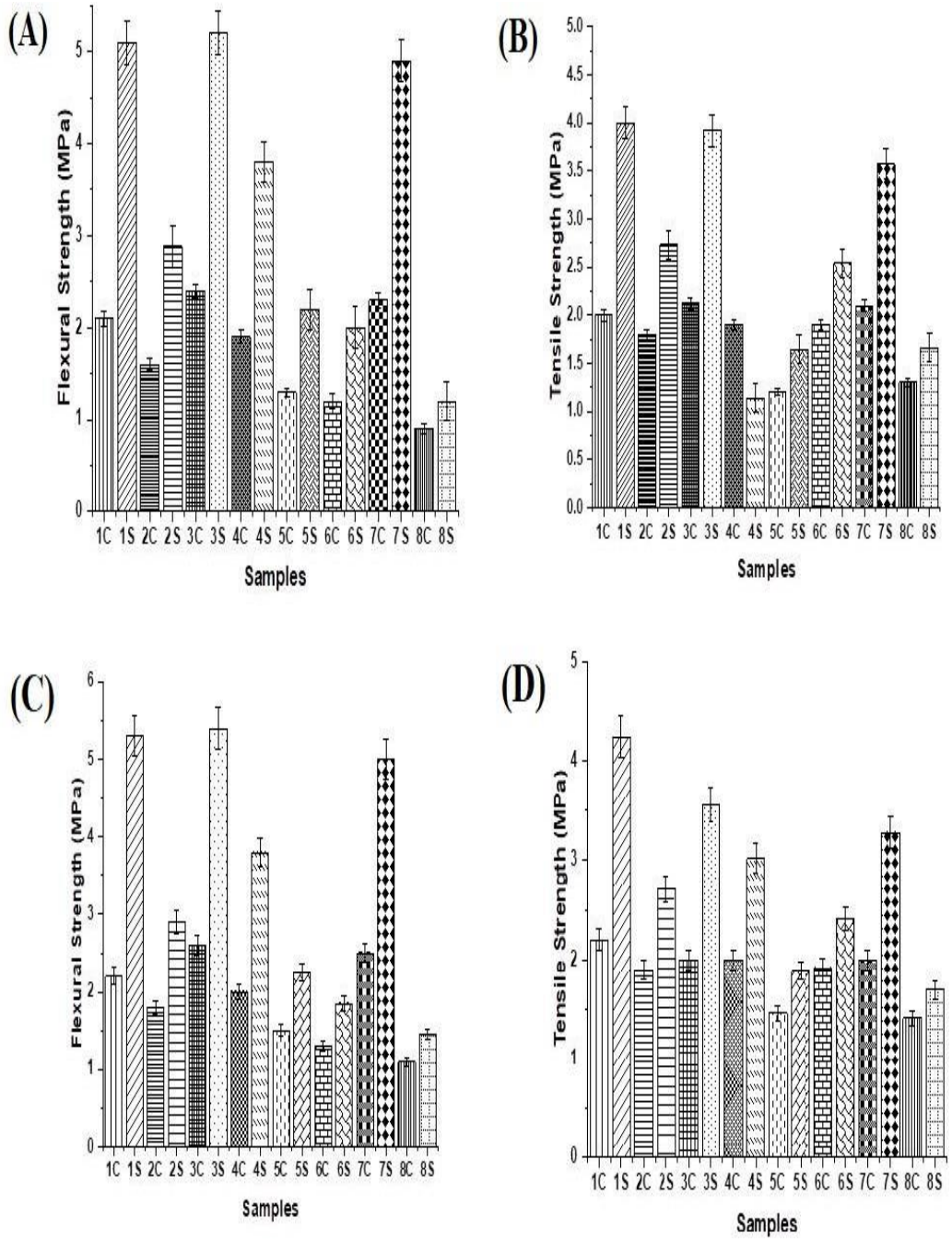

Figure 8: Mechanical strength analysis of bacterial incorporated self-healed geopolymer. (A) Flexural Strengths of self-healed geopolymer mortars cured at different curing conditions. (B) Tensile Strengths of self-healed geopolymer mortars cured at different curing conditions. (C) Flexural Strengths of crack-repaired geopolymer mortars cured at different curing conditions. (D) Tensile Strengths of crack-repaired geopolymer mortars cured at different curing conditions.

Table 12: Water Absorption of self-healed geopolymer mortars

	Water Absorption				
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 controland shown within the parenthesis.

. 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 9; Figs. 8C and 8D) at ambient temperature air curing. The UPV of the self-healed (Table 10) and the crack-repaired (Table 11) 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 12) and crack-repairing samples (Table 13).

Table 13: Water Absorption of crack-repaired geopolymer mortars

	Water Absorption				
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 controland shown within the parenthesis.

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The minimum water ingress was noted in self-healing samples (1.5%; Table 12) and crack-repairing samples (1.09%; Table 13) when cured at ambient temperature air curing condition.

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 14). 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. 9A and 9B), which was maximized at ambient temperature air curing.

Table 14: Sulphate Resistance Activity of self-healed and crack-repairedgeopolymer mortar samples

	SELF-HEALING			CRACK-REPAIRING		
Samples	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.

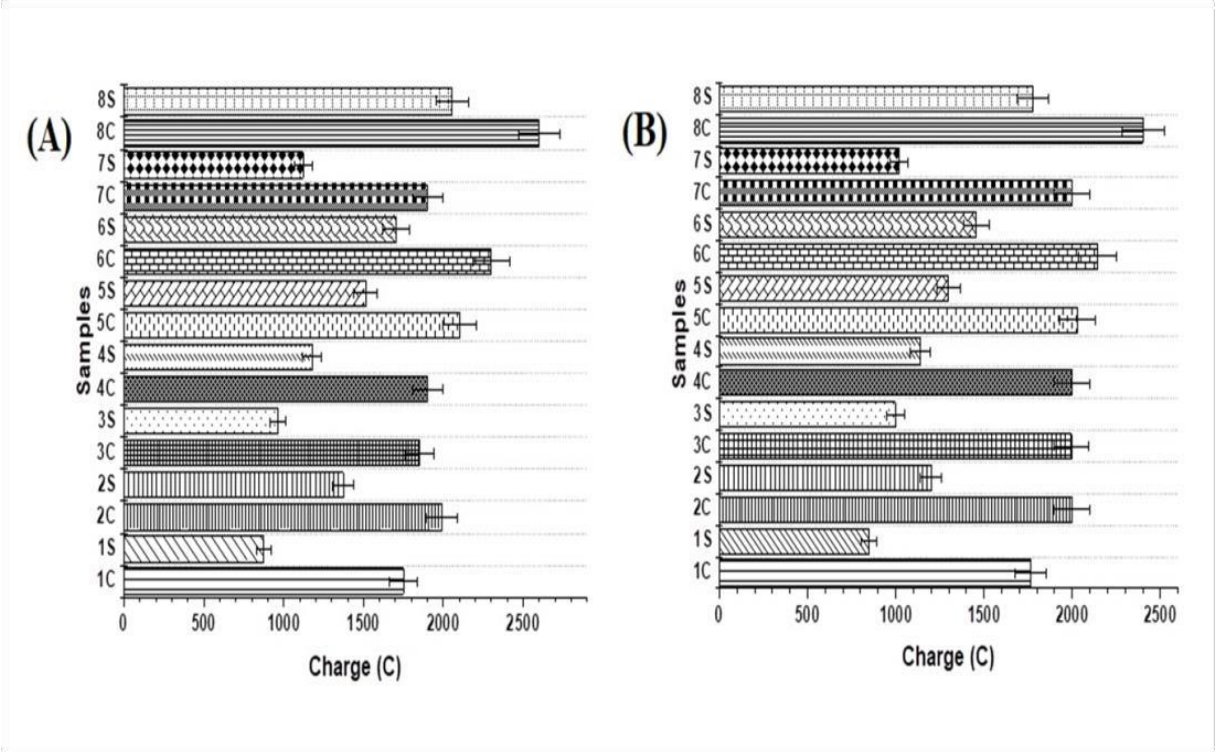

Figure 9: Chloride Permeability Test results (A) Self-healed geopolymer mortars cured in different curing conditions. (B) Crack-repaired geopolymer mortars cured in different curing conditions.

The results of water absorption test for self-healing study (Table 12) and crack-repairing study (Table 13), sulfate resistant study (Table 14) and rapid chloride ions permeability test (Figs. 9A and 9B) 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 likeprotein gene is responsible for gehlenite and different themostable novel phases (Mullite, Albite, Alite etc.) production inside the geo-polymeric material . 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 [18, 19]. The extended stability of the bacterium incorporated geopolymeric material is therefore basically due to the self-healing attribute of the incorporated bacterial cells.

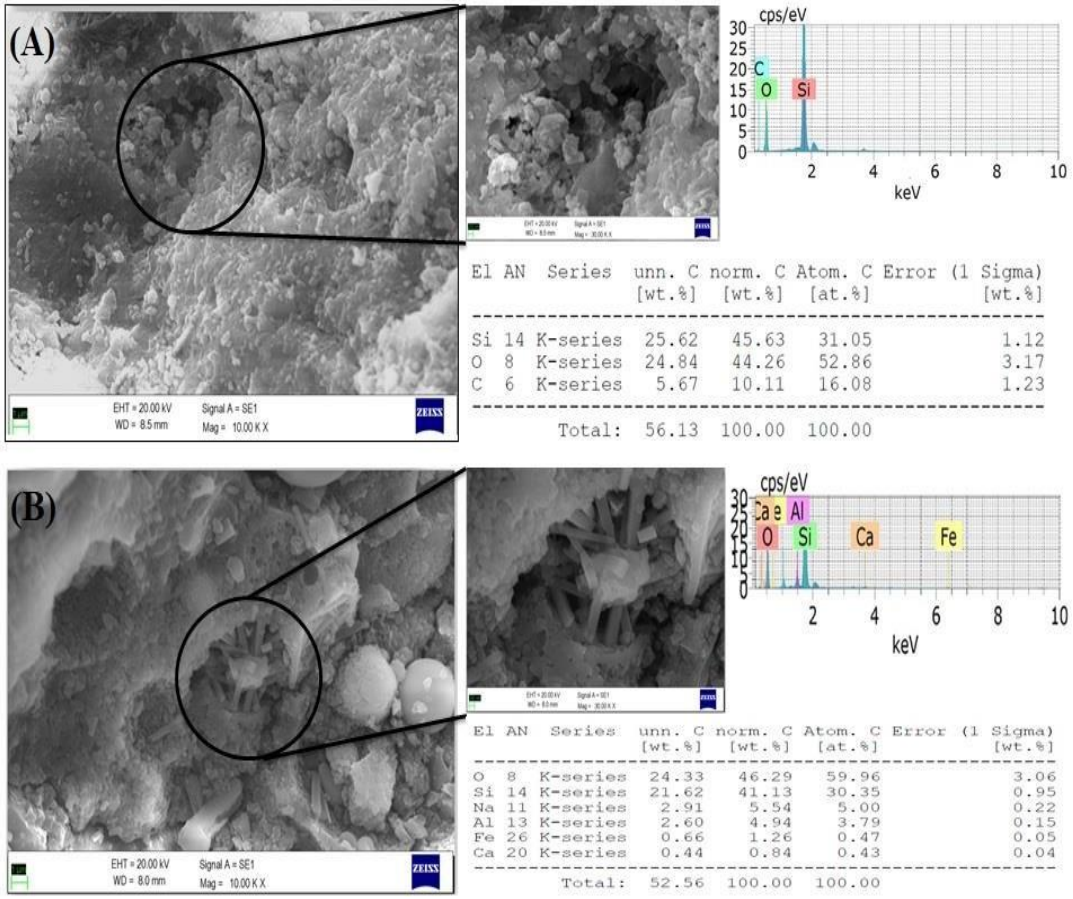

Figure 10: Microstructural analyses of geopolymer samples (A) FE-SEM image and EDAX analysis of geopolymer mortar cured in ambient temperature. (B) FE-SEM image and EDAX analysis of self-healed bacterial amended geopolymer mortar cured in ambient temperature.

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. 10 B) were appeared inside the matrix of the microbial-amended samples, which was absent in the control samples (Fig. 10A). 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. 11.

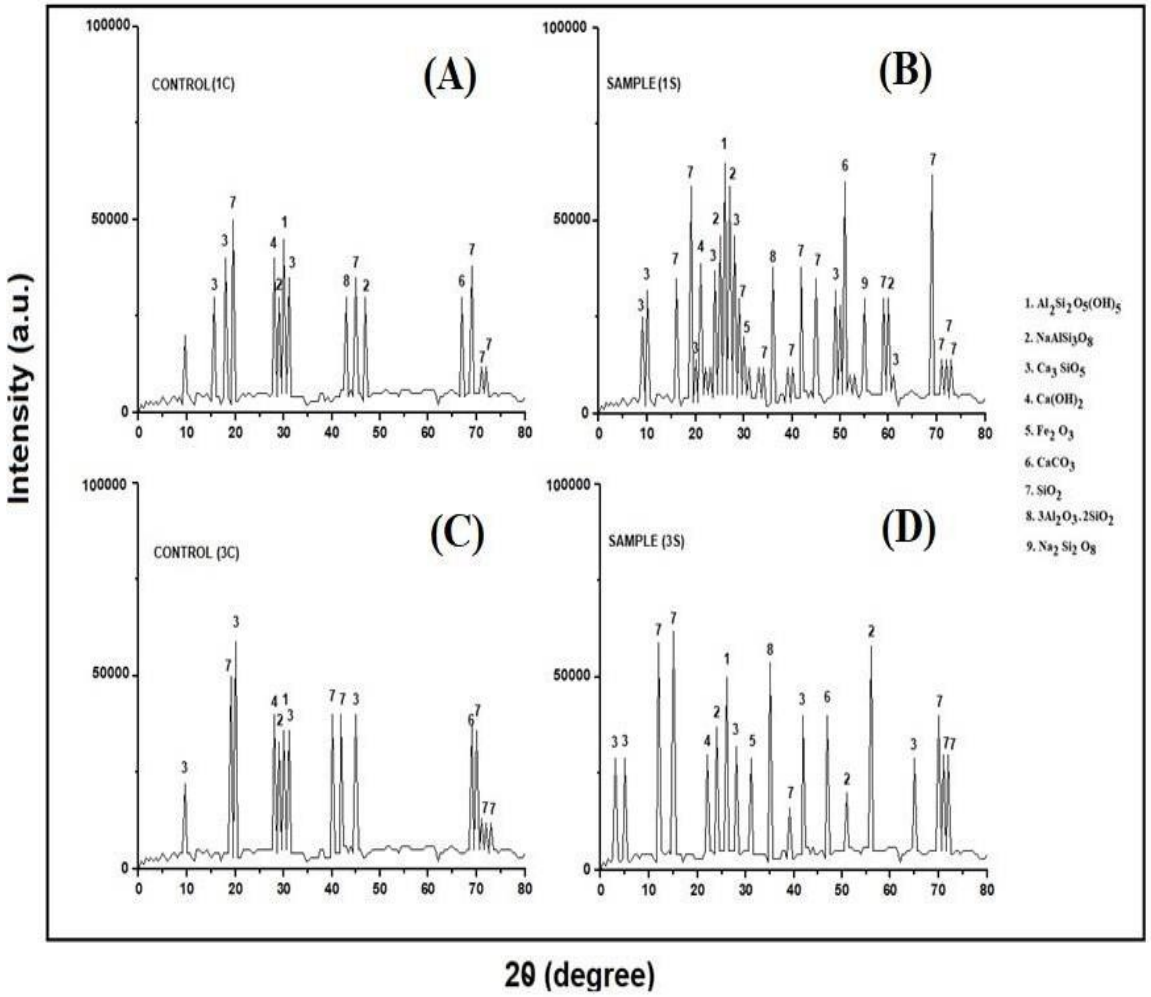

Figure 11 X-ray Diffraction study (A) XRD analysis of geopolymer mortar cured in ambient temperature. (B) XRD analysis of self-healed bacterial amended geopolymer mortar cured in ambient temperature. (C) XRD analysis of geopolymer mortar cured at 50 ℃ for 72 h. (D) XRD analysis of self-healed bacterial amended geopolymer mortar cured at 50 ℃ for 72 h.

Table 15 Survibility Test of bacterium amender self-healed geopolymer mortars cured at ambient temperature on different ages

No. of Days	Optical Density at 620 nm
7	0.85
28	0.84
60	0.73
90	0.65
120	0.62
240	0.41
365	0.39

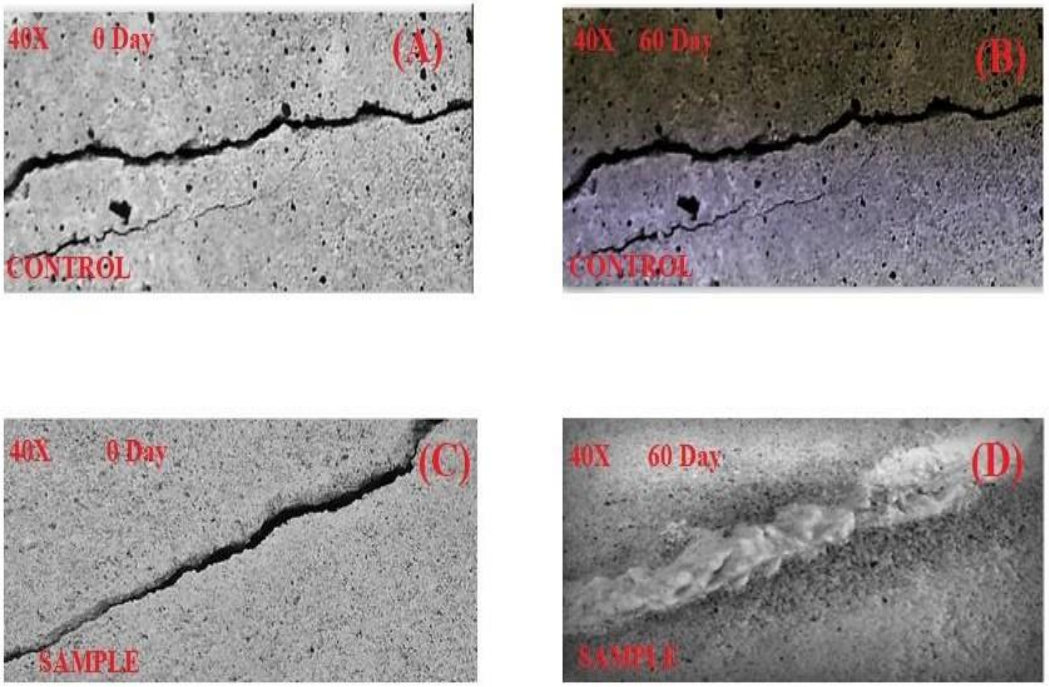

Figure 12 Microscopic images of the mortar samples viewed by Crack Detection Microscope. (A) Image of the crack of geopolymer mortar at 0 days. (B) Image of the crack of geopolymer mortar at 60 days. (C) Image of the crack of bacterial amended geopolymer mortar at 0 days. (D) Image of the crack of self-healed bacterial amended geopolymer at 60 days.

Table 16: Biosilicification Assay of bacterium amender self-healed geopolymer mortars cured at ambient temperature on different ages

No. of Days	Optical Density at 405 nm
7	0.39
28	0.38
60	0.37
90	0.36
120	0.33
240	0.29
365	0.25

Table 17: Biochemical parameters of bacterial treated rats at different levels

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

Table 18: 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

The bacterial survivability(Table 15) and biosilicification (Table 16) results showed that the incorporated bacterial cells remained active for long time (1 year) within the self-healed 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 18). The results of toxicity study of bacterial cells on rats were shown in Table 17. 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 17)

The transformed *Bacillus* bacterial cells neither produced any toxic effects on animals (Table 17) nor on human cell lines (Table 18). 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 17). 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. .

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CONCLUSION

❖ Based on the experimental work reported in this study,the following conclusions are drawn:

- The study shows a higher-strength (Figure 1) and more durable eco-efficient geopolymer material can be developed by incorporating transformed *Bacillus subtilis* bacterial cells (at a concentration of 10^5 cells/ml alkali activator solution) in 100% fly ash.

- The newly developed 100% fly ash-based geopolymer can be used as cement alternative for construction purposes which will be more sustainable at higher temperature as the bacterium incorporated geopolymer mortar shows more mechanical strength compared to cement mortar with and without bacteria (Figure 1).

- The flexural strength and split tensile strength of the bacterium amended geopolymer cured at ambient temperature is found to be 165 % and 142 % more than that of corresponding geopolymer without bacteria. (Table 2).

- The bioremediase-like gene is responsible for the leaching of nano-silica from various silicate phases present within the concrete environment due to its biosilicification activity which forms different phases (e.g. gehlenite or calcium aluminium silicate) by reacting with the different oxides inside the mortar matrix .

- The transformed *Bacillus subtilis* bacterium thus becomes useful for the development of high-performance geopolymer because calcite and gehlenite synergistically fill the micropores of the biopolymer mortars thereby increasing the compressive strength.

- The water absorption and the amount of charge passed in RCPT are also less in bacterium incorporated cement and flyash geopolymer mortars compared to the one without bacteria incorporation (Table 3 and Figure 4). Such improvements in the strength and durability of bacterium amended geopolymer mortar are mainly due to the transformation of amorphous to crystalline compound.

- The cementitious mortar samples fail to maintain its structural integrity at very high temperatures due to which the compressive strength of the samples is decreased by 44.4% with increasing the curing temperatures from 100 to 400 °C (Table 5).

- Incorporation of bacterial cells to the cementitious mortars, the thermal tolerance of the samples is increased to some extent as revealed by the compressive strength (23.1% reduction only) of the OPC + bacteria samples because bioremediase protein helps the formation of more calciumsilicate-hydrate (CSH) gel within the matrix by enhancing the hydration of the unused cement particles. Due to this, less pore water is available within the matrix and thus causes less volume expansion at elevated temperature.

- In this study, the strength of geopolymer mortar specimens is found to increase with the increase in the temperature, attaining peak strength at 250 °C. On the other hand, the compressive strength of the bacterial cells-incorporated fly ash geopolymer specimen after curing at ambient temperature for 28 days shows significant increment (15.9%) of compressive strength at elevated temperature (400 °C) due to the increased formation of various high-temperature stable phases (e.g. mullite or Al_2O_3–SiO_2, hematite or Fe_2O_3, etc.) (Table 5). **The newly developed 100% fly ash-based geopolymer can be used as cement alternative for construction purposes which will be more sustainable at higher temperature.**

- Microstructural analysis infers abundance of silicon atoms (almost 5 times greater as compared to its control) within the pores of the bacterium-amended fly ash geopolymer. XRD spectra exhibit some new peaks as well as some higher-intensity minor peaks in the bacterium-incorporated mortar samples compared to the their respective control samples (Fig. 6) due to enhanced formation of silica , mullite albite and alite , etc., by the action of bioremediase-like protein and calcite ($CaCO_3$) by the action of urease protein.
- In case of self healing and crack repairing study , 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 (Table 7).
- 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 8).
- The crack repaired mortars show remarkable improvement of compressive strength (63.5%; Table 9), split tensile strength (93%; Fig. 8) and flexural strength (140.9%;

Fig. 8) respectively compared to controls after ambient temperature air curing (28 days). The highest increment of UPV was also noted in such case (Table 11 and 12).

- The bacterium was seen to fill the cracks of 1 mm width completely in ambient temperature water curing (60 days; Fig. 12). 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.

- In terms of durability also, minimum water ingress was noted in self-healing samples (1.5%; Table 12) and crack-repairing samples (1.09%; Table 13) when cured at ambient temperature air curing condition also the sulfate resistance test showed that minimum increment of weight in bacterial assimilated self-healed (1.3%) and crack repaired (1.0%) geo-polymeric at ambient temperature air curing (Table 14).
- 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 of rod like structures (approx. 80 nm; Fig. 10 B).
- 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. 11.
- **The genetically enriched alkailophilic *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.

❖ Importance of the thesis related to my work

As we all know that due to increase in carbon di-oxide emissions, global warming is increasing day by day. So the use of 100% fly ash geopolymer as a cement alternative will reduce the use of cement to some extent which will indirectly reduce the emission of green house gases and prevent from air,,soil as well as water pollution too. Before many researchers have used fly ash as a cement replacement to certin extent, but use of 100% fly ash based geopolymer without heat curing after casting was a very challenging work which was been possible due to incorporation of transformed bacteria. Heat curing after casting is a very crucial step for polymerisation in geopolymers but the process was not energy efficient which has been carbed to certain extent by heat activation for 45 minutes only before casting in case of bacterium amended geopolymer. **This energy-efficient curing condition makes the modified geopolymer eco-friendly and easy to use.**

Earlier many researchers have used bacterium and various other chemicals as crack repairing and self-healing agents in cementitious materials, but my work has shown autonomous bioremediation in case of 100% fly ash based geopolymer which hasn't been shown earlier. Moreover the work focuses not only in **usage of genetically modified bacterium as self healing agent but also ensures its survivability as well as workability inside the geopolymer matrices for more than a year.**

Usage of bacteria in concrete has always been a cause of fear for the users to use in brick manufacturing and cement industries (specially for the labours who work there). My work is the first approach which deals with the toxicity study of the bacteria in rat model as well as human cell line. And the results of the toxicity study clearly states that the bacteria is very much safe to use in the concentration required for usage in bio-concrete. High concentration of the genetically modified bacteria is being injected in rat for studying the chnge in bio-chemical parameters like Urea, Alkaline phosphatise, HDL, LDL, Triglyceride etc and it is been seen that high concentrations of bacteria when injected in rat model for a month (for every alternate days) didnot affect the biochemical parameters of the rat. The transformed bacterial cells were also used to treat on two human cell lines (WI38 and HaCaT cells) which also did not exhibit any marked cell death on the two different human cell lines. **The bacterium incorporated material is thus eco-friendly and safe towards human populations.**

❖ FUTURE SCOPE OF THE STUDY :

- ✓ The long term properties of bacterium amended fly ash based geopolymer mortar cured in various curing conditions may be studied in detail.
- ✓ Similar studies can be executed on bacterium amended slag based geopolymer mortar instead of fly ash and its comparison with other geopolymer mortar.
- ✓ A combination of fly ash and slag based geopolymer may be also an area of research.

Clean Technologies and Environmental Policy
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ORIGINAL PAPER

Bacterium-incorporated fly ash geopolymer: a high-performance, thermo-stable cement alternative for future construction material

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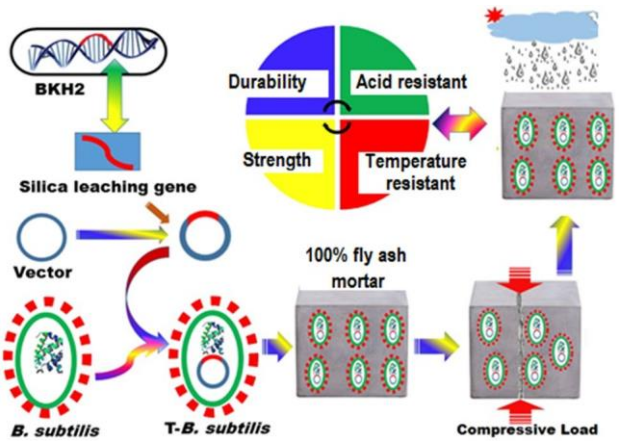
Abstract

Cement, the primary construction material, releases a substantial quantity of CO_2 (about 5–7% of total CO_2 emission) in the atmosphere during its production, thereby causing global warming. Fly ash is a coal combustion by-product that leads to many environmental problems like ground water contamination, spills, heavy metal contamination, etc. To overcome the serious ecological problems and health hazards of cement industries and thermal power plants, development of clean binding materials for construction purposes has become an interesting and new aspect of research to the scientific communities. This study has been designed to develop and characterize 100% fly ash-based geopolymer by incorporating improved *Bacillus subtilis* cells which may be used for cement alternative in future. The designed geopolymer showed significantly increased compressive, flexural and tensile strengths, reduced water absorption capacity and increased sulphate and chloride resistance attributes. It also possesses enhanced durability with high-temperature tolerance (400 °C) compared to cementitious material. Microstructure analysis showed more compactness, reduced porosity and development of new phases inside the geopolymer matrix. The newly developed 100% fly ash-based geopolymer is an eco-efficient material which will reduce the pollution caused by fly ash and be used for sustainable construction purposes in the near future.

Graphic abstract



Keywords Compressive strength · Fly ash · Geopolymer · Microbes · Thermal stability

Introduction

Cement production releases a significant amount of solid waste and gaseous substances in the environment. It is estimated that about 5–7% of the total CO_2 anthropogenic

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emissions comes from cement industries (Chen et al. 2010). In addition to that, the cement manufacturing process produces million tons of waste product (cement kiln dust) each year which affects our respiratory system and health (Huntzinger and Eatmon 2009). Though continuous efforts are being made by cement industry to reduce CO_2 emissions through improvements in process and efficiency, it has some limitations for further improvements because the basic calcinations process generates huge CO_2 gas inherently. Several research groups are therefore working to develop bio-inspired concrete which will require less cement but will have higher mechanical strength; more durability and self-healing attribute so that it would minimize cement-based environmental pollution to some extent (Silva et al. 2015; Vijay et al. 2017; De Belie et al. 2017).

Thermal power plants generate huge quantity of fly ash due to coal combustion which spreads over the cultivated lands, mixes with the ground water and significantly increases the heavy metal contamination in the environment leading to many ecological problems (Verma et al. 2016). It has been estimated that more than 112 million tons of Fly ash is disposed off as a waste material that covers several hectors of valuable cultivated land per annum in India (Cheerarot and Jaturapitakkul 2004; Dwivedi and Jain 2014). Several processes have been developed to use fly ash for reducing environmental pollution caused by it. Fly ash is used to produce more environmentally friendly concrete for partial replacement the amount of ordinary Portland cement (OPC) in concrete. High-volume fly ash (HVFA) concrete uses approximately 40% of OPC and yet possesses excellent mechanical properties with enhanced durability performance (Malhotra 2002). Fly ash bricks, road construction fly ash material, etc., are the other examples which are used to reduce fly ash-related problems. Recently, application of 100% fly ash-based geopolymer as construction material has become an important area of research in concrete technology. Low-calcium fly ash (ASTM Class F)-based geopolymer is shown to have suitable binding property when used in concrete (Xiaolu et al. 2010; Vargas et al. 2011; Richard et al. 2012). The factors on which the strength and durability of fly ash-based geopolymer concrete depend are the concentration of alkali activator, mix proportion, curing temperature and curing time, etc. It has been noted that higher molarities of NaOH used as an alkali activator appear to provide higher compressive strength at early age (Alehyen et al. 2017). The sodium hydroxide leaches the silicon and aluminium in the amorphous phase of fly ash geopolymer, and the sodium silicate acts as a binder. Also, the mechanical strength of geopolymer mortar depends on the ratio of sodium hydroxide versus sodium silicate (Somna et al. 2011). In general, heat activation is needed for the development of geopolymer mortar in the presence of alkali activator because geopolymer mortar shows poor strength when cured at ambient temperature (about 27 ± 2 °C) due to its slow polymerization process (Malkawi et al. 2016; Somna et al. 2011; Ryu et al. 2013; Rashad 2014). Thus, the scope of geopolymer concrete is supposed to be limited to the precast member due to the requirement of heat activation after casting. Most of the research works on fly ash geopolymer are on the mix proportion and strength variation of geopolymer concrete cured at different temperature ranges of 45–80 °C for about 2–3 h. There are limited studies available on geopolymer to eliminate the shortcomings of ambient temperature curing.

Our previous studies have reported that the compressive strength and durability of concrete can be increased substantially (> 30%) by using some specific hot spring bacteria (BKH1 and BKH2) or their extra-cellular protein (e.g. bioremediase, M.W. ~ 28 kDa) (Ghosh et al. 2005; Sarkar et al. 2013, 2015a). Those bacteria possess silica leaching (biosilicification) activity which can be used to develop new phase (Gehlenite) inside the mortar matrices for getting higher strength and more durability in concrete structures (Biswas et al. 2010; Chowdhury et al. 2015). The use of *Bacillus pasteurii* bacteria in concrete is associated with mineral precipitation (calcium carbonate) that helps to fill micropores and cracks, thus reducing its permeability and increasing its strength and durability (Ramachandran et al. 2001). However, the highly alkaline pH environment within the concrete matrices restricts the growth of the bacteria (Pacheco-Torgal and Labrincha 2013). To overcome this problem, different authors have suggested the use of different immobilization solutions (clay capsules, silica gel or polyurethane encapsulation). De Beile et al. (2018) have shown the first in situ applications of encapsulated bacterial spores which have the ability to self-heal cracks in concrete. Inagaki et al. (2003) have shown that thermophilic or hyper-thermophilic microorganisms living in geothermal environments are involved in the formation of biogenic siliceous deposits (siliceous sinter, geyserite and silica scale). The slow growth rate of the hot spring bacteria may restrict them to use in the concrete industry. The problem has been overcome by transferring the bioremediase gene into *E. coli* JM 107 (Sarkar et al. 2015a) and *Bacillus subtilis* bacterial strains and used those transformed bacteria in concrete/mortar mix to increase the strength and durability of the cementitious material in short time period (Sarkar et al. 2015b). *Bacillus subtilis* is a spore-forming bacterial strain which can remain in dormant form within the concrete/mortar matrices for quite long time and becomes active when water ingresses within the concrete (Chislett and Kushner 1961).

The main challenge of our study is the development of ambient temperature-curing clean geopolymer mortar with the addition of transformed *Bacillus subtilis* bacterial cells. The newly designed geopolymer should have higher compressive, flexural and tensile strengths and increased

durability compared to cementitious material and also should be able to reduce environmental pollution by replacing cement to some extent. Our results have shown that bacterium-incorporated 100% fly ash geopolymer concrete is suitable for construction purposes. The LCA analysis of this study is beyond our scope due to which LCA analysis is not performed here.

Materials and methods

Materials

Low-calcium Class F dry fly ash [specific gravity: 2.05; grain size: 150–300 µM (6.02%), 90–150 µM (33.32%), 45–90 µM (53.40%), < 45 µM (6.21%)] was obtained from the National Thermal Power Corporation Ltd, Farakka Plant in India, and used as the base material. The sodium hydroxide (NaOH) used in this investigation was the commercial grade in pellet forms with 99% purity and obtained from local market. Liquid sodium silicate (Na_2SiO_3) was also a commercial grade having 45% solid content and specific gravity of 1.53 gm/cc. ordinary Portland cement (specific gravity 3.1) of 43 grade (IS 8112 1989) and standard Ennore sand (specific gravity 2.64) (IS 650 1991) were used for the study. The bacterial strain, i.e. transformed *Bacillus subtilis*, was obtained from our laboratory stock culture (Sarkar et al. 2015b). The bacterium was grown in the Luria broth (LB, pH 10.0).

Mix proportion and curing

There were 4 categories samples (1—OPC mortar; 2—OPC mortar + bacteria; 3—Fly ash geopolymer and 4—fly ash geopolymer + bacteria) prepared for the study. Mixing proportions of 4 categories are shown in Table 1.

The NaOH solution (12 molar) was mixed with commercially available Na_2SiO_3 solution in the proportion of 1:1.75 (v/v) to make alkali activator fluid. The effective bacterial cells concentration (10^5 cells/ml) was prepared by diluting the bacterial cells with water or activator fluid for preparing bacteria-incorporated cementitious mortar or fly ash geopolymer samples, respectively. A 2% LB medium (v/v) (pH 7.0) as was added during bacteria-incorporated mortar specimens' preparation. Ordinary Portland cement of 43 grade mixed with sand properly and was used for making mortar samples of categories 1 and 2. After 24 h of casting, all the specimens were removed from moulds and cured under water for several days until testing. For the preparation of category 3 and 4 samples, the fly ash- and bacteria cells-containing activator fluid was mixed properly for 2 min and the mixture was heat-cured at 60 °C for 45 min before casting as described earlier (Adak and Mandal 2015). After 24 h of casting, all the specimens were removed from moulds and cured at ambient temperature for several days until testing.

Mechanical strength and ultrasonic pulse velocity (UPV) study

The standard mortar cube specimens (5 for each category) of size 70.6 mm × 70.6 mm × 70.6 mm were cast for different categories to determine the compressive strength of mortar as per IS 4031-1988 standard. After respective curing for 3 days, 7 days, 14 days and 28 days, the ultrasonic pulse velocity and compressive strength of all the mortars were determined. The ultrasonic pulse velocity was determined by using PUNDIT plus PC 1007 UPV machine, UK as per ASTM C597-02 (ASTM 5977-02 2002). The compressive strength of each UPV-tested sample was measured by using Digital Compression Test machine, India

Mortar cylinder specimens (5 in each) of size 100 mm diameter × 200 mm height were cast for 4 different category samples to determine the split tensile strength. After respective curing, the cylinder of each category was placed horizontally between the loading surfaces of compression testing machine and the load is applied perpendicularly to the axis of the cylinder. The maximum breaking load applied to the specimen was recorded to calculate the split tensile strength of the specimen as per IS: 5816 (1999).

The flexural strength testing was carried out on 50 mm × 50 mm × 200 mm geopolymer mortar bar for all 4 categories samples. The centre point loading method was adopted for determination of flexural strength (AASHTO-T-67 2005).

Table 1 Mixing ratio of different category samples

Sample category	Fly ash/ cement:sand	Activator fluid:fly ash	Water:cement	Bacterial cells/ ml	Curing conditions
OPC	1:3	Nil	0.4	Nil	Water curing
OPC + bacteria	1:3	Nil	0.4	10^5	Water curing
Fly ash geopolymer	1:3	0.4	Nil	Nil	Ambient temperature curing
Fly ash geopolymer + bacteria	1:3	0.4	Nil	10^5	Ambient temperature curing

Durability test

The water absorption test, sulphate resistance test, chloride ion permeability test and sulphuric acid (10%) resistance test of the mortar samples were done for durability testing purpose.

Water absorption test—After 28 days of respective curing, all the specimens (5 for each category) were air-dried for 24 h at room temperature and their initial masses were determined. The samples were then kept under deionized water for 30 min. The samples were then removed from water and cleaned with tissue paper, and their wet masses were registered again immediately. Then, samples were kept under water again for another 24 h, and after that, the samples were removed from water and cleaned with tissue paper, and their final wet masses were measured. Water absorption capacity was determined as per Neville's method (Neville 1996).

Sulphate resistance test—After 28 days of respective curing, the initial masses of the mortars (5 numbers for each category) were determined. The samples were then immerged in sulphate solution (5% $MgSO_4$, pH 7.0 in deionized water)-containing tank. The samples were kept under sulphate solution for 90 days. After curing, the samples were removed from the tank and air-dried, and their masses were again determined. The test was carried out as per the guideline of ASTM STP663 (1997).

Chloride permeability test—For rapid chloride ion permeability test (RCPT), mortar cylinders (3 in each) of each category (100 mm diameter × 200 mm height) were prepared. After 28 days of respective curing, each cylinder was cut into three small cylinders (100 mm diameter × 50 mm height) and epoxy-coated along with theirs edges and left under water for 24 h before measuring the chloride ion permeability by Rapid Chloride Ion Penetration Cell. The test was done as per ASTM C1202 (2000).

Acid resistance test—Mortar samples (20 samples for each category) were prepared. After respective curing for 28 days, 5 samples from each category were taken and their average compressive strengths were determined. The rest of the 15 samples of each category were then immerged in 10% sulphuric acid solution for different days of curing (4, 8 and 12 weeks, respectively). After each curing period, 5 samples of each category were taken and their average compressive strengths were determined.

Thermal resistance test—After respective curing for 28 days, the samples from 4 different categories were kept in the oven for heating at different temperatures (100–400 °C, respectively) for 4 h each. After cooling, the compressive strength of the heat-treated mortar sam- ples was measured to observe the thermal tolerance of the samples.

Microstructure analysis

After measuring the compressive strength, the fragmented mortar sample was ground into fine powder and sieved to make the size less than 5 micrometres for analysis. For FESEM and EDX analyses, the fine powder was dispersed with ethanol (99.9%) to make a film on carbon tape and then kept under vacuum desiccators for evaporation. Finally, the dried samples were gold-coated for field emission scanning electron microscope FESEM (HITACHI S-4800, JAPAN) and EDX (EDX-equipped Philips XL30) analysis.

XRD analysis of the powder mortar sample was done by X-ray diffractometer (Bruker AXS Inc, Model D8, WI, USA). The experiment was conducted with a scan speed 0.5 s/step at 40 kV. The XRD spectrum was analysed in the range 2Θ = 10–80 degree, and the peak positions were marked and analysed by using JCPDS data file.

Mercury intrusion porosimetry (MIP) analysis was done to observe the modification of pore size distribution on 4 different categories samples. Liquid nitrogen-frozen fragmented samples (collected after measuring the compressive strength) were cryovacuum-dried for several days prior to analysis.

Bacterial survivability inside the geopolymer samples

Transformed *Bacillus subtilis* bacterium-incorporated mortar samples of different ages (3, 7, 14, 28, 60, 120, 240, 360 days) were made to powder form by pestle-mortar and a pinch of each powdered bacteria-incorporated mortar sample (approximately 10 mg) was added in 5 ml of sterile liquid LB medium and kept some time for precipitation of the heavier dust particles. One millilitre supernatant of each was inoculated to the 5 ml sterile LB medium (pH 10.0) and kept for 24 h at 37 °C in shaker-incubator followed by observation for growth. The growth of the bacterium was detected by measuring the optical density of the growth medium at 620 nm with respect to control growth medium. Biosilicification assay was done to confirm the presence of bioremediase-like protein leached by the active bacterial cells as described elsewhere (Biswas et al. 2010).

Statistical analysis

Since the cementitious mortars or geopolymer mortars are heterogeneous substances, the experimental data may vary from sample to sample. For our experimental purposes, 4 category samples (5 samples in each) as described in methodology section were prepared. Each experimental data were presented as average ± SD. Each set of experiment was repeated at least three times.

Results

Mechanical strength of the different category samples

The 100% fly ash geopolymer mortars possessed higher compressive strength than that of cement-based mortars (Fig. 1). The incorporation of bacteria increased the compressive strength of both cementitious (OPC + bacteria) and geopolymer (Fly ash geopolymer + bacteria) samples at all ages of curing as seen from Fig. 1. It was noted that the transformed *Bacillus* subtilis also increased the flexural strengths (25.6% for OPC + bacteria and 165% for fly ash geopolymer + bacteria) and tensile strengths (50% for OPC + bacteria and 142% for Fly ash geopolymer + bacteria) both with respect to their control samples (Table 2). A significant increase in ultrasonic pulse velocities was observed in OPC + bacteria and fly ash geopolymer + bacteria mortar at all ages of curing in comparison with their respective controls (Fig. 2).

Durability performance of different category samples

The water absorption results showed that the percentage increments of mass of the OPC + bacteria samples were less compared to OPC samples (Table 3). Minimum increment of mass (2.1%) was noted in the case of fly ash geopolymer + bacteria samples. It was observed that the bacterial cells-amended fly ash geopolymer mortar samples had minimum increment of weight (1.40%) compared to the fly ash geopolymer mortar samples (3.29%) during sulphate absorption test (Fig. 3). The results of chloride permeability test also showed that bacterial cells increased the chloride resistance of the samples (Fig. 4). The chloride ions permeability was decreased 47.3% in OPC + bacteria samples compared to OPC samples and 45% in fly ash geopolymer + bacteria samples compared to fly ash geopolymer samples as seen from Fig. 4. Acid resistance activity of the mortar samples showed that transformed *Bacillus subtilis* also protected the bacteria-incorporated samples significantly (Table 4).

It was noted that fly ash geopolymer + bacteria mortar samples exhibited comparatively much better acid-resistant activity than the other mortar samples (Table 4). The compressive strength of the OPC mortars was decreased by 44.4% with increasing the curing temperatures (100–400 °C) as shown in Table 5, whereas the

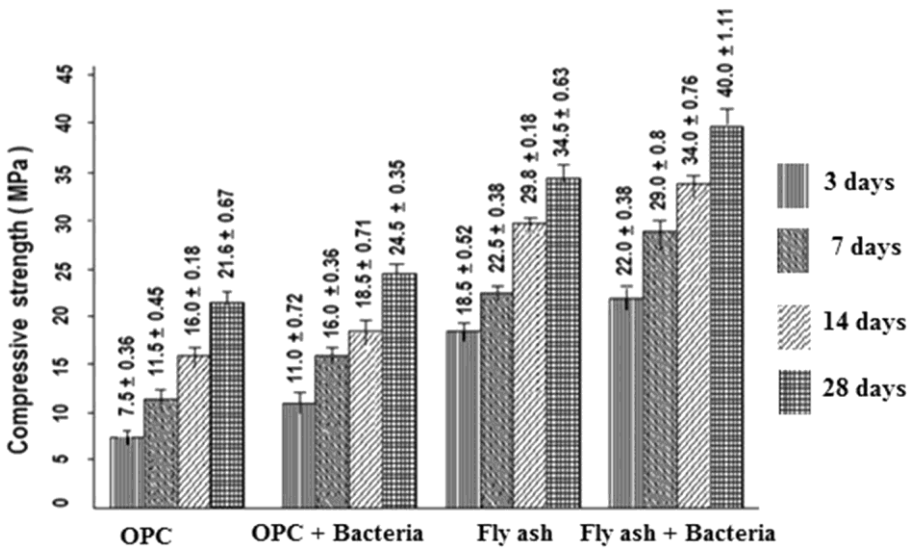

Fig. 1 Compressive strength of different category mortar samples

Table 2 Effect of bacterium on flexural and tensile strength

Sample	Flexural strength (MPa)	Tensile strength (MPa)
OPC + bacteria	5.4 ± 0.69 (25.6↑)	3.0 ± 0.41 (50↑)
Fly ash geopolymer	2.0 ± 0.97	1.59 ± 0.20
Fly ash geopolymer + bacteria	5.3 ± 0.68 (165↑)	3.86 ± 0.20 (142↑)

Data are presented mean ± S.D. (n = 9). The value within parenthesis indicates the % of increment (↑) with respect to its control

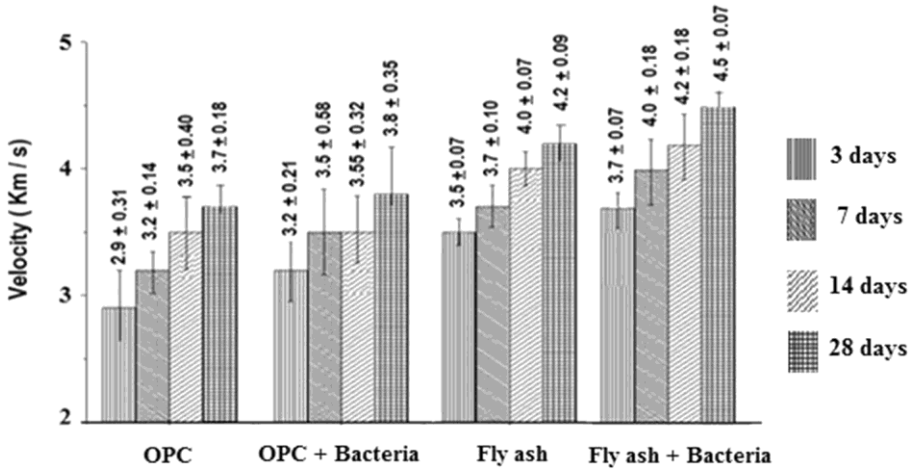

Fig. 2 Ultrasonic pulse velocity of different category mortar samples

Table 3 Water absorption test of bacteria treated samples

Sample	Mass (g)				
	Initial	After 30 min	% Increase	After 24 h	% Increase
OPC	730 ± 3.5	750 ± 9.4	2.7	760 ± 7.0	4.1
OPC + bacteria	725 ± 11.1	740 ± 7.90	2.0	749 ± 10.5	3.3
Fly ash geopolymer	730 ± 7.9	745 ± 5.00	2.0	753 ± 8.3	3.1
Fly ash geopolymer + bacteria	715 ± 7.9	720 ± 3.53	0.7	730 ± 7.0	2.1

Data are presented mean ± SD. (n = 15). The increased percentage was calculated with respect to the corresponding control

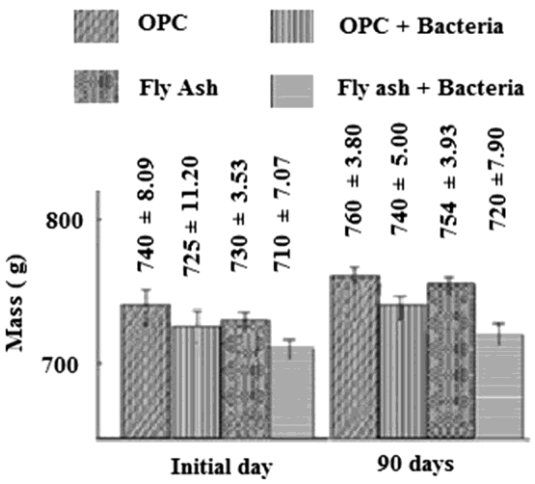

Fig. 3 Sulphate resistance activity of different category mortar samples

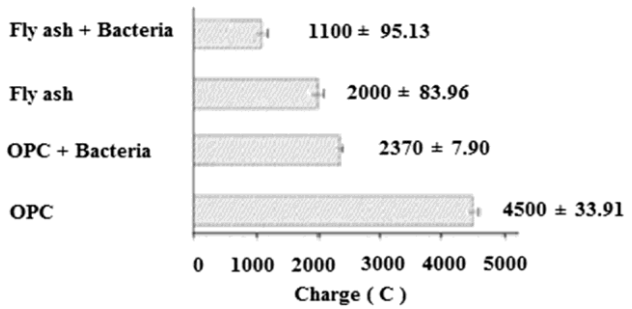

Fig. 4 Rapid chloride permeability of different category mortar samples

compressive strength of the OPC + bacteria samples was decreased only by 23.1% compared to OPC mortar samples. The fly ash geopolymer + bacteria samples showed a significant increase in compressive strength (15.9%) at elevated curing temperature as seen from Table 5.

Microstructures analysis of different category samples

Microstructures analysis by environmental scanning electron microscope equipped with EDX showed that formation of various phases inside the pores of the fly ash geopolymer + bacteria mortar samples. A significant increased concentration of silicon was observed within the phases of micropores of fly ash geopolymer + bacterial samples (Fig. 5). Increased formation of silica (SiO_2) and calcium aluminium silicate ($Ca_2Al_2SiO_7$) was observed in OPC + bacteria mortar samples. The XRD analysis similarly

Table 4 Acid resistance of mortar samples in 10% sulphuric acid

Samples	Compressive strength (MPa)			
	Initial	4 weeks	8 weeks	12 weeks
OPC	21.60 ± 0.67	12.96 ± 0.90 (40.0)	9.20 ± 1.20 (57.4)	6.30 ± 1.20 (70.8)
OPC + bacteria	24.50 ± 0.35	16.21 ± 0.75 (33.8)	13.50 ± 1.00 (44.8)	10.90 ± 1.35 (55.5)
Fly ash geopolymer	34.50 ± 0.63	26.00 ± 1.20 (24.6)	20.70 ± 1.00 (40.0)	18.00 ± 0.90 (47.8)
Fly ash geopolymer + bacteria	40.00 ± 1.11	36.10 ± 1.40 (9.7)	34.00 ± 1.90 (15.0)	31.00 ± 1.60 (22.5)

Data are presented mean ± SD (n = 15). The value within parenthesis indicates the % decrement with respect to its control

Table 5 Effect of temperature on compressive strength

Temperature (°C)	OPC	OPC + bacteria	Fly ash	Fly ash + bacteria
100	21.6 ± 0.6	26.0 ± 0.9 (↑20.00)	34.5 ± 0.4	41.0 ± 1.6 (↑18.80)
150	22.0 ± 0.4	26.5 ± 0.6 (↑20.00)	36.0 ± 0.3	42.0 ± 0.9 (↑16.60)
200	20.0 ± 0.8	25.0 ± 0.5 (↓25.00)	37.5 ± 0.5	42.5 ± 1.0 (↑13.30)
250	20.0 ± 0.6	24.0 ± 0.9 (↓20.00)	38.0 ± 0.8	44.0 ± 0.9 (↑15.70)
300	16.0 ± 0.9	23.0 ± 0.4 (↓43.75)	36.0 ± 0.6	45.0 ± 1.2 (↑25.00)
350	14.0 ± 0.8	21.0 ± 0.3 (↓50.00)	35.0 ± 0.8	46.0 ± 0.5 (↑31.40)
400	12.0 ± 0.6	20.0 ± 0.6 (↓66.60)	33.0 ± 0.6	47.5 ± 0.8 (↑43.90)

Data are presented mean ± SD (n = 5). The value within parenthesis with ↑ symbol indicates the % of increment, and that with ↓ symbol indicates the % of decrement with respect to its control

suggested that new phases (e.g. $3Al_2O_3$, $2SiO_2$, $Na_2Si_2O_3$) along with the increased concentration of sodium aluminium silicate ($NaAlSi_3O_8$), calcium silicate (Ca_3SiO_5), calcium carbonate ($CaCO_3$) and silica (SiO_2) were appeared in the bacterium-incorporated OPC and fly ash geopolymer samples (Fig. 6). From the results of MIP test, it was noticed that specific density within micropore region (pore diameter < 50 µm) of the bacteria-incorporated mortar samples was less in both OPC + bacteria and fly ash geopolymer + bacteria samples compared to their respective controls (Fig. 7).

Bacterial survivability

The bacterial survivability and biosilicification results showed that the incorporated bacterial cells remained active for long time (1 year) within the cementitious and geopolymer mortar samples which are provided in the supplementary section.

Discussion

The transformed *Bacillus subtilis* bacterial strain when incorporated to the alkali activator fluid-treated 100% fly ash to prepare geopolymer material is found to increase the compressive strength of the geopolymer mortars (Fig. 1). The compressive strength increment is maximized at a particular bacterial cells' concentration (10^5 cell/ml alkali-activated fluid used) when incorporated to the fly ash-based geopolymer (Supplementary data). It is already established that genetically improved *Bacillus* bacterial strain (transformed *Bacillus subtilis*) has the ability to increase the compressive strength and durability of the cementitious mortar samples when incorporated within the samples (Sarkar et al. 2015b). The bacterium (*Bacillus subtilis*) possesses urease gene which is responsible for the formation of calcite ($CaCO_3$) in the matrix of cementitious mortars (Ramachandra et al. 2001). On the other hand, the bioremediase-like gene is responsible for the leaching of nano-silica from various silicate phases present within the concrete environment due to its biosilicification activity (Biswas et al. 2010; Chowdhury et al. 2015; Sarkar et al. 2015b). The leached nano-silica forms different phases (e.g. gehlenite or calcium aluminium silicate) by reacting with the different oxides inside the mortar matrix (Sarkar et al. 2015b). The transformed *Bacillus subtilis* bacterium thus becomes useful for the development of high-performance geopolymer because calcite and gehlenite synergistically fill the micropores of the biopolymer mortars thereby increasing the compressive strength. The increments of flexural strength (25.6% for OPC + bacteria and 165% for fly ash geopolymer + bacteria) and tensile strengths (50% for OPC + bacteria and 142% for fly ash geopolymer + bacteria) of the transformed bacterium-incorporated specimens also can be explained similarly.

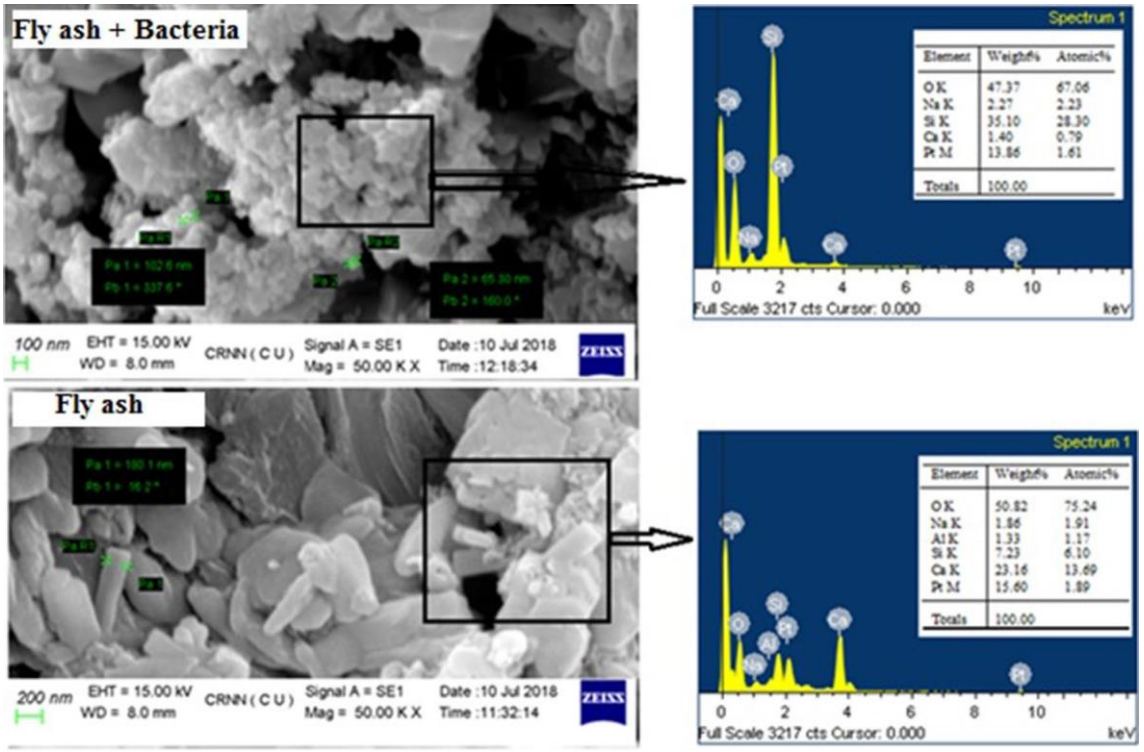

Fig. 5 SEM image with EDX analysis of fly ash geopolymer and fly ash geopolymer + bacteria-incorporated mortar sample

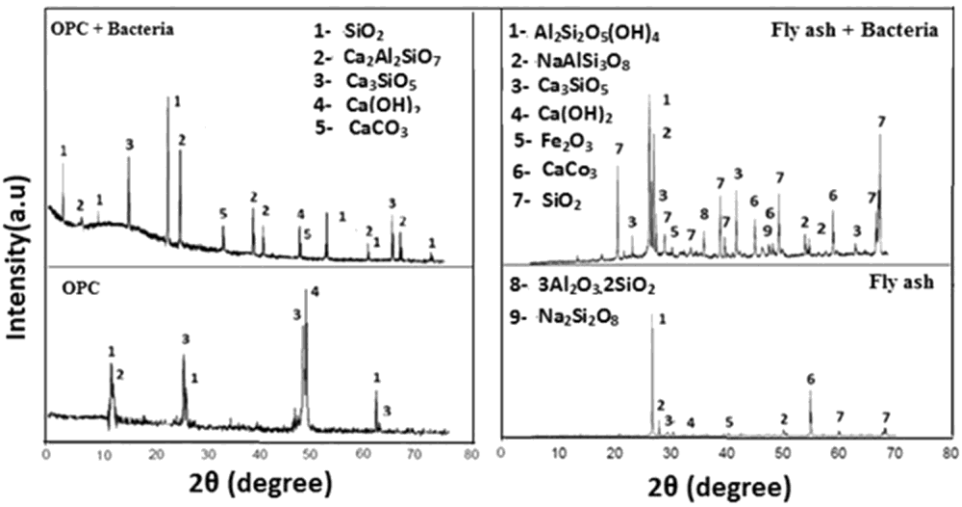

Fig. 6 XRD analyses of different category mortar samples

The durability of the bacterium-incorporated geopolymer specimen is also increased as reflected by the results of water absorption (Table 3), sulphate resistance (Fig. 3) and chloride permeability (Fig. 4) and acid resistance tests (Table 4). The overall increased strength and ultrasonic pulse velocity of the bacterium-incorporated cementitious mortar or geopolymer samples are the clear indication of less porosity present within matrix of the samples. This makes the specimens to allow less water, sulphate or chloride ions whatever may be to ingress within the samples. This effect is more prominent in the case of bacterium-incorporated geopolymer samples. Our results thus suggest that bacterium-incorporated 100% fly ash geopolymer mortars are comparatively more durable material than the cementitious mortar samples.

It is noted that the cementitious mortar samples fail to maintain its structural integrity at very high temperature due to which the compressive strength of the samples is decreased by 44.4% with increasing the curing temperatures from 100 to 400 °C (Table 5). The pore water that remained within the concrete/mortar specimen expands at

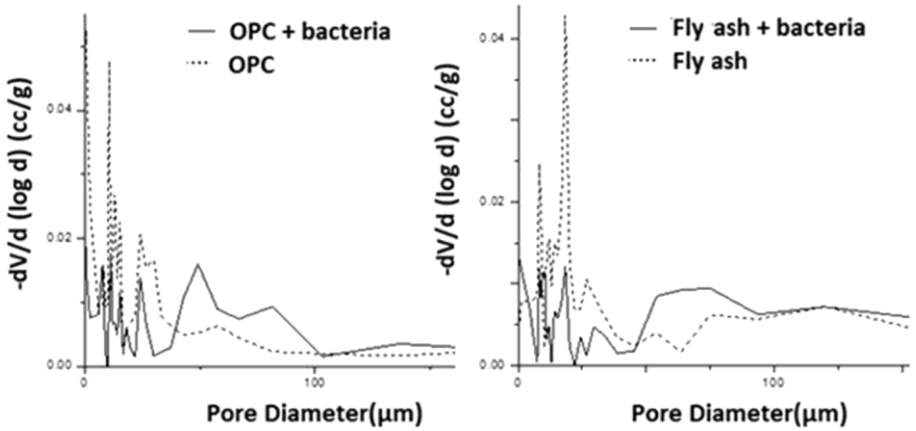

Fig. 7 MIP analysis of different category mortar samples

elevated temperature and causes volume expansion of the samples resulting in the generation of cracks. The cracks lead to reduction of compressive strength. Also the compressive strength deterioration is attributed to the $Ca(OH)_2$ decomposition that occurs at about 400 °C (Lea and Stradling 1922). Whereas, incorporation of bacterial cells to the cementitious mortars, the thermal tolerance of the samples is increased to some extent as revealed by the compressive strength (23.1% reduction only) of the OPC + bacteria samples. Chattopadhyay et al. (2010) have demonstrated that bioremediase protein helps the formation of more calcium-silicate-hydrate (CSH) gel within the matrix by enhancing the hydration of the unused cement particles in the bioremediase protein-amended cement-paste samples. Due to this, less pore water is available within the matrix and thus causes less volume expansion at elevated temperature. This may be one of the causes for high-temperature tolerance of the OPC + bacterial mortar samples.

Higher compressive strength and greater ultrasonic pulse velocity of the bacteria-incorporated fly ash geopolymer samples indicate more compactness and lesser micropores of the samples in comparison with its control or cementitious mortar samples. It is known that water released during the formation of geopolymers was expelled from the geopolymer matrix during heat curing, which causes discontinuous nano-pores within the matrix resulting in improvement of strength of geopolymers (Kong and Sanjayan 2010). In this study, the strength of geopolymer mortar specimens is found to increase with the increase in the temperature, attaining peak strength at 250 °C. Subsequently, this strength is observed to reduce gradually to some extent for the remainder of the heating regime. This result supports the finding of early studies (Kong and Sanjayan 2010). On the other hand, the compressive strength of the bacterial cells-incorporated fly ash geopolymer specimen after curing at ambient temperature for 28 days shows significant increment (15.9%) of compressive strength at elevated temperature (400 °C). This may be explained due to the increased formation of various high-temperature stable phases (e.g. mullite or Al_2O_3–SiO_2, hematite or Fe_2O_3, etc.) inside the bacteria-incorporated geopolymer matrices during the curing for several days.

Microstructure analysis of fly ash geopolymer mortar and bacteria cells-incorporated fly ash geopolymer mortar samples by environmental scanning electron microscope showed that there is a significant difference between the elementary compositions in the mortar matrices (Fig. 5). Abundance of silicon atoms (almost 5 times greater as compared to its control) is seen within the pores of the bacterium-amended fly ash geopolymer. The powder crystal XRD spectra exhibit some new peaks as well as some higher-intensity minor peaks in the bacterium-incorporated mortar samples compared to the their respective control samples (Fig. 6). An intricate investigation of all those peaks from JCPDS data file clearly confirms that those peaks are due to the newly formation of silica (SiO_2) and gehlenite ($Ca_2Al_2SiO_7$) by the action of bioremediase-like protein and calcite ($CaCO_3$) by the action of urease protein within the OPC + bacteria samples. Similarly, the new peaks that appeared in the fly ash geopolymer + bacteria are also due to enhanced formation of silica (SiO_2), mullite ($3Al_2O_3$,$2SiO_2$), albite ($NaAlSi_3O_8$) and alite (Ca_3SiO_5), etc., by the action of bioremediase-like protein and calcite ($CaCO_3$) by the action of urease protein within the fly ash geopolymer + bacteria samples. These newly formed phases are responsible for higher strength more thermostable as explained earlier. These newly developed phases also reduce the porosity of bacteria-amended samples as revealed by the MIP test results (Fig. 7). The MIP test results clearly indicate that the specific densities of the bacteria treated mortars are significantly lesser in both bacteria-amended OPC and fly ash specimens as compared to their respective controls. The MIP test results thus corroborate with the findings of UPV test and durability test.

Conclusion

Cement production is one of the most polluting processes of the environment. Thermal power stations using pulverized coal or lignite as fuel generate huge amount of CO_2 and large quantities of fly ash as waste products, which causes serious ecological problems. Our study shows a higher-strength and more durable eco-efficient geopolymer material can be developed by incorporating transformed *Bacillus subtilis* bacterial cells (at a concentration of 10^5 cells/ml alkali activator solution) in 100% fly ash. The newly developed 100% fly ash-based geopolymer can be used as cement alternative for construction purposes which will be more sustainable at higher temperature. The spore-forming ability of the *Bacillus subtilis* bacterium will be an added advantage as it will remain active within the geopolymer matrices and provide the desired effect for quite long time. The use of newly developed geopolymer in construction purposes will certainly improve the ecological footprint because it will reduce the cement and fly ash-related environmental pollution to some extent.

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Compliance with ethical standards

Conflict of interest There is no conflict of interest of any kind related to this work.

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Bacterium amended 100% fly ash geopolymer

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Bacterium amended 100% Fly ash geopolymer

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Abstract. The production of cement results in the release of a significant amount of solid waste materials and gaseous emissions. Actually this industrial sector is thought to represent 5–7% of the total CO2 anthropogenic emissions. In addition to the generation of CO_2, the cement manufacturing process produces millions of tons of the waste product cement kiln dust each year contributing to respiratory and pollution health risks. All significant environmental impacts in their life cycle can be addressed by evaluating the Life cycle assessment (LCA) of the processes or products on the environment. It have been estimated the green house gas emissions from Portland cement concrete pavement construction where they have shown that the main greenhouse gas is CO_2 , which accounts for more than 98% of the total emissions in the process. Fly ash is a coal combustion by-product that leads to many environmental problems like ground water contamination, spills, heavy metal contamination etc. The release of considerable amount CO_2 by cement industries and large quantity of fly ash by thermal power plants are both undesirable for environment. A geopolymer material was developed by incorporating genetically-transformed *Bacillus subtilis* bacterium to an alkali- activator-treated 100% Fly ash to minimize environmental pollution. The use of bacteria in concrete is associated with mineral precipitation (Calcium carbonate and Gehlenite) that helps to fill micro pores and cracks thus reducing its permeability and increasing its strength and durability. The self-bioremediation in bacterial amended geopolymer material is one of the most interesting avenues relating to damage management and self-life of constructions, which needs to be cogitated. The self-bioremediation of bacterial amended thermostable geopolymer material has been explored in this work. The main challenge of this study is to development of ambient temperature curing geopolymer mortar with the addition of transformed *Bacillus subtilis* bacterial cells and spores followed by self-healing. Artificial cracks were generated within the mortar samples by applying partial breaking load (50 %) and the samples were cured for different days. Image analysis by Crackscope and microstructure analysis by field emission scanning electron microscope ascertained the formation of irregular crystalline healing material within the cracks of the test samples. Increase of ultrasonic pulse velocity and compressive strength, augmentation of sulphate resistance, decrease of chloride permeability and water absorption capacity revealed that there were overall improvement of mechanical properties and durability of the bacteria-incorporated mortar samples compared to the control (without bacteria incorporation) mortar samples. This cost effective and eco-friendly self-bioremediation phenomenon observed in geopolymer mortar is evolved due to the biosilicification activity of bioremediase protein which is being formed by the transformed bacteria. The exceptional potential of the microbial bioremediase protein for self -bioremediation attribute may add a new dimension in self-healing construction technology in near future.

INTRODUCTION

Production of cement results in huge amount of CO2 emissions and tons of the waste product cement kiln dust each year contributing to respiratory and pollution health risks and global warming [1]. Fly ash, a coal combustion product also leads to many environmental problems like ground water contamination, spills, heavy metal contamination etc. [2]. Due to the increase in constructions and increased amount of cement usage day by day,

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several researchers have tried to partially replace the cement with cement alternatives such as fly ash, slag, rice husk ash etc. in order to reduce the CO2 emissions that is being generated during cement production [3]. Moreover geopolymer needs heat activation in order to gain high mechanical strength due to polymerization process [4]. Cracks are intrinsic concrete characteristics and cracking can endanger the durability of a structure, because it eases the ingress of aggressive gasses and liquids. Scientists inspired by nature have created self-healing concrete able to self-repair as a result of the metabolic activity of bacteria [5]. In this work, a spore forming genetically enriched bacterium (Bacillus sp.) has been used which have been transformed with biosilicification gene (bioremediase-like protein gene) of a facultative anaerobic hot spring bacteria BKH2 [6]. Our aim is to develop a bacterium (alkaliphilic spore forming genetically enriched Bacillus sp.) amended geopolymer material that can be used as a cement alternative in future construction purposes which can be cured at ambient temperature and will be having self-healing attributes.

LITERATURE REVIEW

There are limited literatures available on eliminating the short comings of ambient temperature (about 27 ± 2 °C) curing as due to slow polymerization process it leads to poor strength of the geopolymers. Adak et al. (2014) have demonstrated the ambient temperature curing of Fly ash based geopolymer with usage of different concentrations of nano-silica. Sarkar et al. (2015) have investigated on structural behavior and self-healing attributes of bio-mortars prepared by incorporating genetically enriched bacteria to cementitious material.

METHODOLOGY ADOPTED

Materials

Low Calcium Class F dry fly-ash was obtained from the National Thermal Power Corporation Ltd; Farakka Plant in India. Ordinary Portland Cement 43 grade [8] and standard Ennore sand [9] were used for the study. The bacterial strain i.e., transformed Bacillus subtilis was obtained from our laboratory stock culture [6]. The bacterium was grown in a specific mineral media (pH 10.0) [6]

Mix proportion and curing

There were 4 categories samples (1- OPC mortar; 2- OPC mortar + bacteria; 3- Fly ash geopolymer and 4- Fly ash geopolymer + bacteria) prepared for the study. Mixing proportions of 4 categories are shown in Table 1.

TABLE 1. Mixing ratio of different category samples

Sample Category	Fly ash /Cement : Sand	Activator Fluid : Fly ash	Water : Cement	Bacterial cells/ml	Curing conditions
OPC	1:3	Nil	0.4	Nil	Water curing
OPC + bacteria	1:3	Nil	0.4	10^5	Water curing
Fly ash geopolymer	1:3	0.4	Nil	Nil	Ambient temperature curing.
Fly ash geopolymer + Bacteria	1:3	0.4	Nil	10^5	Ambient temperature curing

Mechanical strength and Ultrasonic pulse velocity (UPV) study

After respective curing for 28 days, the ultrasonic pulse velocity and compressive strength of all the mortars were determined. The ultrasonic pulse velocity was determined by using PUNDIT plus PC 1007 UPV machine, UK Make as per ASTM C597-02 [10]. The compressive strength of each UPV-tested sample was measured by using Digital

Compression Test machine, India Make. After 28 days of respective curing, the average UPV and breaking load of each category mortars (n=10) were measured. After measuring the breaking load, 50% of the corresponding breaking load was applied to the rest bio-mortars (n=10) of each category samples. The maximum breaking load applied to the specimen was recorded to calculate

Durability test

Water absorption capacity was determined as per Neville's method to the 50% breaking load applied mortars [11] and sulphate and acid resistance test was carried out as per the guideline of ASTM STP663 [12]. For rapid chloride ion permeability test (RCPT), test was done as per ASTM C1202 [13]

Statistical analysis

There were 4 categories samples (20 samples in each) as described in methodology section were prepared. Each experimental data were presented as average ± S.D. Each set of experiment was repeated at least three times.

RESULTS

Compressive test and UPV test results shows an increment after application of 50% load in case of bacterial amended geopolymer mortars as shown in Tables 2 and 3. The durability of the bacterium incorporated geopolymer specimen is also increased as reflected by the results of water absorption (Table 4), sulphate resistant (Table 5) and chloride permeability (Table 6) and acid resistance tests (Table 7).

TABLE 2. Compressive Strength of Bacteria incorporated mortars

Samples	Compressive Strength (MPa)					
	28 Days	50% Load	3 Days	7 Days	14 Days	28 Days
OPC	21.6 ± 0.67	10.8 ± 0.9	11.5 ± 1.2	12.00 ± 1.0	12.05 ± 0.91	13.00 ± 1.5
OPC + Bacteria	24.5 ± 1.35 (13.4↑)	12.25 ± 1.2	13.3 ± 0.86 (15.6↑)	15.6 ± 1.0 (30↑)	17.5 ± 1.2 (45.2↑)	20.0 ± 1.2 (53.8↑)
Fly ash	34.5 ± 1.63	17.25 ± 1.09	18.0 ± 1.2	18.5 ± 0.95	19.0 ± 0.9	19.89 ± 1.0
Fly ash + Bacteria	40.11 ± 1.11 (16.2↑)	20.00 ± 0.98	22.5 ± 0.85 (25.0↑)	27.2 ± 1.2 (47.0↑)	30.8 ± 1.0 (62.1↑)	34.0 ± 1.6 (70.9↑)

TABLE 3. Ultrasonic Pulse Velocity Results of Bacteria incorporated mortars

Samples	Velocity (km/sec)					
	28 Days	50% Load	3 Days	7 Days	14 Days	28 Days
OPC	3.7 ± 0.18	1.85 ± 0.9	2 ± 0.9	2.2 ± 0.8	2.2 ± 1.2	2.3 ± 1.0
OPC + Bacteria	3.8 ± 0.35 (2.7↑)	1.9 ± 0.17	2.2 ± 1.1 (10.0↑)	2.5 ± 0.9 (13.6↑)	2.7 ± 0.9 (22.7↑)	3.2 ± 0.8 (39.1↑)
Fly ash	4.2 ± 0.9	2.1 ± 0.4	2.8 ± 0.8	2.8 ± 0.6	2.9 ± 1.0	3.0 ± 1.0
Fly ash + Bacteria	4.58 ± 0.7 (9.0↑)	2.29 ± 0.3	3.5 ± 1.0(25.0↑)	3.9 ± 0.7 (39.2↑)	4.0 ± 1.0 (37.9↑)	4.2 ± 0.9 (40.0↑)

TABLE 4. Water absorption test of Bacteria incorporated mortars

Samples	Mass (gms)				
	Initial mass	Mass after 30 minutes	% increase	Mass after 24 hours	% increase
OPC	740 ± 3.67	752 ± 4.6	1.6	763 ± 6.2	3.1
OPC + Bacteria	725 ± 6.3	735 ± 4.2	1.3	744 ± 3.8	2.6
Fly ash	731 ± 5.2	743 ± 5.3	1.6	750 ± 7.1	2.5
Fly ash + Bacteria	716 ± 6.9	724 ± 3.5	1.1	732 ± 4.2	2.2

TABLE 5. Sulphate Resistance test of Bacteria incorporated mortars

Samples	Mass (gms)		
	Initial mass	Mass at 120 days	% Increase of mass
OPC	740 ± 3.67	764 ± 5.9	3.2
OPC + Bacteria	725 ± 6.3	746 ± 4.6	2.8
Fly ash	731 ± 5.2	755 ± 5.1	3.2
Fly ash + Bacteria	716 ± 6.9	729 ± 5.4	1.8

TABLE 6. Rapid Choride Permeability test of Bacteria incorporated mortars

Samples	Charge (C)
OPC	4590 ± 63.90
OPC + Bacteria	2675 ± 77.50 (↓41.7)
Fly ash	2450 ± 53.41
Fly ash + Bacteria	1520 ± 62.65 (↓37.9)

TABLE 7. Acid Resistance (4 weeks of acid curing) of Bateria incorporated mortar

Samples	Compressive Strength (MPa)					
	28 Days	50% Load	3 Days	7 Days	14 Days	28 Days
OPC	12.96 ± 0.98	6.48 ± 0.9	7.2 ± 1.0	7.3 ± 1.2	7.5 ± 0.91	8.0 ± 1.5
OPC + Bacteria	16.21 ± 1.25 (25.0↑)	8.10 ± 1.0	9.1 ± 0.86 (26.3↑)	10.7 ± 0.87 (46.5↑)	11.5 ± 1.0 (53.3↑)	13.0 ± 1.2 (62.5↑)
Fly ash	26.0 ± 1.43	13.0 ± 1.0	15.0 ± 1.2	16.5 ± 0.9	17.0 ± 0.9	18.89 ± 1.0
Fly ash + Bacteria	36.1 ± 1.0 (38.8↑)	18.05 ± 0.98	19.2 ± 0.85 (28.0↑)	24.5 ± 1.2 (48.4↑)	27.4 ± 1.0 (61.1↑)	31.9 ± 1.6 (68.87↑)

CONCLUSIONS

This study shows a higher strength and more durable geopolymer material can be developed by incorporating transformed Bacillus subtilis bacterial cells (105 cells/ml) with self healing attributes. The newly developed 100% Fly ash based geopolymer with autonomous bioremediation property can be used as cement-alternative for construction purposes. The spore forming ability of the Bacillus subtilis bacterium will be an added advantage as it will remain active within the geopolymer matrices and provide the desired effect for quite long time.

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

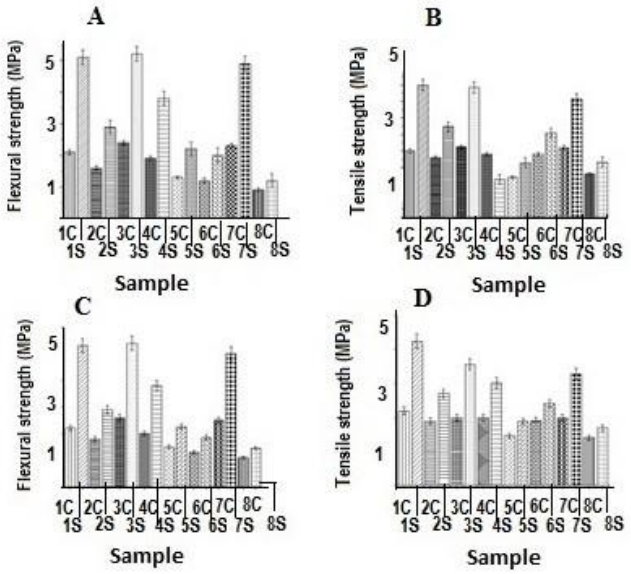

Figure 1. Mechanical strength analysis of bacterial incorporated geopolymer cured at different curing conditions. (A) Flexural strengths of self-healed samples, (B) Split Tensile strengths of self-healed samples, (C) Flexural strengths of crack-repaired samples, (D) Split Tensile strengths of crack-repaired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

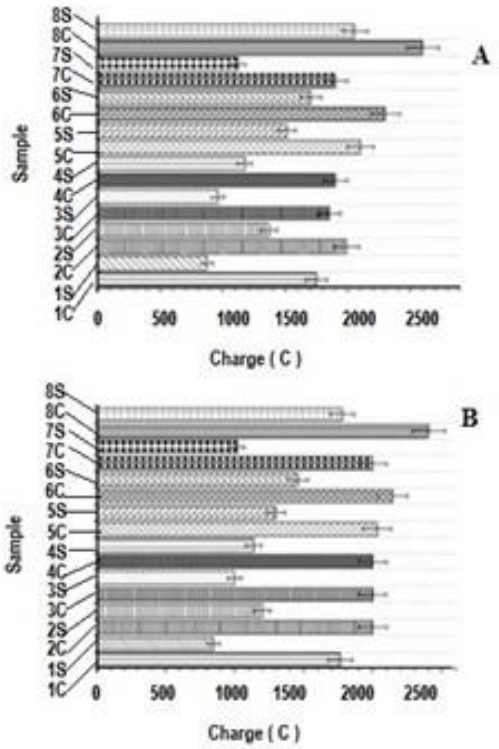

Figure 2. Rapid chloride permeability test results of bacterial incorporated geopolymer cured at different curing conditions: (A) Self-healed geopolymer mortar samples, (B) Crack-repaired geopolymer mortar samples.

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).

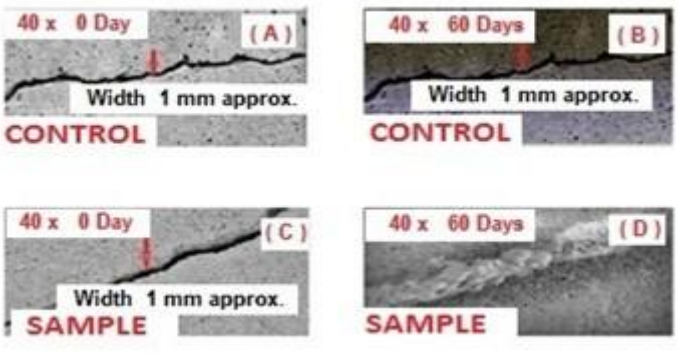

Figure 3. Crackscopic image of self-healed bacterial incorporated geopolymer mortar ample.

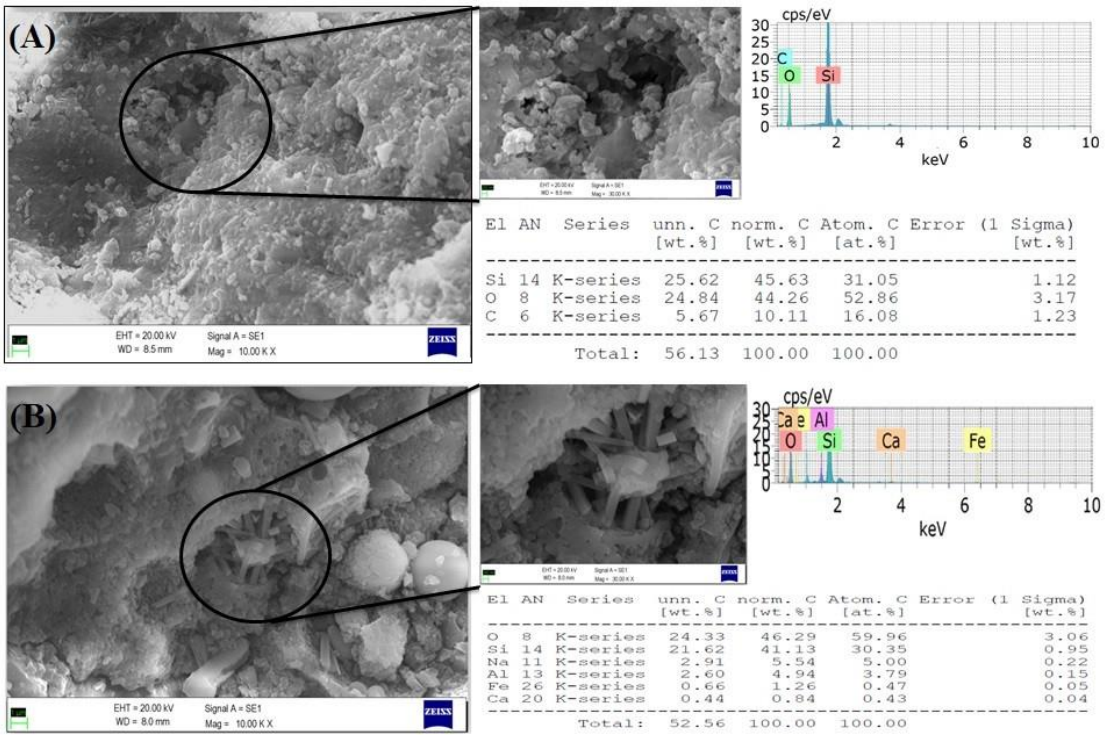

Figure 4. Microstructure analysis of geopolymer samples cured at ambient temperature air curing: (A) FE-SEM image and EDAX analysis of control geopolymer mortar, (B) FE-SEM image and EDAX analysis of bacterial cells amended geopolymer mortar.

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

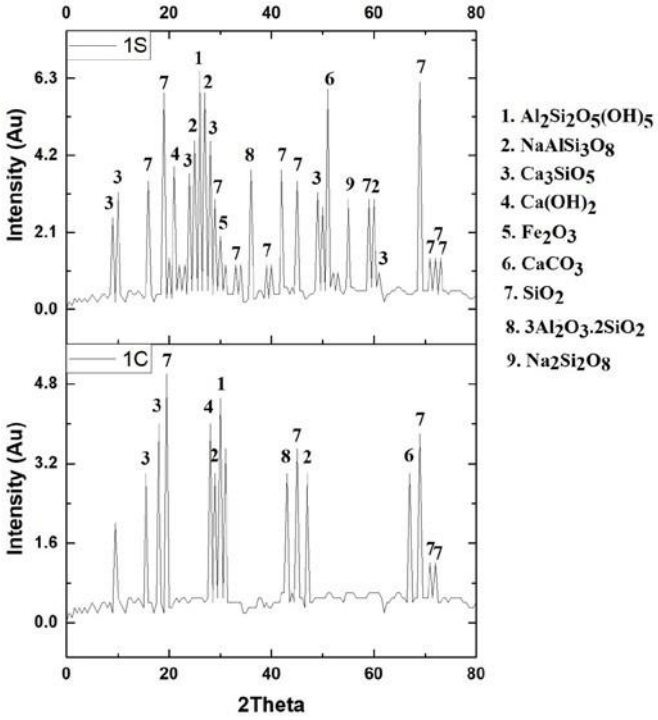

Figure 5. X-ray Diffraction study of geopolymer mortar cured in ambient temperature air curing: (1C) XRD analysis of control mortar sample, (1S) XRD analysis of bacterial cells amended mortar sample.

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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ORIGINAL ARTICLE

An alkaliphilic bacterium BKH4 of Bakreshwar hot spring pertinent to bioconcrete technology

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Abstract

Aims: Hot springs have always drawn attention due to their unique chemical richness and the presence of different microbial communities. The use of hot spring bacteria in concrete technology is our primary focus; isolation of an alkaliphilic bacterium from the Bakreshwar hot springs having longer survival and better efficacy towards cementitious environment was the basis of our study's design.

Methods and Results: A novel facultative anaerobic and highly alkaliphilic bacterial strain (BKH4; GenBank accession no. KX622782) belonging to the family 'Bacillaceae' and homologous (99%) with *Lysinibacillus fusiformis* was isolated from Bakreshwar hot springs. The isolated coccoid-type Gram-positive bacterium grows well in a defined semi-synthetic medium (pH 12·0 and 65°C). This bacterium survives for more than a month and shows better efficacy in enhancing compressive strengths (>50%), ultrasonic pulse velocity (>25%) and durability of the cementitious mortar when incorporated at a concentration of 10^4 cells per ml of water used.

Conclusion: The novel bacterium BKH4 is more effective for the enhancement of the bioconcrete properties.

Significance and Impact of the Study: BKH4 bacterium will add a new dimension to future concrete technology for its usefulness in strength enhancement and durability due to its alkaliphilic nature and longer survival within a cementitious environment.

Introduction

Micro-organism-incorporated modified mortar/concrete is a composite material which not only possesses higher compressive strength but is also more durable in nature (Hoffmann *et al.* 2006; Sarkar *et al.* 2014). It also exhibits a self-healing attribute due to the augmentation of a new phase within the concrete/mortar matrices. As the bacterial cell wall is anionic in nature, so various metallic accumulations (e.g., calcite) occur on the cell surface and substantial deposition on the cell surface makes the cell crystalline and they eventually plug the pores and repair the cracks in mortar/concrete (Ramakrishnan *et al.* 2005). Experimental observations of Kim *et al.* (2001) and Rodriguez-Navarro *et al.* (2003) have shown that there are some aerobic bacteria (*Bacillus pseudomonas and Bacillus pasturi*) which induce calcite precipitation inside the cracks and increase the strength and durability of the structure when injected into the concrete crack. There are number of studies showing the potential application of some specific micro-organisms in concrete technology, for example, cleaning of concrete surfaces (Couturier *et al.* 2005), improvement of mortar/concrete's compressive strength (Hoffmann *et al.* 2006). Moreover, bacterial treatment of degraded limestone, ornamental stone and concrete structures for durability improvement has been one of the key topics for a number of recent studies (Nicol and Macfarlane Dick 2006).

A hot spring provides favourable conditions for the development of microbial mats, which contain physiologically and phylogenetically different groups of prokaryotes depending on the temperature, pH and some other environmental conditions (Stal and Caumette 2013). Natural hot springs are distinguished by the minerals dissolved in

water and give rise to the development of different bacterial communal mats (Noffke 2010). Bakreshwar, located at 23·88°N 87·37°E in the Birbhum district of West Bengal, India, contains nine hot springs harbouring different types of thermophilic and hyperthermophilic microbial communities (Ghosh 2016).

The silica-leaching attribute of some of the mystifying bacteria present in the cluster of hot springs at Bakreshwar has provided some significant advancement in construction technology. Researchers have demonstrated the strength and durability increment properties along with the self-healing attribute of Bakreshwar hot spring bacteria (BKH1 and BKH2) in mortar samples due to the deposition of Gehlenite (calcium-aluminium-silicate) within the cementitious matrices (Ghosh *et al.* 2008; Majumdar *et al.* 2012; Sarkar *et al.* 2014, 2015). Unfortunately all of those isolated hot spring strains grow at pH 8·0 and survive only for 10–15 days within the cementitious matrices owing to very high alkaline environment. A long-term effect particularly for the self-healing attribute is not possible while incorporating the isolated bacteria (BKH1 and BKH2) within the cementitious material. However, Sarkar *et al.* (2015) have shown that the spore-forming *Bacillus subtilis* bacterial strain when genetically transformed by incorporating the bioremediase-like protein gene, can survive for quite a long time within the cementitious environment and provides a true self-healing attribute to the cementitious samples. The transformed Bacillus bacterium needs adequate food to grow from sporulation stage to vegetative form which may affect the strength of the cementitious samples. With this background in view, an attempt has been made to isolate a novel bacterial strain (BKH4) from one of the hot springs of Bakreshwar, West Bengal, India, which would not only possess better strength increment property but also survive at a very high pH (12·0) similar to that of the cementitious environment for a quite longer time.

Materials and methods

The crude soil specimen was taken out along with some water from one of the hot springs in Bakreshwar (65°C) and cultured in our laboratory by using a specific synthetic medium (pH 12) as described by Ghosh *et al.* (2009). The culture was maintained by subculturing the mixed population culture on a regular basis. A purified bacterial strain was obtained from the mixed population culture through the serial dilution technique. The 16S rRNA gene sequences for identification, the optimum medium's pH and temperature for the growth condition, the morphology and Gram's staining for phenotypic characters etc. of the isolated strain (designated as BKH4) were determined to characterize the bacterium.

Phylogenetic study of the pure strain

Genomic DNA was isolated from the bacterial cells. The ~1·5-kb 16S rRNA gene fragment was amplified using high-fidelity PCR polymerase. The PCR product was cloned at the Not I site in pBlueScript vector. Positive clones were screened by colony PCR. The clones were sequenced bidirectionally using the forward and reverse primer. The 16S rRNA genes were amplified by using the primer 5^0-GACTTGCATGTGTTAGGCCTG-3^0. The partial 16S rRNA gene sequence was determined by the dideoxy chain termination method using the Big Dye Terminator ver. 3·1 and ABI 3500 Genetic Analyzer (Applied Biosystem Micro Amp Optical 96-Well Reaction plate, ThermoFisher Scientific, Waltham, USA). To construct a phylogenetic tree, the Neighbor sequence aligned method was used. A distance matrix was generated using the Jukes–Cantor corrected distance model (Price *et al.* 2009). The sequence similarity was searched on (BLAST) and phylogenetic analysis was done by the neighbour-joining method where distances between sequences were determined by Kimura's two-parameter model (Altschul *et al.* 1997). The confidence limits of the branching were performed by Bootstrap analysis. The details of phylogenetic analysis is given in Supplementary section (S1).

Morphological identification of the strain

For scanning electron microscope (SEM), slides were prepared by fixing bacterial cells with 2·5% (v/v) gluteraldehyde for approximately 24 h at room temperature. Samples were dehydrated by incubation at 65°C for 15 min, air-dried and transferred onto SEM alumina supports and sputtered with gold. Slides were observed under SEM (JEOL-JSM 5200) and photomicrographs of bacterial cells were taken.

To visualize the external appearance of bacteria through inverted fluorescence microscope, one drop of anaerobic bacteria cell culture was placed on the slide, air-dried, covered with a cover slip and bound with paraffin on both the sides. Slides were observed under the fluorescence microscope of LEIKA DFCC450C, Model DMI8 manual (SIN 446714) under a DAPI (emission wavelength: 417–477 nm and excitation wavelength: 352– 402 nm) filter at 20X resolution and photomicrographs of bacterial cells were taken.

Optimization of bacterial growth pH and growth temperature

The growth curve of the bacterium BKH4 at different pHs of the medium (6–13) at 65°C was plotted. Sufficiently grown bacteria culture of 1 ml (10^7 cells per ml) was

inoculated to each culture vials containing semi-synthetic medium having different pH and incubated at 65°C for several days. Three millilitres of bacterial culture was taken out from each vial on each alternate day and their optical density was measured at 620 nm against a blank medium. For each pH of the growth medium, at least three cultures were prepared. A growth curve for 10 days of incubation was plotted (OD *vs* pH). Similar growth curves were drawn for the bacterium at three different temperatures (42, 50 and 65°C).

Compressive strength and ultrasonic pulse velocity of bacteria-incorporated mortar samples

Standard mortar cubes (70·6 mm 9 70·6 mm 970·6 mm) were prepared by mixing different bacterial cell concentrations (10^2–10^7 cells per ml of water used) with cement–sand mixture (1 : 3 w/w ratio) as described by Ghosh *et al.* (2005). Ordinary Portland cement (43 grade; IS 8112: 1989) (IS 8112, 1989) and standard Ennor sand (IS650-1991) (IS650, 1991) having a well-graded coarse aggregate with a maximum size of 10 mm were used for sample preparation. The water to cement ratio was taken as 0·4 (w/w). The samples without bacteria (control) and with bacteria (experimental) were cured under water at ambient temperature for different days. Ultrasonic pulse velocity (UPVs) of each sample was measured first by using Pundit plus meter (PC1007) according to the standard test method (A.S.T.M. Norm C597, 2002) and then the compressive strength of the samples was measured. Each experiment was repeated three times with five samples each and average data SD (n = 15) was presented.

Water absorption capacity

After 28 days of water curing, the mortar samples were air-dried at room temperature for 24 h and their initial masses were recorded. The samples were then cured under deionized water for 30 min, cleaned with tissue paper and their masses (wet mass) were recorded immediately. Then, samples were kept again under water for 24 h. After that the samples were removed, cleaned with tissue paper and their wet masses were measured. Water absorption capacity of the samples was determined by using those weights as per Neville's method (STP663 A.S.T.M, 1977).

Sulphate resistance capacity

Masses were registered after 28 days of water curing, from each category of the respective samples. Then, the samples were immerged in sulphate solution (5% MgSO4, pH 7·0 in deionized water) and cured for a further 90 days. After curing, the samples were removed from the solution, air-dried and their masses were determined. The test was performed according to the guide line of ASTMC1012 (Neville 2011).

Rapid chloride permeability test

Mortar cylinders (100 mm diameter and 200 mm height) were prepared by using cement–sand mixture along with the bacterial cells at a concentration of 10^4 ml^{-1} water. The cement to sand ratio and water to cement ratio was kept the same as described earlier. The cylinder was immerged under deionized water for 28 days. Then three small cylinders (100 mm diameter 9 50 mm height) were cut from the original cylinder prepared earlier. The samples were epoxy coated along with theirs edges and left under water for 24 h before measuring their chloride ion permeability using a rapid chloride ion penetration cell. The test was done on three samples for each category as per ASTM C1202 (ASTM C1202, 2000).

Microstructure analysis of bacteria-incorporated mortar samples

After measurement of compressive strengths (28 days water curing), fragmented mortar samples with and without bacteria were crushed into dust powder and examined under SEM (INSPECT F50 SEM, FEI Europe BV, Eindhoven, the Netherlands). Energy-dispersive spectra analysis was also done by using QUANTAX ESPRIT 1.9 software. For X-ray diffraction (XRD) analysis, dry powder samples were sieved (5 1m) to obtain uniform particle size and examined in powder XRD (Bruker AXS Inc., Model D8, WI) at 40 kV with a scan speed 0·2 s per step. The XRD spectrum was taken from 2h = 20° to 80°. The peaks in the new positions of the spectrum were marked and detected from the JCPDS data File (JCPDS ASTM, 1941).

Survivability of the bacterium within mortar specimen

Fragmented mortar samples of different days of water curing (3–30 days) were collected and crushed into powder form. A pinch of powder sample was added to the culture vial containing the bacterial growth medium (pH 12·0) and kept in an incubator at 65°C for several days to observe the growth of the bacterium as described in the section of optimization of bacterial growth pH and temperature. The growth was monitored by measuring the optical density of the cultured medium as demonstrated earlier.

Statistical analysis

For each category of testing, five samples were prepared. Each experiment was repeated at least three times. Data were presented as an average (n = 15 samples) with SD.

Results

The isolated pure bacterium (BKH4) is shown in Fig. 1a, b. The coccoid-like morphology of the bacterium is depicted in Fig. 1a. The dimension of the bacterium was 1·5–3·1m long (Fig. 1b). The bacterium also exhibited fluorescence property when examined under a fluorescence microscope under the DAPI filter. Gram staining suggested that the bacterium was Gram positive. The growth curve showed that the bacterium grew well at 65°C (Fig. 2a) and over a wide pH range (Fig. 2b) when grown in the specific semi-synthetic medium. The bacterium was found to be a facultative anaerobic, thermophilic and highly alkaliphilic, whose optimum growth was obtained at pH 12·0 and 65°C (Fig. 2b).

Partial 16S rRNA gene sequences (1335 bp) (GenBank accession number KX622782) clearly suggested that the isolate was a novel bacterium which affiliated with the family 'Bacillaceae' and closest similarity (99·0%) with *Lysinibacillus fusiformis* (Fig. 3). The survival ability of the bacterium within the concrete environment suggested that the bacterium could remain alive for more than a month within the cementitious matrices (A. Sarkar, A. Chatterjee, S. Mandal, B.D. Chattopadhyay, unpublished data).

The bacterium could increase the compressive strength of the cement–sand mortars when incorporated at different cell concentrations as shown in Fig. 4. The maximum strength increment (>50%) was observed at 10^4 cells per ml of water used under the 28-day water curing period. A more than 25% increment of UPV at 10^4 cells per ml of water used under the 28-day water curing period revealed that the bacterium was able to increase the compactness of the incorporated mortars under such

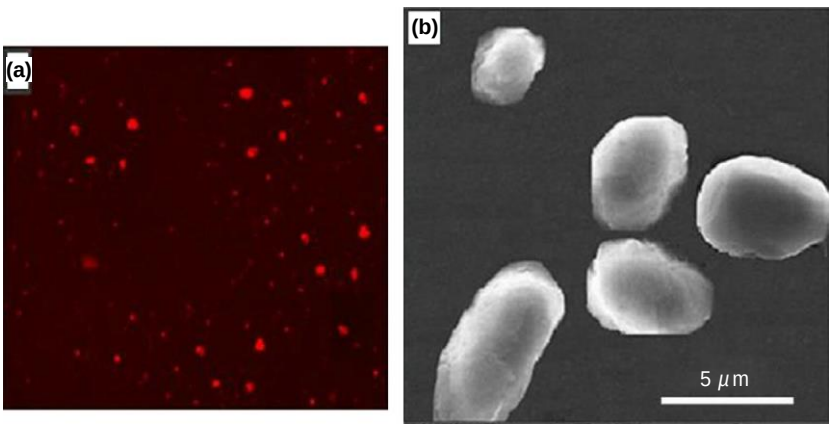


Figure 1 (a) Microscopic view (20X). (b) SEM view of the isolated BKH4 bacterium. [Colour figure can be viewed at wileyonlinelibrary.com]

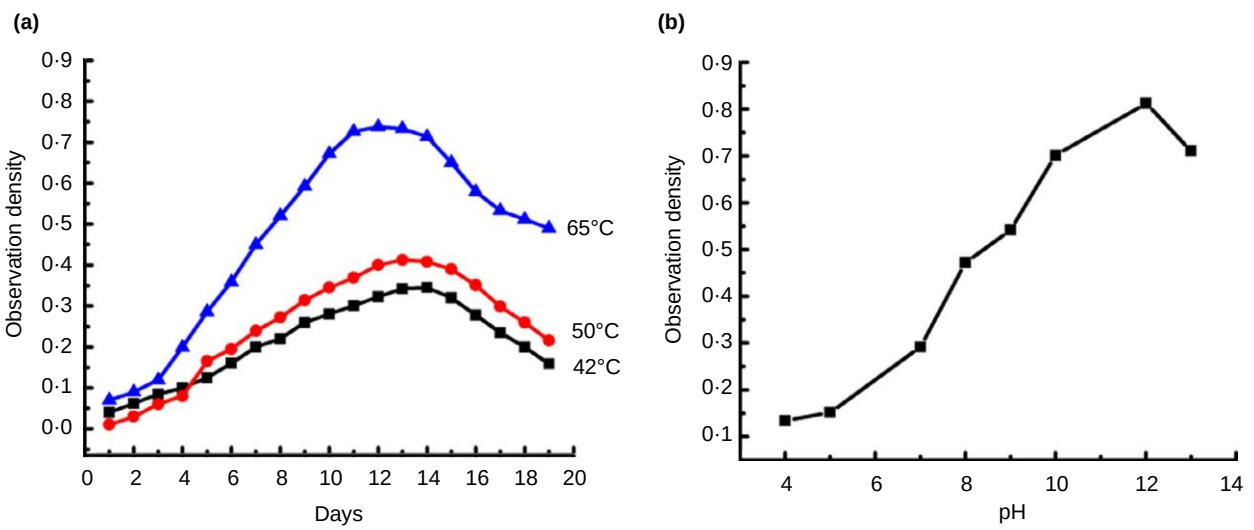


Figure 2 (a) Temperature variation growth curve of BKH4 (n = 3). (b) pH variation growth curve of BKH4 (n = 3). [Colour figure can be viewed at wileyonlinelibrary.com]

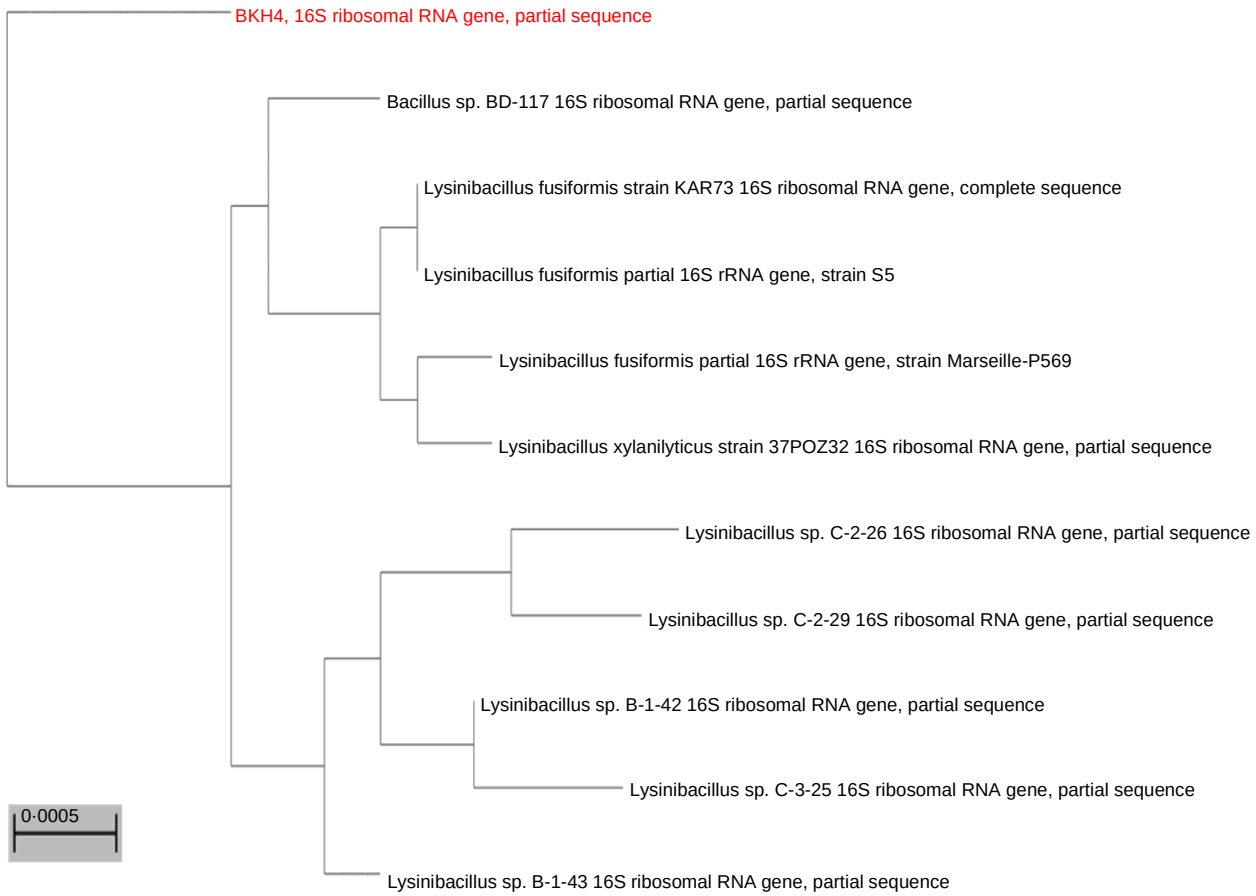


Figure 3 Phylogenetic tree of BKH4 bacterium. [Colour figure can be viewed at wileyonlinelibrary.com]

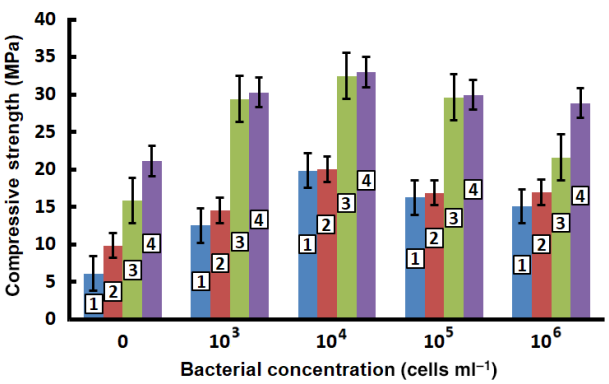


Figure 4 Compressive strength of BKH4 bacterium amended mortar samples at different days of water curing. Where 1: 3 days, 2: 7 days, 3: 14 days and 4: 28 days of curing respectively. [Colour figure can be viewed at wileyonlinelibrary.com]

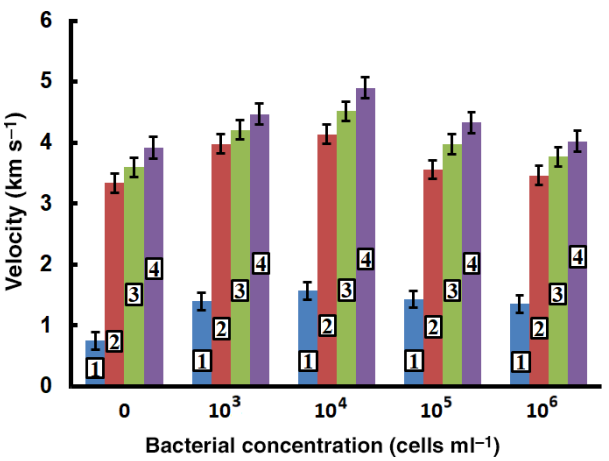


Figure 5 Ultrasonic pulse velocity of BKH4 bacterium-amended mortar samples at different days of water curing. Where 1: 3 days, 2: 7 days, 3: 14 days and 4: 28 days of curing respectively. [Colour figure can be viewed at wileyonlinelibrary.com]

conditions (Fig. 5). The bacteria-incorporated mortar samples were seen to be more water resistant compared to control samples (Table 1). It also showed higher sulphate-resistant activity compared to control mortar samples (Table 2). The result of rapid chloride permeability test (RCPT) suggested that bacterium-incorporated mortars were more resistive against chloride ions' attack than control samples' attack (Fig. 6).

Microstructure analysis of the samples showed that the needle-like structures appeared in the bacterium-incorporated mortars (Fig. 7a) which were absent in control mortars (Fig. 7b). XRD analysis of bacterium-incorporated mortars (inset of Fig. 7a) showed extra new peaks of Gehlenite ($Ca_2Al_2SiO_7$). The EDX analyses of the mortar matrices also suggested the formation of a new phase inside the bacterium-incorporated mortar matrices (Fig. 7a).

Discussion

Micro-organism-modified mortar/concrete is now an important area of research where scientists are searching for different microbial communities to incorporate them into mortar/concrete matrices for the enhancement of its overall properties. Although this technique shows some positive effects on compressive strength, durability, crack repairing ability and self-healing attribute, there are, however, several limitations that restrict the usage of these micro-organisms for practical construction purposes. For example, some bacteria need food to grow inside the cementitious matrices which reduces the strength of the sample. Some cannot grow within a high alkaline cementitious environment. Keeping all these facts in mind, a novel highly alkaline, water-grown bacterium (BKH4) has been isolated from a hot spring in Bakreshwar which may overcome some limitations of this study.

The isolated bacterium has been found to be coccoid in morphology, Gram positive and possesses fluorescence property (Fig. 1a,b). The culture condition reveals that the bacterium is a facultative anaerobic that grows under anaerobic conditions but survives under aerobic conditions. The optimum growth temperature at 65°C and pH at 12·0 implies that the isolated BKH4 strain from a thermophilic and highly alkaliphilic micro-organism. Several bacterial strains (e.g. BKH1, BKH2, BKH3, etc.) have been isolated from Bakreshwar hot springs previously, but none of them were found to be highly alkaliphilic as reported earlier (Sarkar *et al.* 2014, 2015; Chaudhuri *et al.* 2016). The growth of the bacterium can be revived from a 30-day-old bacterium-incorporated mortar sample which indicates that the isolated bacterium is able to survive more than a month within the cementitious environment. The intramatrix environment of cementitious concrete/mortar is highly alkaline (pH 13–14) which restricts the growth of several bacteria. Also some acid-producing bacteria alter the high alkaline environment of the cementitious material which affect the strength and durability of the structures (Dong *et al.* 2018). Incorporation of BHH4 will be suitable for strength and durability enhancement of the cementitious materials due to its highly alkaliphilic nature. This is encouraging as previously isolated bacteria from a Bakreshwar hot spring failed to survive for more than 10 days in a concrete environment (Sarkar *et al.* 2014, 2015). The phylogenetic tree based on partial 16S rRNA gene sequences of the isolated strains clearly indicates that the bacterium is a novel one which is closely associated with the *L. fusiformis*, under phylum 'Bacillaceae' (BKH4; GenBank accession no.: KX622782). *Lysinibacillus fusiformis* tests positive for oxidase and is an obligate aerobe (Hendricks *et al.* 2009). The bacterium, *L. fusiformis* can hydrolyse urea to produce ammonia and CO_2 due to the presence of urease gene (Benita 2014). *Lysinibacillus fusiformis* ZC1 showed resistances to multiple metals (Cu, Ni, Co, Hg, Cd and Ag) and a metalloid (As) (He *et al.* 2011). Our isolated strain BKH4 also can reduce magnesium and grow well in the presence of magnesium ions in the medium (A. Sarkar, A. Chatterjee, S. Mandal, B.D. Chattopadhyay, unpublished data).

The bacterium BKH4 possesses an effective compressive strength increment property because more than 50% of the compressive strength increment is noted by using the BKH4 bacterial cells at a concentration of 10^4 cells per ml of water used (Fig. 4). Previous studies showed that 25–40% compressive strength could be increased by incorporation of BKH1 and BKH2 bacteria within the cementitious mortar/concrete (Ghosh *et al.* 2009; Sarkar *et al.* 2014). The increase in the strength of BKH4 bacteria-incorporated mortars is due to the development of new filamentous material within the mortar matrices, that is, formation of Gehlenite material as revealed by XRD analysis which supports our earlier findings (Sarkar *et al.* 2014). EDX analysis similarly shows that there is remarkable

Table 1 Water absorption test of mortar samples

Bacterial cells per ml of medium	Initial mass (g)	Mass after 30 min (g).	% of increasing	Mass after 24 h (g)	% of final increment
0	751·00 1·35	765·75 2·17	1·96	776·75 2·69	3·42
10^2	755·00 2·38	757·50 1·44	0·33	765·50 2·10	1·39
10^3	754·00 2·45	755·00 1·41	0·13	763·50 2·36	1·26
10^4	729·75 0·63	734·00 1·35	0·58	738·00 1·35	1·13
10^5	734·50 0·86	742·50 1·89	1·08	750·00 1·87	2·11
10^6	749·50 1·97	749·50 2·10	1·73	765·50 2·10	2·13
10^7	757·00 3·13	763·75 2·39	0·89	773·75 3·12	2·21

N = 15 for all measurements.

variation in the distribution of silicon atoms within the matrix of bacteria-incorporated mortar samples compared to that of control mortar sample. It was reported earlier that some hot spring bacteria (BKH1 and BKH2) possess biosilicification activity due to the presence of a secretary protein (named bioremediase) in their cell walls (Biswas *et al.* 2010; Sarkar *et al.* 2014; Chowdhuri *et al.* 2015). The catalytic activity of bioremediase can leach silica (SiO_2) from a silicate compound in the form of silica nanoparticles. The released silica reacts with oxides of calcium and aluminium present in the cement form Gehlenite within the cementitious matrices. The bacterium BKH4 is also seen to secrete a similar protein in the growth medium that might have similar role in strength and UPV increment of the mortar samples (A. Sarkar, A. Chatterjee, S. Mandal, B.D. Chattopadhyay, unpublished data). The newly isolated BKH4 bacterium would be a better strain than BKH1 and BKH2 in terms of the compressive strength increment attribute and longer survivability when incorporated within the cementitious mortars.

Table 2 Sulphate resistance test of mortar samples

Bacterial cells per ml of medium	Initial mass at 0 day (g)		Final mass at 90 days (g)		% of increment
Control	751·00	1·35	783·25	1·37	4·29
10^2	755·00	2·38	779·25	1·49	3·21
10^3	754·00	2·45	778·00	1·41	3·18
10^4	729·75	0·63	738·25	1·18	1·16
10^5	734·50	0·86	754·00	1·35	2·65
10^6	736·75	1·97	758·00	1·78	2·88
10^7	757·00	3·13	783·25	1·97	3·46

N = 15 for all measurements.

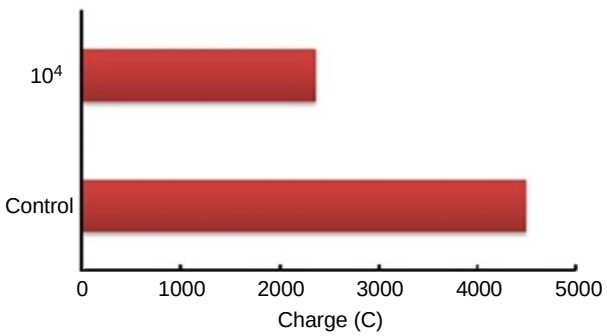


Figure 6 Result of RCPT test of control and bacterium-amended mortar samples. [Colour figure can be viewed at wileyonlinelibrary.com]

Besides the increment of the compressive strength and compactness of the bacteria-incorporated cementitious samples, the durability of the sample in another highly important criterion which provides the longevity of the cementitious material. Durability can be inferred from the experimental results of the water absorption test, sulphate resistance test and RCPT. The experimental findings of the water absorption test (Table 1), sulphate resistance tests (Table 2) and RCPT (Fig. 8) suggest that the bacterium BKH4-incorporated mortar samples are more durable compared to the control mortar samples. The addition of BKH4 cells to the cement–sand mortar shows less water absorption and greater sulphate- and chloride-resistant activities which are maximized at 10^4

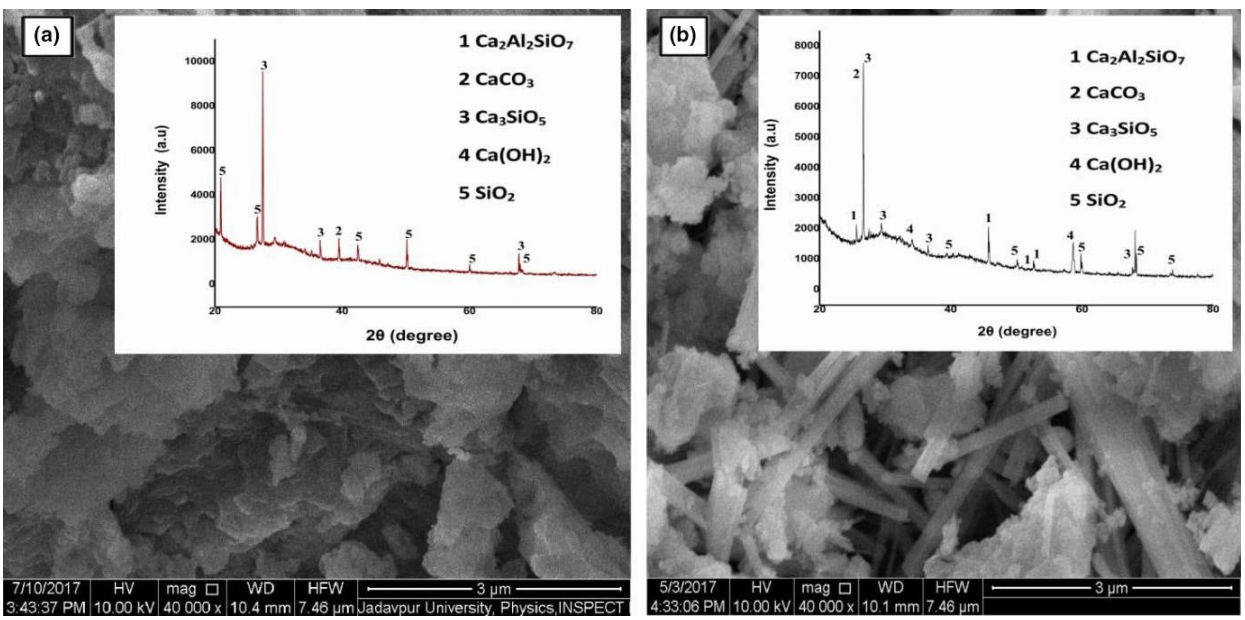
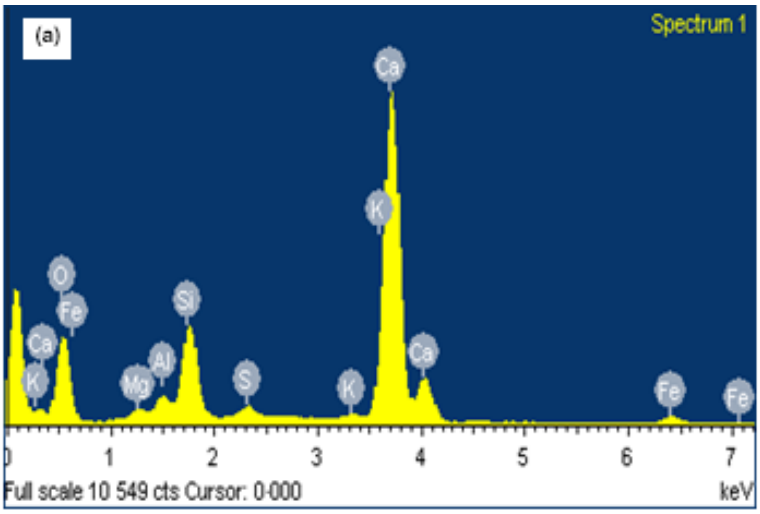
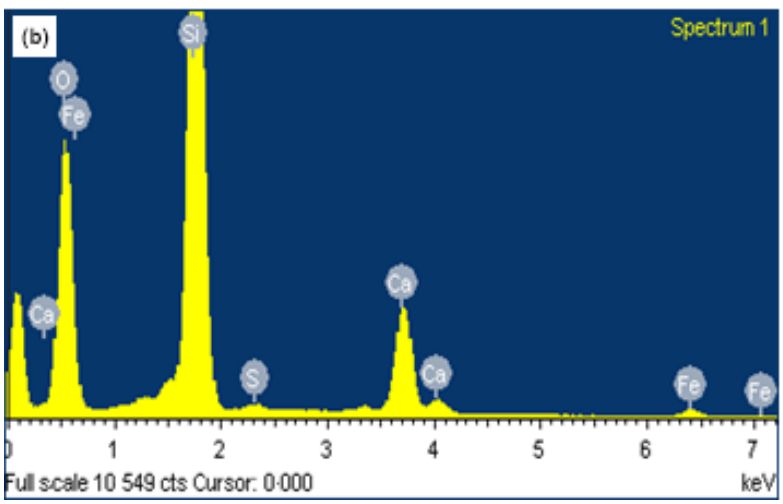


Figure 7 SEM photographs of control mortar matrix (a) and BKH4 bacterium-amended mortar matrix (b); XRD analysis of the matrix are shown in the inset of the corresponding figure. [Colour figure can be viewed at wileyonlinelibrary.com]



Element	Weight%	Atomic%
O K	54·94	73·73
Mg K	0·84	0·75
Al K	1·64	1·30
Si K	6·84	5·23
S K	0·81	0·54
K K	0·43	0·24
Ca K	32·71	17·52
Fe K	1·79	0·69
Totals	100·00	



Element	Weight%	Atomic%
O K	61·88	75·54
Si K	28·87	20·07
S K	0·32	0·19
Ca K	7·81	3·81
Fe K	1·13	0·39
Totals	100·00	

Figure 8 EDX analysis of control mortar matrix (a) and BKH4 bacterium-amended mortar matrix (b). [Colour figure can be viewed at wileyonline library.com]

cells per ml water used. Higher water absorption capacity and lesser sulphate resistance activity damages the concrete structure and affects its shelf life. Similarly, the ingress of enhanced chloride ions deteriorates the mortar/concrete structures and reduces their lifetime. Our results thus demonstrate that thermophilic and highly alkaliphilic BKH4 bacterium would be an ideal and effective micro-organism which would be used for the development of higher strength and more durable concrete/mortar material in the near future.

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Conflict of Interest

The authors declare that there is no conflict of interest of any kind in this work.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Data S1. Phylogenetic analysis (in details).

Evaluation of Self-Healing Attribute of an Alkaliphilic Microbial Protein in Cementitious Mortars

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Evaluation of Self-Healing Attribute of an Alkaliphilic Microbial Protein in Cementitious Mortars

Atreyee Sarkar[1]; Avishek Chatterjee[2]; Trinath Chowdhury[3]; and Brajadulal Chattopadhyay[4]

Abstract: Unavoidable cracks cause a significant reduction in the strength and longevity of concrete. Water and several harmful ions seep through the cracks, initiate corrosion of the reinforcement, and affect the self-life of concrete. Self-repaired concrete will stand for a longer period and thus is gaining interest for constructions purposes. This work was an attempt to design a microbial protein (~28 kDa) that incorporated self-healing cementitious material for future construction needs. The protein was isolated from an alkaliphilic hot spring bacterium (BKH4) of Bakreshwar, West Bengal, India. The prepared control and protein-amended cementitious mortar samples were subjected to simulate cracks and cured under water for several days. Images and microstructures of the control and protein-incorporated samples were analyzed, which established that there was a tiny fingers-like crystalline substance developed on the cracked surfaces. The developed substance was identified as a silicate phase (Gehlenite) by energy dispersive X-ray spectroscopic analysis. The microbial protein enhanced the mechanical strengths and durability of the protein-incorporated samples that were supported by the increments of ultrasonic pulse velocity, compressive strength, and sulfate resistance as well as reduction of water permeability and slow water movement (sorptivity test) of the experimental samples. This self-healing phenomenon is eco-efficient and developed due to the bio-silicification action of the microbial protein that was incorporated in mortar samples. DOI: 10.1061/(ASCE)MT.1943-5533.0004197. © *2022 American Society of Civil Engineers.*

Author keywords: BKH4 bacterium; Cracks; Microbial protein; Mortar; Self-healing.

Introduction

Concrete is one of the most essential construction materials for modern civilization. Unfortunately, the formation of cracks is natural in cement-based structures and weakens the strength and longevity of concrete. Water and several damaging ions usually enter the concrete through the tiny cracks and corrode the steel reinforcement, making the whole structure susceptible. This phenomenon hampers the longevity of concrete structures. According to Schlangen and Joseph (2009), the strength of concrete progressively reduces due to cracks when the first repair is needed. Relatively low tensile strength and not timely repair of concrete structure facilitate the development of various forms of microcracks in concrete. Currently, scientists and civil engineers have focused their approaches toward the self-healing phenomenon of cementitious materials for restoring the mechanical property and longevity of concrete. The self-healing in concrete is a characteristic feature that will prolong the service life of infrastructures and reduce its repairing cost. The microbial self-healing approach particularly prevails the other techniques due to its cost efficacy, competent bonding capacity, and compatibility with concrete compositions (Seifan et al. 2016). Several efforts are explored to ascertain the self-governing repairing activity by incorporating several mineral-producing microbes in the concrete mix, but the efficient self-governing repairing phenomenon through the process of bacteria-assisted bio-mineralization occurs at the initial stage of concretization, as suggested by Chattopadhyay (2020). The reaction between the unused cement and the dissolved carbon dioxide in water results in the formation of calcite (calcium carbonate) within the cracks of concrete structures, which is the primary cause for the self-healing phenomenon in concrete (Guthrie et al. 2017). Sometimes, cementitious properties and environmental conditions endorse a stronger self-healing response. The self-healing event also occurs for a wide range of Ca: Si ratios in cement as well as for various reservoir fluid compositions (Guthrie et al. 2017, 2018). White et al. (2001), and Kessler et al. (2003) did revolutionary work on the self-healing phenomenon of polymeric material using encapsulated chemicals. Zamani et al. (2020) has introduced polyuria material for the encapsulation of bacteria as a self-healing agent in cement paste. Bang et al. (2001) and Rodriguez-Navarro et al. (2003) have shown that inserted microorganisms could stimulate calcite precipitation resulting in the repairing of cracks inside the concrete structures. Some reports state the incorporation of calcite-forming bacteria increases the strength and self-healing performance of building materials by superseding the effect of climatic conditions (Jeong et al. 2017). Renovation of cementitious structures should be dealt sincerely for damage management, as suggested by Han et al. (2017). Wiktor and Jonkers (2011) used mineral-producing concrete-immobilized bacteria for self-healing in concrete. Several research works are going on in the field of self-healing concrete because of the associated huge financial and ecological impacts on construction materials (Sarkar et al. 2015). The biologically induced self-healing phenomenon aims to revive the original characteristics features of concrete by recovering the water stiffness that was lost by cracking (Tziviloglou et al. 2016). Sometimes fibers are used in construction industries to

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enhance the strength and durability of the concrete structure (Vijay and Murmu 2019). Fibers reduce the crack width by a bridging action and bacteria that form a filling material in that bridge portion. Scientists have proposed that self-repairing consists of additional biological and chemical agents to the concrete mix along with shape memory alloys (SMA) to obtain an improved self-healing effect (Insaurralde et al. 2016).

A group of scientists is engaged in developing high-performance self-governing repairing bio-concrete material by using several novel hot spring bacteria. There are different types of bacteria (BKH1, BKH2, BKH3, and BKH4) fished out from hot springs of Bakreshwar, West Bengal, India, and their particular proteins are being used to increase the mechanical strength as well as longevity of cementitious material, as described by Sarkar et al. (2014), Ghosh et al. (2005, 2008, 2009), Majumdar et al. (2012), Chaudhuri et al. (2016), Sarkar et al. (2019), Biswas et al. (2010), and Chowdhury et al. (2015). Concrete is highly alkaline in nature and hence alkaliphilic or alkali-tolerant microbe would be the best choice for bacterial-induced self-healing concrete (Mamo and Mattiasson 2019). In this study, the microbial protein was getting isolated from a highly alkaliphilic (pH 12.0) BKH4 cultured medium and getting used instead of the whole bacterium cells to achieve the desired results. The optimistic consequences of all the previous studies inspired us to develop a novel self-governing repairing mediator by using a bacterial protein that possesses the independent self-repairing character at very high alkaline pH (pH 12.0 to 13.0) and anaerobic conditions. This paper is a clear demonstration that shows the ability of a bacterial protein toward the self-healing phenomenon due to which the simulated cracks in mortar samples are effectively healed by the action of the protein when impregnated in the sample. Several experiments were performed to determine the bacterial protein-associated self-healing phenomenon within the mortar samples, e.g., the measurement of compressive strength, determination of ultrasonic pulse velocity (UPV), sulfate resistive activity, water permeability, and microstructure of cementitious mortar analysis.

Materials and Methods

Bacterium BKH4

The pure strain of BKH4 (Fig. 1) was obtained from our laboratory stock culture. The bacterium was originally isolated from the soil samples taken from the bottom of one of the hot springs (water temperature 65°C) of Bakreshwar, West Bengal, India. The serial dilution technique was applied to get pure BKH4 bacterial strain, as described previously (Sarkar et al. 2019). BKH4 bacterium is closely related to *Lysinibacillus fusiformis* (GenBank Accession No. KX622782). It is a facultative anaerobic and highly alkaliphilic in nature and also possesses an iron-reducing property. It grows well at high pH (pH 12.0) and 65°C temperature, as observed by Sarkar et al. (2019).

The Protein from BKH4 Cultured Medium

The bacterial cells (BKH4) release a small number of proteins in the growth medium during the culture of the bacterium. These proteins were separated from the cultured medium (10-12 days old). The supernatant of the cultured medium was collected by discarding the bacterial cell pellet at the bottom through a centrifugation process (6,000 × g for 10 min.). The supernatant was concentrated by lyophilization (Eyela FDU-1200 Lyophilizer, Tokyo Rikakikai, Japan), and then the protein part of the supernatant was separated using a Sephadex G-100 chromatographic column associated with Eyla Fraction collector (Column length 100 cm and diameter 1 cm;

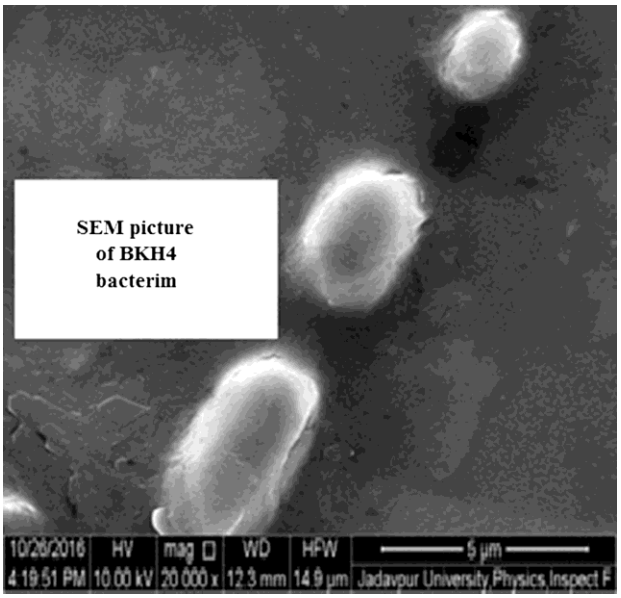


Fig. 1. FESEM image of BKH4 bacterium.

eluted volume 1 mL). There was 100 μL of each eluted fraction used for a biosilicification assay to ensure the fraction that possesses the silica leaching activity as described by Biswas et al. (2010). Those fractions containing the desired protein (having silica leaching activity) were pooled and further concentrated by lyophilization and kept at −20°C for other experimental purposes.

Mortar Sample Preparation

Mortar samples were prepared by using ordinary 43 grade Portland cement [IS 8112: 1989 (BIS 1989)] and locally available sand (specific gravity 2.52, water absorption 0.50%, and fineness modulus of 2.38; sieved by passing through 850 μM IS Sieve). Standard mortar cubes having dimensions (70.6 × 70.6 × 70.6 mm) were cast for all samples preparation as per IS 4031 (BIS 1988). For each experiment, five cubes of each category were cast to achieve the best averaged result. Isolated bacterial protein with three different doses (2, 3, and 4 μg protein/g of cement used) was mixed as the experimental samples. No protein was used for control samples preparation. The cement versus sand ratio was 1:3, and the cement vs. water ratio was maintained at 1:0.4 for all types of samples preparation. All samples were removed from the molds after 24 h of casting and initially kept under water or air for curing for 28 days. After 28 days of initial curing, the samples were used for different experiments. The results of water curing are shown in the manuscript, and the same of air curing are presented in the Supplemental Materials. Except for the microbial protein, no other admixture was used in mortar samples preparation.

Study of Compressive Strength and UPV of Cracked Mortar Samples

A sufficient number of mortar samples for the control category and experimental categories were prepared similarly and cured under water for this study. After 28 days of water curing, the mortar samples were air-dried, and an average breaking load of each category sample (5 in number) was estimated separately. 50% of the estimated average breaking load of particular category samples was applied (at a rate of 0.5 kN=s) to the rest of the corresponding category samples for determining the compressive strength and UPV

of stimulated-crack mortar samples studies. One set of samples (5 samples) from each category was kept under deionized water, and another set of samples (5 samples) from each category was kept in air for different days of curing (3, 7, 14, 28 days). After the curing periods, a digital compressive strength testing machine was used to measure the compressive strength of the samples, and a Pundit plus PC1007 UPV meter was used to determine the ultra-pulse velocity as per ASTM C597-02 (ASTM 2002). UPV measurement was done first and then followed by a compressive strength study.

Self-Healing of Mortar Samples

A sufficient number of control mortar samples and experimental mortar samples were prepared similarly for self-healing study. Only water was used here to cure the samples for 28 days. After the curing period, the samples were taken out from water and dried in air for 24 h. Average breaking loads were similarly determined, and 50% load of particular category samples was applied to the rest of the respective category samples for creating microcracks on the samples. The photographs of the cracks regions were taken and the widths of the individual cracks were measured by Crackscope. One set (5 samples of each category) of the induced-crack mortar specimens were dipped in distilled water in a covered plastic container for 60 days to keep the samples away from oxygen and carbon dioxide circulation during the curing period. Some control mortar specimens (where breaking load was not applied) were also immersed in a solution that contained bacterial protein (0.03 μg=mL water) and similarly kept for 60 days water curing. After the curing period, the samples were removed from water, and photographs of the cracked surfaces were taken. Additionally, the widths of the cracks were measured for comparison.

Microlevel Analysis of Self-Healing Substance

The substance deposited on the induced-crack surfaces of the bacterial protein-impregnated mortars cured for 60 days under water was gathered by scraping the surface with a sharp steel knife and left for air drying. The scraped substance was then made into fine powder by pestle mortars. Substance from the crack region of control samples was gathered in the same manner and treated similarly. The powder samples were examined by FESEM (INSPECT F50 SEM, FEI Europe BV, Eindhoven, the Netherlands) equipped with energy dispersive X-ray spectra analysis (EDS). QUANTAX ESPRIT 1.9 software was used for elemental quantification studies. X-ray spectroscopy analysis (Bruker AXS, Model D8, WI, USA) was done with monochromatic Cu-Ka radiation of wavelength at 40 kV. The experiment was performed by varying the diffraction angle (2θ) from 10° to 80°, and the x-ray peaks were recognized by comparing the data from Joint Committee on Powder Diffraction Standards (JCPDS) files.

Evaluation of Water Absorption Capacity

For water absorption by mortar samples experiment, the samples of each category (5 in numbers) were immersed in water for 28 days. After the curing period, the samples were removed from water and dried by keeping the samples in air for 24 h at room temperature. After that, their initial masses were noted. Then, 50% of the corresponding predetermined average breaking load was applied to the respective set of samples and kept under distilled water for 30 min. The samples were taken out from water, cleaned with soft paper, and their wet masses were measured again. The samples were again dipped in distilled water for another 24 h, and their final wet masses were measured after the similar treatment mentioned. The percentage of water absorption capacity of the mortar samples was estimated according to Neville's procedure (Neville 1996) to observe the effect of how the bacterial protein works against water permeability in protein amended samples.

Sulfate Resistive Activity

For sulfate resistive activity, the as-prepared mortar samples were cured under water for 28 days and then subjected to receive 50% of the corresponding breaking load, as determined earlier. The masses of each category sample were recorded. The samples were then placed under 5% sulfate solution (5% $MgSO_4$, pH 7.0 in distilled water) in a covered tank for 120 days. After curing days, the samples were taken out from the solution, air-dried, and their masses were noted similarly. The experiment was performed according to the procedure of ASTM C 1012 (ASTM 1977).

Sorptivity Test

The sorptivity of the control and protein incorporated mortar cubes (five samples for each category) was determined by the measurement of the water absorption rate of the samples that occurred due to capillary action only of water rise. The samples were first cured under water for 28 days. The samples were then removed from the water tank, air dried, subjected to the application of 50% corresponding breaking load, and followed by heat curing at 65°C in an oven. Afterward, plastic paint was coated carefully to all sides of the mortar cubes except for the exposure face. This coating of paint not only sealed all peripheral surfaces but also maintained the unidirectional capillary flow of water through the exposed surface of the samples. The masses of the paint-sealed samples were measured and recorded as initial mass values set for water absorption calculations. The samples were placed on a wire-gauge kept inside the water bath that helped the exposed surface to contact the water properly. Tap water was poured into the container slowly until the water level reached approximately 3 mm above the level of the exposed surface. The water absorption rate of the samples was noted in different intervals of time. The sorptivity of the control and protein incorporated mortar cubes was determined by the standard procedure.

Statistical Analysis

For each category of testing, five samples have been tested, and each experiment was repeated at least three times. Data are presented as average (over 15 samples) and ± SD.

Results and Discussions

The formation of cracks within the concrete made structures results in the deterioration of strength and durability. However, the self-healing technology in concrete can effectively extend the initial repair period. It also leads to a longer material lifetime and involves less repair and maintenance costs. Therefore, growing trends in the development of self-governing repairing properties of cementitious materials by augmenting some specific bacteria have created enormous interest to the researchers and also given rise to develop several smart materials with versatile properties and high sustainability in construction technology (Sarkar et al. 2014; Jonkers 2011; Wiktor and Jonkers 2011).

Here, the study shows that a new hot spring bacterium BKH4 is beneficial for the development of self-healing and eco-efficient bioconcrete mortar. The cells of the BKH4 bacterium release some proteins in the medium during their culture. One of the particular proteins possesses silica leaching action like the bioremediase

Table 1. Biosilicification activity of isolated microbial protein

Sample	Volume (mL)	Total protein (mg)	Activity (units)	Specific activity (units/mg)
Crude protein	1	4	221	55.25
Purified protein	1	2	408	204

Note: One unit activity of microbial protein is expressed as μg of silica released/mg of protein.

protein. This property has been confirmed by the biosilicification reaction of the protein, as shown in Table 1. The silica leaching quality of the protein is found to be accountable for the higher compressive strength as well as more longevity of the protein incorporated mortars, as reported earlier (Sarkar et al. 2019). There are some novel hot spring bacteria (e.g., BKH1 and BKH2) that likewise have similar silica-releasing ability. These bacteria are used for the development of eco-efficient bio-concrete materials (Sarkar et al. 2014, 2015; Ghosh et al. 2008; Majumdar et al. 2012). BKH1 or BKH2 bacterial cells amended cementitious mortars exhibit not only increased strength and higher longevity but also show the remarkable self-governing repairing quality that appears due to the development of a new calcium-aluminium-silicate (Gehlenite) phase in the cracked regions of cementitious matrices as described by Sarkar et al. (2014, 2015), Ghosh et al. (2008), and Majumdar et al. (2012). Under favorable conditions, some well-known aerobic and active alkaliphilic soil bacteria (e.g., *Bacillus sp*., *Pseudomonas sp*.) show their self-healing features by continuously precipitating solid calcium carbonate (calcite) over the surface of the existing concrete layer. The precipitated substance fills the microcracks and therefore acts as a self-healing mediator for the cracked-concrete structures (Wiktor and Jonkers 2011; Ramachandran et al. 2001; De Muynck et al. 2008).

Unlikely, all of those aforementioned bacteria usually grow at a normal pH range (pH 7.0 to 8.0) and are able to survive for few days (10-15 days) inside the highly alkaline (12.0 to 13.0 pH) cementitious environments. This fact is certainly a disruption against the long-term self-healing action that occurred to the bacteria incorporated cementitious material. However, reports state that spore-forming *Bacillus subtilis* bacteria can survive for a longer time inside the extreme concrete environment (Ulrich et al. 2018). The bacterium could be a true self-healing mediator if the bioremediase gene is incorporated within the cells through transformation by some suitable plasmid vector (Sarkar et al. 2015). However, the transformed Bacillus bacterium needs some food to grow from the sporulation stage to a vegetative form inside the concrete matrices, which will affect the strength of the concrete or mortar. Surprisingly, the bacterium BKH4 being highly alkaliphilic (grows well at optimum pH 12.0 at 65°C) can survive more than a month within the cementitious matrices without any supplementation of food, as stated by Sarkar et al. (2019). It is experimentally verified that the growth of the bacterium BKH4 can be revived from the bacterial cells amended old mortars (more than 2 months old), as described earlier (Sarkar et al. 2019). This phenomenon certainly is advantageous to the self-governing repairing process because it facilitates the bacterium BKH4 to repair the cracks and fissure of the cementitious structures for a long period.

The proteins released by the bacterium BKH4 are separated through the Sephadex G-100 column chromatographic technique. One particular protein [molecular weight (MW)~28 kDa] reveals biosilicification action, as reported previously. The protein was separated in pure form from BKH4 cultured medium, as shown in Fig. 2. The mechanistic action of silica leaching ability of bioremediase protein (isolated from BKH1) was already discussed by Chowdhury et al. (2015). Similarly, we would like to explain that the purified protein is entangled within the cell wall of the bacterium. The enzymatic action of the protein can leach silica (in the form of nanosilica) from any silicate substance. Thus, nanosilica is formed by the interaction between the protein with tetra-ethyl-ortho-silicate (TEOS), as shown in Supplemental Materials Fig. S1. Similar silica leaching proteins have been found in several hot spring bacterial strains (e.g., BKH1 and BKH2). The nanosilica is very reactive due to which it interacts with calcium oxide and aluminum oxide present within the cementitious matrix and thereby forms a new phase viz, calcium-aluminium-silicate (Gehlenite) inside the concrete matrices. The tiny finger-like structure of Gehlenite heals the cracks and micropores of the cementitious structures and thus increases the strength and overall self-life of the structures.

The purified BKH4 bacterial protein was added to the mortar samples at three different concentrations (e.g., 2, 3, and 4 μg

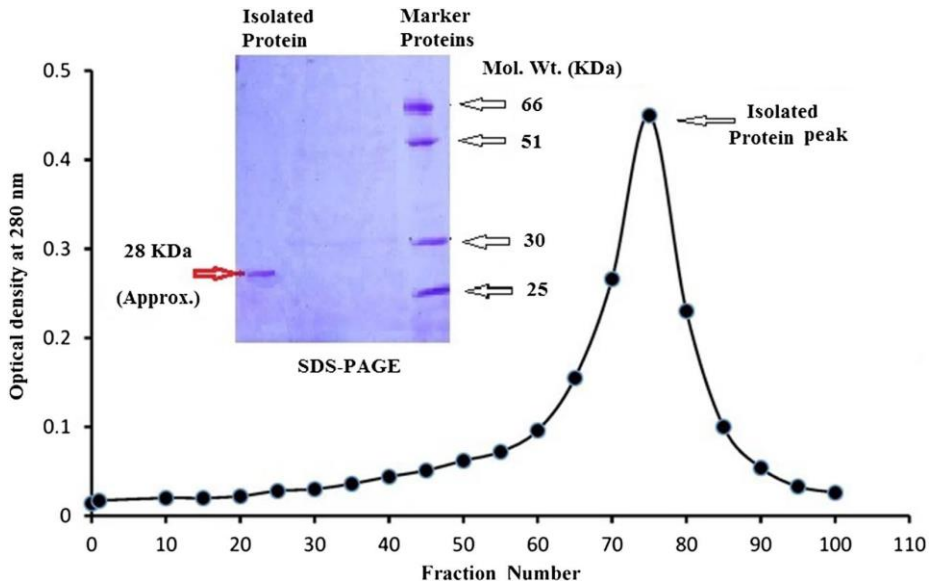


Fig. 2. Purification and SDS-PAGE (inset) of secretory protein of BKH4 bacterium.

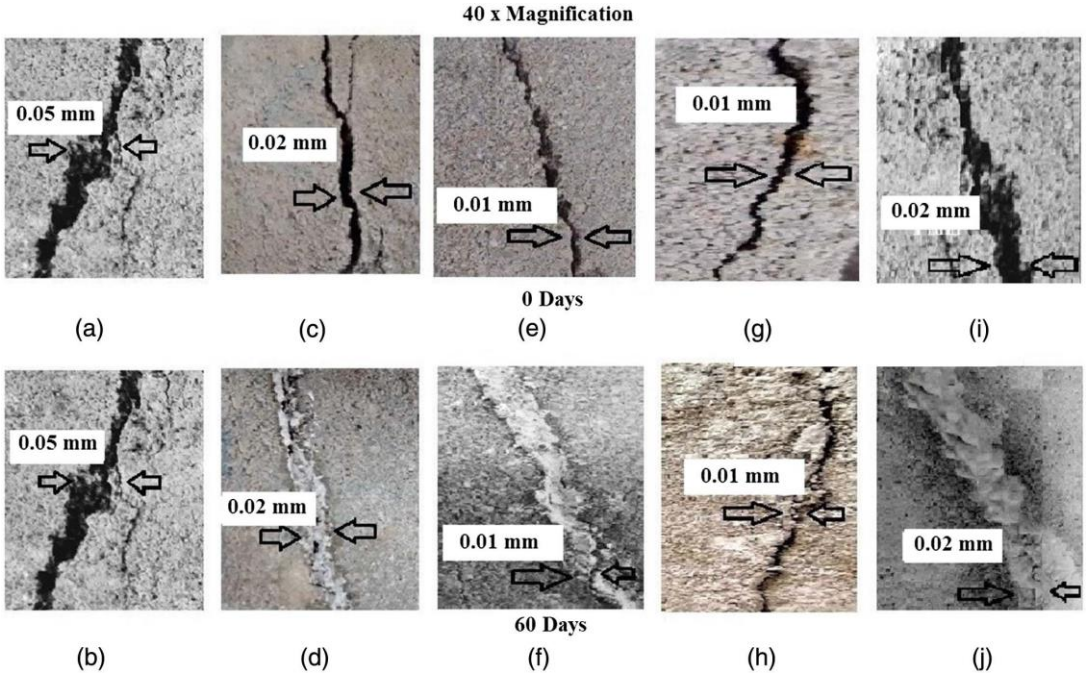


Fig. 3. Images of the cracked mortar samples viewed by Crack scope: (a) control mortar at 0 days; (b) control mortar at 60 days; (c) test mortar at 0 days when 2 μg protein/g cement was used; (d) test mortar at 60 days when 2 μg protein/g cement was used; (e) test mortar at 0 days when 3 μg protein/g cement was used; (f) test mortar at 60 days when 3 μg protein/g cement was used; (g) test mortar at 0 days when 4 μg protein/g cement was used; (h) test mortar at 60 days when 4 μg protein/g cement was used; (i) control mortar at 0 days when immersed in protein (0.03 μg/mL) solution; and (j) control mortar at 60 days when immersed in protein (0.03 μg/mL) solution.

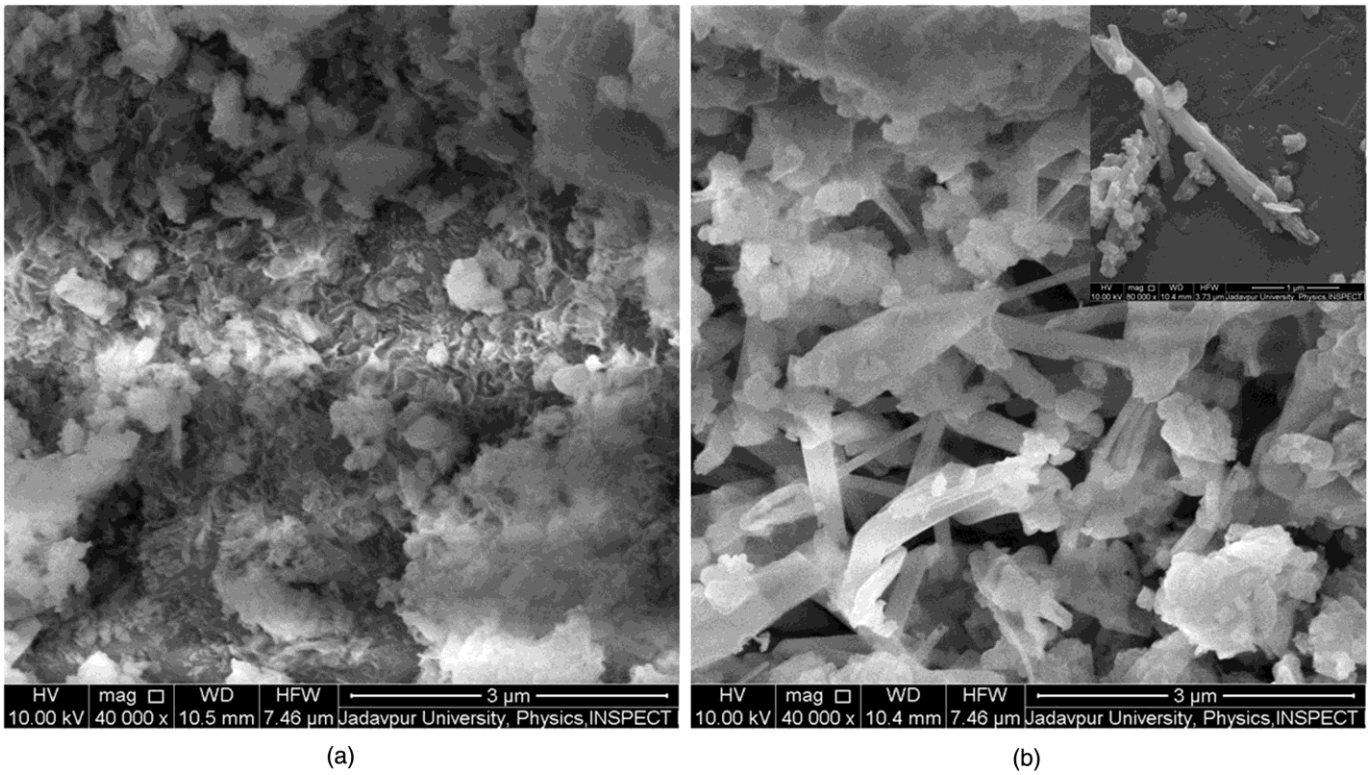
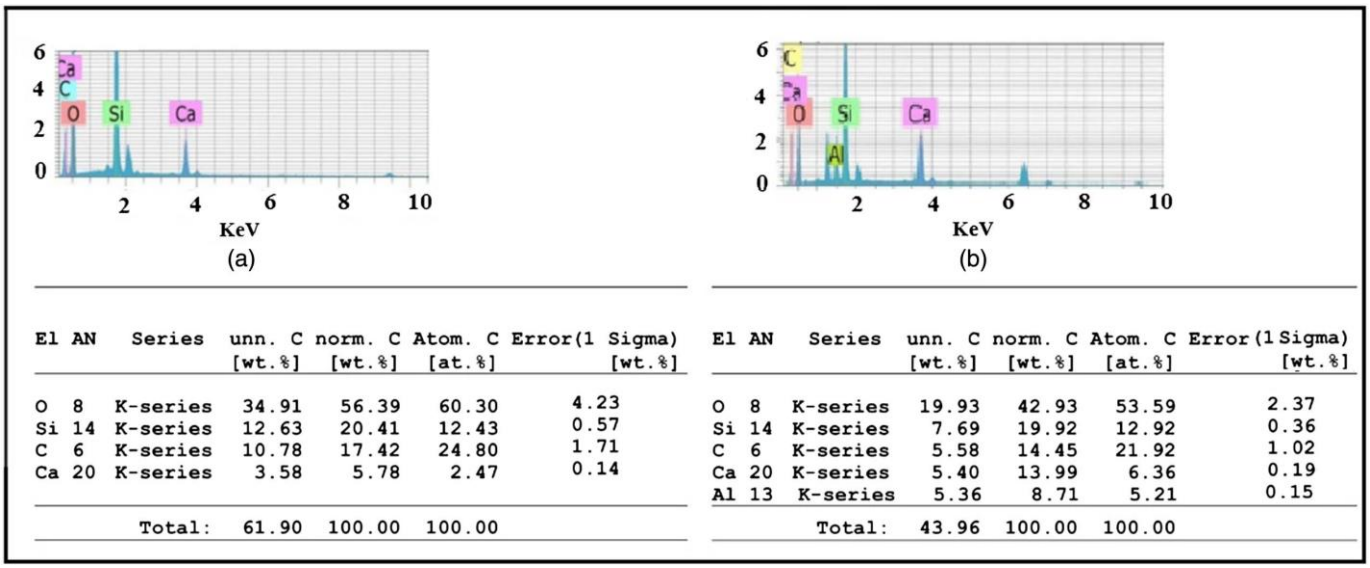


Fig. 4. FESEM image of the powder healing materials: (a) control mortar; and (b) test mortar with single rod-shaped structure (inset).

protein/g cement) for evaluating the mechanical strength, longevity, and self-healing characteristics of the prepared samples. It is noted that cracks (0.01 to 0.05 mm width) of the protein-amended mortars as developed due to the application of 50% of breaking load to the samples are sealed by some tiny fingers like material after water curing [Figs. 3(d, f, and h)]. No such sealing substance is observed on the cracks of control mortar samples when similarly cured under water [Fig. 3(b)]. Whereas, a significant sealing effect is seen on the

cracks of control (without protein amended) mortar samples when immersed under protein (0.03 μg protein/mL water) containing solution [Fig. 3(j)]. This is clear evidence for supporting the fact that the bacterial protein actively participates in the crack healing process of the samples. Several scientists have examined the microbial induction of calcium carbonate preparation (CCP) in self-healing concrete and the bioremediation process (Kim et al. 2016; Seifan and Berenjian 2018). Various enzymatic proteins (e.g., urease) present in the cell surface or secreted by the cells mainly take active roles in the deposition of various substances on the cell surfaces. Our result has also sustained the earlier findings. Ramakrishnan et al. (2005) have shown that bacterial cell walls gather various metallic ions (e.g., calcite) on the cell surface due to its anionic character and thereby makes the cell crystalline. The crystalline ions in the long run fill the pores and repair the cracks in cementitious structures.

The materials as deposited on the cracked regions of mortar were gathered and made powder form. The investigation of the powdered sample taken from protein-impregnated mortars clearly shows that the sample contains tiny finger-like uneven crystalline substances. The crystalline substances are deposited in the cracked portion of the bacteria-incorporated mortars [Fig. 4(b)]. Whereas, no such appearance is seen in the powdered sample taken from cracked regions of control mortar samples [Fig. 4(a)]. The analytical result for elemental identification of the healing material as done by energy-dispersive X-ray spectroscopy is shown in (Fig. 5). It basically confirms the formation of a novel phase only consisting of calcium, aluminum, oxygen, and silicon atoms. The energy-dispersive X-ray spectroscopic analysis of the self-repairing material deposited on the cracked area of the protein-impregnated samples displays some additional minor and major peaks as compared to the control samples (Fig. 6). The additional new peaks are



El	AN	Series	unn. C [wt.%]	norm. C [wt.%]	Atom. C [at.%]	Error (1 Sigma) [wt.%]
O	8	K-series	34.91	56.39	60.30	4.23
Si	14	K-series	12.63	20.41	12.43	0.57
C	6	K-series	10.78	17.42	24.80	1.71
Ca	20	K-series	3.58	5.78	2.47	0.14
		Total:	61.90	100.00	100.00	

El	AN	Series	unn. C [wt.%]	norm. C [wt.%]	Atom. C [at.%]	Error (1 Sigma) [wt.%]
O	8	K-series	19.93	42.93	53.59	2.37
Si	14	K-series	7.69	19.92	12.92	0.36
C	6	K-series	5.58	14.45	21.92	1.02
Ca	20	K-series	5.40	13.99	6.36	0.19
Al	13	K-series	5.36	8.71	5.21	0.15
		Total:	43.96	100.00	100.00	

Fig. 5. Elemental analysis by FESEM of (a) control; and (b) test sample (healing material) obtained from the mortar samples.

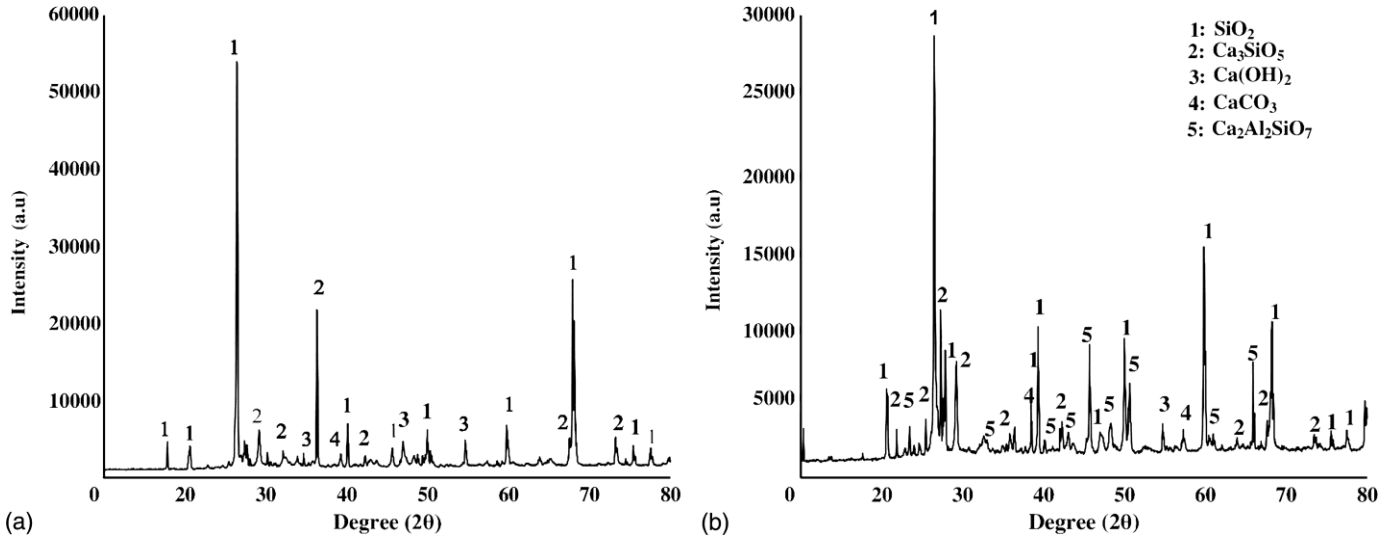


Fig. 6. XRD analysis: (a) control mortar powdered healing material; and (b) test mortar powdered healing material.

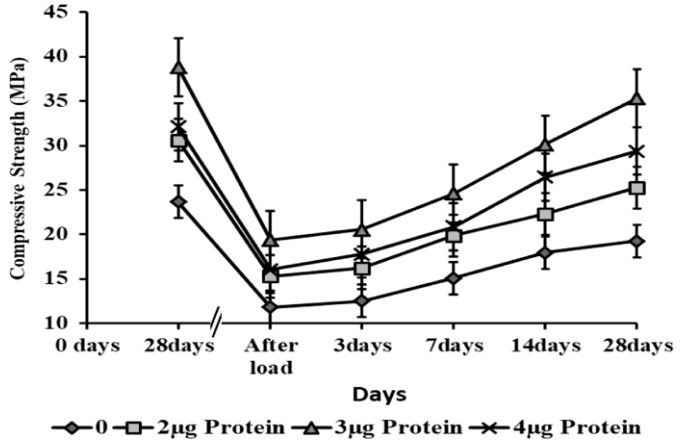


Fig. 7. Compressive strength of the loaded mortar samples at different days of curing.

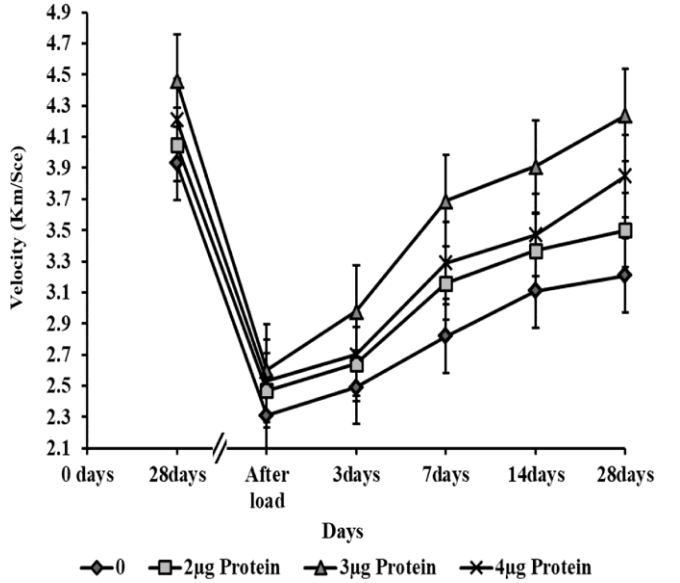


Fig. 8. Ultrasonic pulse velocity of the loaded mortar samples at different days of curing.

the correspondent peaks of a pure calcium-aluminum-silicate phase (Ca_2Al_2-SiO_7 or Gehlenite), as discussed in JCPDS data file. Majumdar et al. (2012) and Chaudhuri et al. (2016) reported previously that the addition of 2 μg protein/g cement produced the maximum effect on mechanical properties of the protein-amended concrete/mortar samples for Portland cement and that of 3 μg protein/g cement produced the maximum effect on mechanical properties of the protein incorporated samples for Pozzolana Portland cement. From Fig. 7, it is noted that the compressive strengths of the cracked mortar samples are decreased for all categories after the application of 50% breaking load. The strengths are seen to increase when cured under water for all categories of samples (Fig. 7). Similar results are obtained in air-cured samples (Supplemental Materials Fig. S2). The rate of increment of strength is always greater in bacterial protein-incorporated samples than that of control mortar samples. The highest increment is seen at the bacterial protein concentration of 3 μg protein/g cement used that also substantiates the previous results (Majumdar et al. 2012; Chaudhuri et al. 2016). The self-repairing activity of the impregnated protein is the primary cause behind the compressive strength increment of the cementitious mortar samples. Mondal and Ghosh (2018) observed that the optimum bacterial cells concentration for achieving the highest compressive strength in concrete was not necessarily a high value, though higher cell concentration led to greater Gehlenite formation. Our results also support the other observations that arise due to the formation of healing material (e.g., calcite) within the bacterial protein-incorporated mortar samples. It heals the microcracks as well as micropores of the cementitious matrices and thereby increases the overall compressive strength of the samples.

The healing of microcracks or filling of pores similarly increases the compactness of the samples that is reflected from the increased UPVof the protein-incorporated mortar samples when cured in different conditions (Fig. 8; Supplemental Materials Fig. S3). Besides the compressive strength and compactness, the self-governing repairing action of the protein also increases the longevity of the material as verified from the experimental results of the water absorption test, sulfate resistance test, and sorptivity test. The water absorption test shows that the water permeability of the protein-mixed mortar samples is decreased compared to control mortar samples (Table 2). It is noted that engrossed water increases the mass of the bacterial protein-amended samples (3 μg=g cement used) only by 1.54%. Whereas, the mass of the control samples is increased by 4.81% due to water engross. This implies that the protein-amended mortars are of less porosity and thus become less water permeable than the control cementitious mortars. Wang et al. (2012) showed substantial improvement of the cementitious material against water absorption in bacteria immobilized in diatomaceous earth. They also observed that the water absorption of the cracked specimen was reduced by one-third and 50% when incubated in a deposition medium and water, respectively. Similarly, it is observed that the bacterial protein-amended mortar samples attain higher sulfate resistance ability when compared to the control mortars (Table 3). The maximum sulfate resistive action was found

Table 3. Sulfate resistance test of the 50% loaded Purified protein incorporated Mortar samples

Sample	Initial mass at 0 day (g)		Final mass at 90 days (g)		Percentage of increment
Control	751.50	1.19	776.00	3.08	3.26
2 μg protein	754.50	1.25	774.25	1.65	2.61
3 μg protein	751.75	1.18	761.25	0.95	1.26
4 μg protein	755.25	0.63	768.25	1.03	1.72

Table 2. Water absorption test of the 50% loaded purified protein incorporated Mortar samples

Sample	Initial mass (g)		Mass after 30 min (g)		% of increasing	Mass after 24 h (g)		% of increasing
Control	727.00	1.23	737.00	1.22	1.37	772.50	1.44	4.81
2 μg protein	715.75	0.25	723.50	0.50	1.08	755.25	1.89	4.38
3 μg protein	710.75	0.48	712.50	0.50	0.24	723.50	0.50	1.54
4 μg protein	717.75	0.25	730.00	0.82	1.70	764.50	1.65	4.72

Table 4. Sorptivity test of the purified Protein incorporated 50% loaded Mortar samples

	Control		2 μg protein		3 μg protein		4 μg protein	
Time (s)	Mass (g)	Percentage increase	Mass (g)	Percentage increase	Mass (g)	Percentage increase	Mass (g)	Percentage increase
0	719	0	717	0	716	0	716	0
60	721	0.27	718	0.13	716	0	716	0
300	724	0.69	720	0.41	717	0.13	719	0.41
600	727	1.11	723	0.83	717	0.13	720	0.55
1,200	730	1.52	725	1.11	718	0.27	723	0.97
1,800	732	1.80	729	1.67	718	0.27	724	1.11
3,600	736	2.36	734	2.37	718	0.27	728	1.67
7,200	741	3.05	741	3.23	719	0.41	733	2.37
10,800	747	3.89	745	3.90	720	0.55	736	2.79
14,400	754	4.86	753	5.02	720	0.55	739	3.21
18,000	759	5.56	756	5.43	721	0.69	740	3.35
21,600	764	6.25	758	5.71	723	0.97	744	3.91
86,400	769	6.95	765	6.69	727	1.53	758	5.86
172,800	770	7.09	768	7.11	729	1.81	759	6.00
259,200	773	7.51	769	7.25	730	1.95	761	6.28
345,600	774	7.64	770	7.39	730	1.95	761	6.28
432,000	774	7.64	770	7.39	730	1.95	761	6.28
691,200	774	7.64	770	7.39	730	1.95	761	6.28

at the protein concentration of 3 μg=g cement used. Majumdar et al. (2012) and Sarkar et al. (2014) also observed the maximum sulfate resistance activity of the bioremediase protein at a concentration of 3 μg=g cement for making of the mortar samples. Table 4 shows that bacterial protein-amended mortar samples are slower in water movement progression than that of control mortar samples. Lesser water permeability and higher sulfate resistivity will protect the concrete structures more efficiently against the corrosion of reinforcement and that will result in increased shelf-life of protein impregnated mortar samples. Xu et al. (2018) has evaluated the self-healing efficiency by visual inspection on crack closure, compressive strength regain, and capillary water absorption. Our results thus demonstrate here that the self-governing repairing ability of the BKH4 bacterial protein not only repairs the cracks but also increases the strength and longevity of the protein-amended mortar materials. It is needless to say that the bacterium BKH4 is a water-grown hot spring bacterium that requires minimal ingredients for its growth (Sarkar et al. 2019). The desired protein is secreted by the bacterial cells and can be easily separated in the pure form through the column chromatographic technique. Also, 3 μg protein is sufficient to achieve the maximum efficacy when used per gram of cement for sample preparation t means 3 g of protein can work on 1 ton of cement. Neither the protein nor the bacterium do not cause any harm to human health. Therefore, this is an ecoefficient and cost-effective methodology (as no additional techniques are required to obtain the protein) that may be used for construction purposes.

Conclusions

It can be concluded here that,

- The quality self-healing in concrete is possible by infusing the highly alkaliphilic bacterial protein (maximum activity ranges from pH 12.0 to 12.5) in the cementitious material that neither requires the supplementation of food nor affects the mechanical properties of the material.
- The maximum effect is achieved by using the microbial protein at a concentration of 3 μg=g cement used.
- The microbial protein increases the ultrasonic pulse velocity and compressive strength, augments the sulfate resistance, reduces water permeability, and slows down water movement (sorptivity test) of the protein amended mortar samples, which reveal that there is overall improvement in mechanical properties and durability of the protein-incorporated mortar samples.
- This would be one of the low-cost and effective measures against concrete deterioration for future construction technology.

Data Availability Statement

All the data obtained from several experiments are included in the article.

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Supplemental Materials

Figs. S1-S3 and Tables S1 and S2 are available online in the ASCE Library (www.ascelibrary.org).

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