Exploration of a Hot Spring Alkaliphilic Bacterium and its Secretory Protein Pertinent to Self-healing Bio-concrete Material

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CERTIFICATE FROM THE SUPERVISOR

This is to certify that the thesis entitled "**Exploration of a Hot Spring Alkaliphilic Bacterium and its Secretory Protein Pertinent to Self-healing Bio-concrete Material**" submitted by Atreyee Sarkar who got her name registered on 31.07.2015 for the award of Ph. D. (Science) degree of Jadavpur University, is absolutely based upon his own work under the supervision of Dr. Brajadulal Chattopadhyay, Professor, Department of physics, Jadavpur university 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 in this thesis entitled **"Exploration of a Hot Spring Alkaliphilic Bacterium and its Secretory Protein Pertinent to Self-healing Bio-concrete Material"** which is being submitted for the degree of Doctor of Philosophy (Science) has been carried out by me under the supervision of Professor Brajadulal Chattopadhyay of the Department of Physics, Jadavpur University, Kolkata. Neither the thesis nor any part thereof has been presented anywhere earlier for any degree or award whatsoever.

Atreyee Sarkar

Atreyee Sarkar

Dated 22/06/2022

This thesis is dedicated to my

Parents

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Acknowledgements are not mere formalities but an expression of deep sense of gratitude and cumulative appreciation. The report is not only being individual endeavor, but teams work with the zeal to achieve something. I feel extremely proud to mention who really helped me a lot during the tenure of my PhD work.

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Place:

Date: *Signature of the Candidate*

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Preface

This PhD dissertation embraces five years of my research work in isolation and characterization of profoundly alkaliphilic, thermophile bacterium (BKH4), bacterial excretory protein and the application of bacterial cells and its excretory protein in concrete technology. Microorganisms are exceptionally modest animals that exclusively are as well little to be seen with the unaided eye. The unicellular microorganisms were the essential frame of life to appear on the earth around four billion, a long time back. Between Of all the distinctive organisms exist in nature, the present study was outlined to insulate a novel bacterium from hot springs at Bakreshwar, India and my center bargains with the characterization of a novel protein named bioremediase separated from a thermophilic bacterium BKH4 for creating an inborn, eco-friendly, strong, and self-healed bio concrete fabric. The test comes about of this ponder, clearly clarify that the bacterium (BKH4) and the novel protein, confined from the bacterium, plays a key role in advancement of mortar properties.

A novel facultative anaerobic and profoundly alkaliphilic bacterial strain (BKH4; GenBank increase no. KX622782) having a place to the family 'Bacillaceae' and homologous (99%) with *Lysinibacillus fusiformis* was obtained through the method of serial weakening strategy to realize an immaculate strain. Phenotype characterization appeared that it is coccoid-type Gram-positive bacterium develops in a profoundly alkaliphilic (pH 12) well characterized semi-synthetic medium at 65°C.

The bacterial strain BKH4 when consolidated with distinctive bacterial cell concentrations (0, 10^3, 10^4, 10^5 and 10^6 cells per ml of water utilized) to the cement-sand mortar increased the compressive

strength of the samples. The most extreme strength increase (>50 %) of the mortar cubes regarding control, was watched at the cell concentration of 10^4 cells per ml of water utilized. Micro structural view of such bacterium consolidated mortar samples by Scanning Electron Microscope (SEM) appeared a few needles like crystalline deposition inside the pores of the mortar matrix regarding control tests. This was affirmed by X-ray diffraction (XRD) investigation that clearly proposed the arrangement of new stages of silicates (Gehlenite) inside the lattice of mortar fabric which was capable for strength enhancement.

Not as it were with the bacterium but too its corresponding protein named as bioremediase has comparable sort of part in strength increase property of mortar/concrete samples. Bacterial protein responsible for such deposition, was at that point separated from bacterial cell supernatant and filtered by gel filtration chromatography utilizing Sephadex G-100. The decontaminated protein "bioremediase" having atomic weight 28 kDa is affirmed by SDS-PAGE. Although it remained dynamic over a wide run of pH and temperature but was ideally receptive at pH 8 and 65 °C.

This filtered protein was too found to extend the compressive strength when blended with the shifting concentration. The bioremediase protein with changing concentration (1µg, 2µg, 3µg and 4µg per gram of cement) when included to the cement-sand mortar paste comes about a greatest strength increase at 3µg of protein utilized regarding control tests. The prepared control and protein-amended cementitious mortar samples were subjected to mimic splits and cured under water for a few days. Pictures and microstructures of the control and protein-incorporated samples were analyzed, which set up that there was a modest fingers-like crystalline substance created on the split surfaces. The created substance was recognized as a silicate stage (Gehlenite) by energy dispersive X-ray spectroscopic investigation. The microbial protein upgraded the mechanical

qualities and solidness of the protein-incorporated samples that were upheld by the increases of ultrasonic pulse velocity, compressive strength, and sulfate resistance as well as decrease of water penetrability and moderate water movement (sorptivity test) of the exploratory samples. This self-healing phenomenon is eco-efficient and created due to the bio-silicification activity of the microbial protein that was consolidated in mortar samples.

Microstructure investigations by Environmental Scanning Electron Microscope (ESEM) study and Energy Dispersive X-Ray (EDX) investigations demonstrated improved hydration of protein revised cement glue and expanded composition of calcium and silicon rates of calcium silicate hydrate (CSH) gel. Subsequently, all these observed changes in micro-structural characteristics of the protein-amended cement glue especially regarding expanded consistency of the framework components were reflected by quality change. This protein moreover increments the strength of the concrete which will aid create feasible structures. This novel concept may speak to inventive pathways to artificially actuating the profoundly alluring self-healing in concrete materials and concrete innovation in close future.

Presently the address emerges why this sort of bacterium was chosen for the ponder. The reason was that this bacterium features a crucial role within the silica leaching action through the method of bio-mineralization. As we know that utilize of microorganisms in concrete technology are recognized and reacted with the work of numerous famous researchers. The word “Bio-mineralization” may be a common and far-reaching complex phenomenon by which living shapes impact the precipitation of different minerals such as carbonate, phosphate, oxalate, silica, press, sulphur-containing minerals etc. These microbial mineral precipitations are come about from metabolic exercises of microorganisms. Proteins/enzymes inside these living beings control this

handle that either coordinates the anticipation or creation of mineral stores. Such bio-mineralogy concept leads to a modern zone of inquire about taken after by a potential development of a modern material called bacterial concrete or bio-concrete, an inborn self-repairing biomaterial that can remediate the splits and crevices in concrete. A comprehensive basic and utilitarian characterization of the profoundly alkaliphilic, thermophile bacterium has been done in this thesis work and investigate the role of BKH4 bacterium excretory protein in concrete innovation. The effective utilize of the bacterium BKH4 and its bioremediase protein within the concrete/mortar will make a new hope for feasible and green concrete innovation.

List of Abbreviations

SEM→ Scanning electron microscope

XRD → X-ray diffraction

KDa → Kilo Dalton

FESEM → Field emission scanning electron microscope

EDS → Energy dispersive X-Ray Spectroscopy

rRNA → Ribosomal ribonucleic acid

IS → Indian Standard

TEOS → Tetraethyl orthosilicate

BSA → Bovine serum albumin

O.D. → Optical density

UV → Ultraviolet

BLAST → Basic local alignment search tool

SDS-PAGE →Sodium dodecyl sulfate – polyacrylamide gel electrophoresis

S.D.→ Standard deviation

MPa → Mega Pascal

UPV → Ultrasonic Pulse Velocity

RCPT → Rapid Chloride Permeability Test

NMR → Nuclear magnetic resonance

INTRODUCTION

Introduction

What is evolution? From each knowledgeable source, we found that, evolution is being define as a change. Change which takes more than an era to reach its present condition. Whatever the changes are heritable changes for living organisms from their ancestors to their present offspring, the qualitative changes of our environment (global warming situation) or the huge changes of our standard of living which includes small toothbrush to construction materials. All are effectively known as evolution. Due to the enormous improvement of our science and technology, its major effect is highlighted in our everyday life. So, we can say, the change or the evolution is not only happened for the living things but also for the inert things.

On the setting of advancement, how come we overlooked the part of microorganisms in our standard life, from old time they are our part of life. Interior of human body to each portion of the biosphere, counting soil, hot springs, "seven miles deep" within the ocean, "40 miles tall" within the air and interior rocks distant down inside the Earth's outside (Science Every day), microbes are lived.

Microbes constitute a huge space of prokaryotic microorganisms. The shape of the microbes extending from a circle to poles and winding shape changes ordinarily some micrometers in length. Bacteria are among the primary life shapes to seem on the Soil and are display in most of its environments. Microscopic organisms occupy in soil, water, acidic hot springs, radioactive squander (Fredrickson et al., 2004) and the profound parcels of Earth's hull. Microbes too live with advantageous and parasitic connections in plants and creatures. They are too known to have

prospered in staffed shuttle (Nasa, Science News). A few microscopic organisms are thermophilic in nature meaning they flourish at a temperature extending from 45 ºC – 122 ºC. Like all other microbes, thermophilic microscopic organisms moreover have different proteins which play a dynamic part in different natural responses. A bacterial protein is either a portion of the structure of it or delivered by a bacterium as portion of its life cycle. Luckily, most of the microbes are simple to culture within the research facility due to which an incredible bargain of investigation on bacterial proteins has been performed with the objective of learning more almost particular proteins and their capacities.

Especially, the microbial chemicals have broad employments in several businesses. The microbial chemicals are moreover more dynamic and steadier than plant and creature proteins. In expansion, the microorganisms speak to an elective source of proteins since they can be refined in colossal amounts in a brief time by maturation and owing to their biochemical differing qualities and susceptibility to quality control. Businesses are seeking out for unused microbial strains to deliver distinctive chemicals to fulfill the current protein necessities. One of the major parts of microbial strain or its corresponding protein is the improvement of compressive quality and strength of the concrete fabric. Concrete could be a development fabric composed of cement (commonly Portland cement) as well as other cementitious materials such as fly cinder, slag cement, total (a coarse total such as rock limestone or rock, furthermore a fine total such as sand), water and chemical admixtures. The word concrete comes from the Latin word "concrētus" which suggests "hardened" or "hard." Concrete sets and solidifies after blending with water and situation due to a chemical preparation known as hydration. The water responds with the cement, which bonds the other components together, inevitably making a stone like fabric. The responses are exceedingly exothermic, and care must be taken that the built-up in warm does not influence the astuteness of

the structure. Concrete is used to create asphalts, structural structures, brick/block dividers and footings for entryways, wall, and shafts. Concrete has numerous properties that make it a well-known construction fabric. The right extent of ingredients, situation and curing are required for these properties to be ideal. Good-quality concrete has numerous points of interest that include to its ubiquity. To begin with, it is conservative when ingredients are promptly accessible. Concrete's long life and negligible upkeep prerequisites increase its financial benefits. Concrete is not as likely to spoil, erode or rot as other building materials. Concrete can be molded or cast into any craved shape. Building of the molds and casting can happen on the worksite which reduces costs. Concrete is an incombustible fabric that produces it fire-safe and can withstand hot temperatures. It is safe to wind, water, rodents, and creepy crawlies. Thus, concrete is extensively using for storm covers.

Concrete does have a few restrictions too despite its various focal points. The impediments are as takes after –

- Low pliable quality (compared to other building materials)
- Low ductility
- Lack of durability
- Low strength-to-weight ratio
- Permeability to fluid and resulting erosion of reinforcement
- Susceptibility to chemical attack
- In expansion to the trouble in solidified concrete, the plastic concrete may endure harm due to plastic shrinkage and settlement cracking.

These are the reasons why concrete falls apart can be attributed to either unique construction blunders and/or natural impacts.

An assortment of added substances or substitutions of cement can be connected to progress the solidness and to repair splits in concrete item. Moreover, certain mechanical squander or reused materials can be utilized to progress the maintainability or natural neighborliness of concrete and a few indeed progress certain properties. Illustrations of mechanical squander items which can mostly supplant and indeed move forward cement properties are fly fiery remains, impact heater slag and silica exhaust. Application of fly fiery debris increments concrete quality because it diminishes the specified water/cement proportion and makes strides resistance against chemical assault because it diminishes the lattice porousness. Additionally, silica rage from silicon industry and impact heater slag from steel businesses can supplant cement within the concrete blend as these are the sources of responsive silica and both receptive silica and calcium, separately. Other commonly connected added substances that progress or alter certain concrete characteristics required for applications are air-containing specialists to progress freeze/thaw resistance, setting or impeding specialists and plasticizers to empower a lower water/cement proportion to extend concrete quality. A few other adjustments have also been made from time to time to overcome the challenges of concrete, but all those forms are not simple and great (Jonkers, 2007). Subsequently, a dynamic self-healing mechanism should be perfect because it does not require labor-intensive manual checking and repair which would spare a gigantic sum of cash.

As of late, an endeavored was made by joining microorganisms inside the concrete lattice by utilizing the special property of biomineralization to repair splits and increment solidness which investigates a curiously investigate range called “Bacterial concrete” or “Bio-concrete.” Bacterial

concrete could be a fabric which can effectively remediate breaks in concrete. This strategy is profoundly alluring since the mineral precipitation actuated since of microbial exercises is contamination free and common. As the cell divider of microbes is anionic, metal aggregation (calcite) on the surface of the divider is considerable, in this way the whole cell gets to be crystalline, and they inevitably plug the pores and splits in concrete (Ramakrishnan, 2005).

In this PhD proposal I am attending to talk about my entire inquire about work which is based on distinguishing proof and characterization of a profoundly alkaliphilic anaerobic microbes and its excretory protein. Application of the highly alkaliphilic microscopic organisms and bacterial protein in concrete innovation is additionally the concerned portion of my proposal.

To begin with portion of my proposition is examined around the confinement and characterization of microbes. The unicellular animal microscopic organisms are advanced on this soil around four billion a long time back and most of these microscopic organisms are infinitesimal in nature. All over differing sorts of existing high-impact and anaerobic microbes my investigate work is based on, a facultative anaerobic bacterium strain named BKH4. The bacterium is separated from the hot spring of Bakreshwar, India. In research facility by utilizing the serial weakening strategy the immaculate bacterial strain BKH4 is confined. To preserve the cocci formed gram-positive BKH4 microbes in a research facility environment, 65°C temperature and a profoundly alkaliphilic (pH 12) press and magnesium come to personalized manufactured media is castoff. The novel facultative anaerobic and profoundly alkaliphilic bacterial strain is (BKH4; GenBank promotion no. KX622782) having a place to the family ‘Bacillaceae’ and homologous (99%) with *Lysinibacillus fusiformis*.

The bacterium BKH4 secretes few proteins in its development medium. In display ponder, I have too centered on the filtration of extracellular rough protein, which was separated from the bacterium, utilizing Sephadex G-100 gel filtration chromatography method. Each division of the eluted protein gotten was planned for biosilicification test to affirm which protein is mindful for the action. The protein has special biomineralization property by implies of which it makes a difference to create higher quality and tough bio-concrete fabric when joined into the tests. It makes a difference to make nano-filament of modern silicate stage (Gehlenite or calcium aluminum silicate) inside the concrete/mortar lattices by collaboration with the oxides of calcium and aluminum coming about upgrade of mechanical quality of the bio-concrete materials. Other than the increase of compressive quality, the self-healing nature of the bioremediase protein moreover repairs the splits of the concrete fabric. In my second portion of the study, I have talked about the part of microscopic organisms (BKH4) and its excretory protein in concrete technology.

In innovative civilization as an essential component of concrete, cement remains the foremost broadly utilized construction fabric. Standard Portland cements (OPC) & Portland Pozzolan cements (PPC) are two sorts of cement which used in developments. The foremost important uses of cement are as a component within the generation of mortar in masonry, and of concrete, a combination of cement and a total to create a solid building fabric.

In later times, a curiously endeavor has been made by joining appropriate microorganisms inside the concrete lattice to utilize the property of bio mineralization for improving the compressive, flexural, malleable quality, split repairing, and strength issue. These recently concocted materials are known as “Bacterial Concrete” or “Bio concrete.” This concept has presently ended up a curiously zone of investigate. The “Bacterial Concrete” can be made by consolidating microscopic

organisms inside the concrete with reasonable cells/protein concentration that can accelerate calcite or a few other minerals. This marvel is called microbiologically initiated calcite precipitation or microbiologically actuated mineralization. It has been appeared in numerous investigate papers that beneath favorable conditions a few common aerobic and dynamic alkaliphilic soil microscopic organisms like *Bacillus pasteurii*, *Pseudomonas aeruginosa* can ceaselessly accelerate/ mineralize impermeable calcite layer over the surface of an as of now existing concrete layer which may work as a self-healing operator inside the concrete structures.

Recently an inalienable and self-repairing thermophilic microorganism-based biomaterial "Bacterial concrete/ bio-concrete" has been created to remediate the micro-cracks and gaps within the concrete structures in Material science research facility, Jadavpur College, Kolkata. An endeavor has been made to ponder the impact of distinctive thermophilic microorganisms (BKH1 and BKH2 confined from hot spring of Bakreshwar, West Bengal) by joining inside the mortar and concrete lattice. It is as of now demonstrated by our senior individual that the expansion of hot spring microscopic organisms (BKH1 & BKH2) or bacterial protein (high pH & elevated temperature tolerant protein) increments the compressive quality and flexural quality when consolidated in cementitious materials in fitting concentrations. Advancement of

Fig. 1: BKH4 Protein-amended Mortar

microbiologically consolidated bio-concrete materials is innovative state of mind in which the arranged materials not as it had higher quality and more strength in character but too can relate to less cement for tall execution development purposes. Thermophilic microorganisms have picked up around the world significance due to their immense potential to create thermostable proteins that have wide applications in pharmaceuticals and industries.

The bacterial strain BKH4 when consolidated with distinctive cell concentrations (0,10^3, 10^4, 10^5 and 10^6 cells per ml of water utilized) to the cement-sand mortar increases the compressive strength of the samples. The most extreme strength increase (> 50 %) of the mortar cubes about control is watched at the cell concentration of 10^4 cells per ml of water utilized. Not as it were with the bacterium but too its comparing protein named as bioremediase has comparable sort of part in quality increase property of mortar/concrete tests. The bioremediase protein with shifting concentration (0µg, 2µg, 3µg and 4µg per gram of cement) when included to the cement-sand mortar glue comes about a most extreme quality increase at 3µg of protein utilized. Smaller scale basic sees of bacterial protein joined mortar tests by Filtering Electron Magnifying lens (SEM) appears presence of a few needles like crystalline statement of nano-filaments inside the pores of the mortar lattice which is truant in control tests. X-ray diffraction (XRD) examination clearly recommends the arrangement of new stages of silicates (Gehlenite) inside the lattice of mortar fabric which plays a key part within the enhancement of compressive strength.

A comprehensive structural and useful characterization of the bacterium (BKH4) and a comprehensive physical and functional property of BKH4 bacterium excretory protein has been tired this thesis work which is the first-time prove of highly alkaliphilic microbes and bacterial

protein-based mortar/concrete. The fruitful utilization of the bacterium BKH4 or its protein within the concrete/mortar will make a modern trust for economical and green concrete technology.

REVIEW OF LITERATURE

Review of Literature

Origin of Bacteria

Microscopic organisms have existed from exceptionally early within the history of life on earth. Bacteria fossils found in rocks date from at slightest the Devonian Period (416 to 359.2 million a long time back) and there are persuading contentions that microbes have been shown since early Precambrian time, almost 3.5 billion a long time back. They were far reaching on earth at least since the middle of the Proterozoic, almost 1.5 billion a long time ago, when oxygen showed up within the atmosphere since of the activity of cyanobacteria. The primary tenants of soil did not require oxygen to breath, in fact oxygen was poisonous to them and this gas was uncommon within

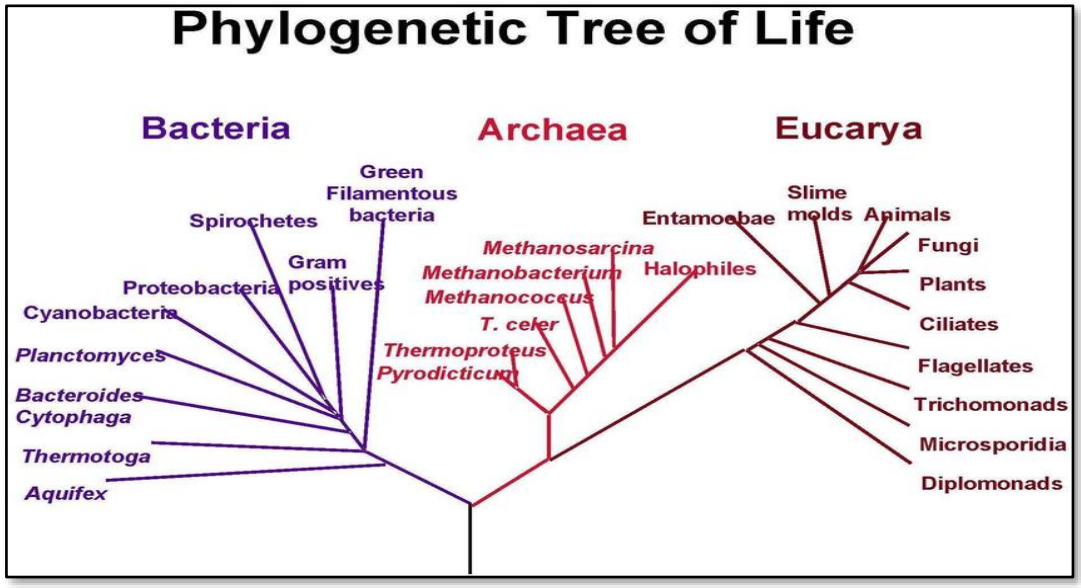


Fig. 1: Phylogenetic Tree of Life

the environment in those days. In any case, the cyanobacteria that occupied earth within the Precambrian produced oxygen as a squander gas and so made a difference build up an aerobic biological system (Evolution and the Origin of Life, online article, 2006). Microscopic organisms have in this way had bounty of time to adjust to their situations and to allow rise to various relative

shapes. The nature of the first forerunner included within the origin of life is subject to significant hypothesis. It has been recommended that the first cell might have utilized RNA as its hereditary material. Hence, depending upon the r-RNA gene sequence the thought of a "tree of life" or "developmental tree" from antiquated ideas of a ladder-like movement from lower to higher shapes of life was first illustrated by Charles Darwin (1859). An evolutionary tree, also called a phylogenetic tree, could be a tree appearing the evolutionary connections among different organic species or other substances that are accepted to have a common precursor.

The tree of life is made up of three branches. The Eukaryotes, Microbes and Archaea. The eukaryotes incorporate animals (humans), plants and fungi and a wealthy assortment of microorganisms moreover known as protists. The protists include parasites, which can be naturally speaking exceptionally effective, and they can compromise the environment of whole nations. The Eukaryotes are recognized and distinguished from other shapes of life by the presence of nuclei and the presence of a cytoskeleton. Microbes are infinitesimal living beings whose single cells don't have a membrane-bounded nucleus or other membrane-bounded organelles like mitochondria and chloroplasts. Another bunch of organisms, the archaea, meet these criteria but are so diverse from the microbes in other ways that they must have had a long, autonomous evolutionary history since near to the daybreak of life. Archaea are organisms and most live-in extraordinary situations; those are called extremophiles. Other Archaea species are not extremophiles and live-in standard temperatures and salinities. Archaea are a much diverse and less complex shape of life. They may moreover be the most seasoned shape of life on Earth, since they require not one or the other daylight for photosynthesis as do plants, nor oxygen as to animals. Archaea retains CO2, N_2 or H_2S and gives off methane gas as a squander item within the same way people breathe in oxygen and breathe out carbon dioxide. Archaean’s may be the only living

beings that can live in extraordinary environments such as greatly hot warm vents or hyper saline water. They show up to be greatly plenteous in situations that are antagonistic to all other life shapes (Science and Evolution, 2007).

Divergence Between Archaea & Bacteria

From the evolutionary tree" it has been seen that microbes and Archaea separated from their common antecedent exceptionally early in time. The two sorts of prokaryotes tend to possess diverse sorts of situations and allow rise to unused species at distinctive rates (Advancement of microscopic organisms- Reference book, 2008).

Although Archaebacteria and microscopic organisms came from the common forerunner, but archaea contrast in certain physiological and hereditary highlights from bacteria, the foremost noticeable prokaryotes (Archaea- Reference book, 2007). Archaea have a few highlights in common with bacteria as well as eukaryotes but prove recommends that archaea are more closely related to eukaryotes than to bacteria. Some scientists, however, have contended that the Archaea and Eukaryotes emerged from a bunch of bacteria (Gupta, 2000). It is conceivable that the final common predecessor of the bacteria and archaea was a thermophile, which raises the plausibility that lower temperatures are "extreme environments" in archaeal terms, and organisms that live in cooler environments showed up afterward within the history of life on Earth (Gribaldo & Brochier-Armanet, 2006). Since the Archaea and Bacteria are no more related to each other than they are to eukaryotes, this has driven to the contention that the term prokaryote has no genuine developmental meaning and should be discarded entirely (Woese, 1994).

Over a century afterward, evolutionary scientists still utilize such tree charts to delineate evolution since the floral similarity successfully passes on the concept that speciation happens through the versatile and arbitrary part of ancestries. Over time, species classification has ended up less inactive and more energetic.

- **STRUCTURAL DIFFERENCES BETWEEN ARCHAEA AND BACTERIA:**

Table 1: Structural difference between archaea and bacteria

Archaea	Bacteria
Archaea contain pseudopeptidoglycan, polysaccharides, glycoproteins, or pure protein in their cell wall.	Bacteria contain peptidoglycan in their cell wall.
The cell membrane of Archaean has lipid bilayer or a monolayer.	The cell membrane in bacteria is a lipid bilayer.
Archaea contain hydrocarbon (phytanyl) on the cell membrane.	Bacteria contain fatty acids on the cell membrane.

Bacterial and archaeal flagella differ in their chemical structure and differ from in the structure of their ribosomal RNAs (Archaea- Encyclopedia, 2007).

- **PHYSIOLOGICAL DIFFERENCES BETWEEN ARCHAEA AND BACTERIA:**

One transcendent distinction is that microbes have broadly adjusted to oxygen consuming conditions, though numerous archaea are committing anaerobes (Bacteria- encyclopedia, 2007). Archaea may be anaerobic or facultative anaerobic in their metabolic necessities. There are three major known bunches inside the archaebacteria: methanogens, halophiles, and thermophiles. The

methanogens are anaerobic bacteria that create methane as a product. They are found in sewage treatment plants, lowlands, and the intestinal tracts of ruminants. Old methanogens are the source of characteristic gas. Halophiles are bacteria that flourish in high salt concentrations such as those found in salt lakes or pools of seawater. Thermophiles are the heat-loving microscopic organisms found close aqueous vents and hot springs (Archaea- Encyclopedia, 2007; Evolution and the Origin of Life, online article, 2007).

Thermophilic archaea favor high-temperature niches (Evolution of bacteria encyclopedia, 2008). They can survive in temperature higher than 45 °C to 50 °C though a few archaea found in oceanic warm vents, developing at temperature higher than 95 °C (Archaea- encyclopedia, 2007). Their inclinations for hot temperatures have given rise to a few theories around their advancement. One hypothesis recommends that the thermophiles were among the primary living things on this planet, creating and advancing amid the primordial birthing days of soil, when surface temperatures were very hot. They have in this way been called the "Universal Ancestor" (Weblife.org: Thermophilic Microorganism).

Revelation of Thermophiles

Microorganisms those are adjusted to develop at high temperatures (over 60ºC) have been confined from high temperature earthly and marine territories. One hypothesis proposes that the thermophiles were among the primary living things on this planet, creating and evolving amid the primordial birthing days of earth, when surface temperatures were very hot. They have hence been called the “Universal Ancestor”. In 1966, Thomas Brock made the momentous disclosure that microorganisms were developing within the bubbling hot springs of Yellowstone National Stop. Since Brock's revelation, thermopiles have been found in geothermal highlights all over the world

counting ranges in Iceland, Kamchatka, New Zealand, Italy, Mt. Lassen, and a few areas in India like Bakreshwar hot spring. Whereas bubbling hot springs are distant past the consolation zone of people and other animals' life, particularly prokaryotic life, can adjust to situations that would demonstrate deadly to most other life-forms. All thermophiles require a hot water environment, but a few flourishes in more than one extraordinary, such as those with high levels of sulfur or calcium carbonate, acidic water, or alkaline springs.

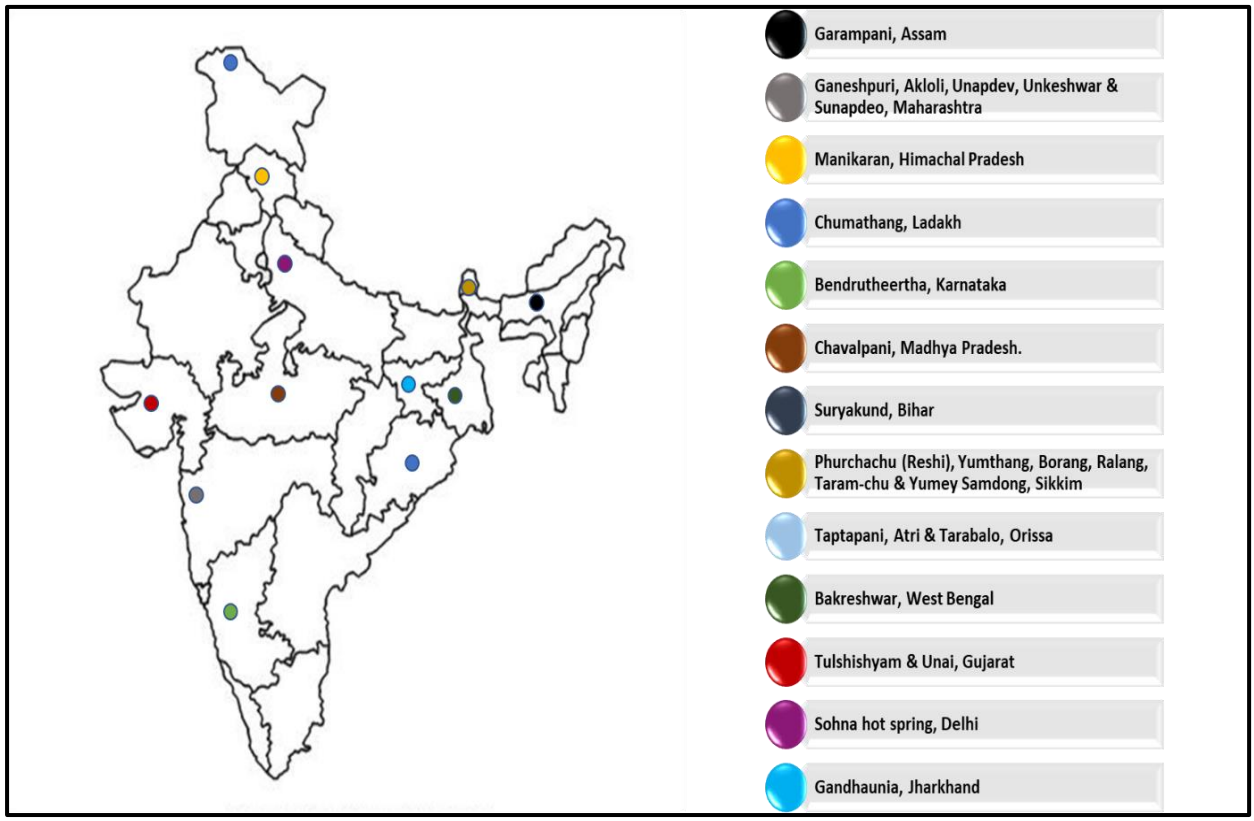


Fig. 2: Different hot springs in India

A hot spring or a hydrothermal spring could be a place where warm or hot groundwater is warmed by geothermal warm, basic warm from earth's insides. Such warm water contains dissolved solids and a few minerals such as calcium, lithium, radium etc. Such geothermal hot spring streams give favorable condition for the improvement of microbial mats, which contain physiologically and

phylogenetically distinctive bunches of prokaryotes, such as chemotropic sulfur microbes, cyanobacteria, and oxygenic phototrophic microbes, depending on the temperature, pH, sulfide concentration and a few other natural conditions (Akira et al., 1999). Natural hot springs are various all over the world which are recognized by the minerals broken up in water and allow rise to the advancement of distinctive bacterial communal tangle (www.science .html). There are 17 hot springs recognized so distant in India where diverse sorts of thermophilic bacterial differing qualities are found. These are –

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 meters (276 feet). It is a place of geological interest with many hot springs. There are ten hot springs which are –

1. **Papharaganga**

2. **Baitariniganga**

3. **Kharkunda:** The spring water is here 66 degrees Celsius.

4. **Bhairavkunda:** The spring water is here 65 degrees Celsius.

5. **Agni kunda:** The spring water is at 80 degrees Celsius and rich in many minerals like; sodium, potassium, calcium, silicates, chlorides, bicarbonates, and sulphates which have medicinal properties in a broader spectrum. There might have few traces of radioactive elements.

6. **Dudhkunda:** Due to ozone concentration, probably the water of this spring attains a dull white hue during early morning. The spring water here is at 66 degrees Celsius.

7. **Surya kunda:** The spring water is here 61 degrees Celsius.

8. **Shwetganga**

9. **Brahma kunda**

10. **Amrita kunda**

These hot springs due to their unique chemical richness have different types of thermophilic and hyper-thermophilic microbial community (Bakreshwar, Wikipedia: The free encyclopedia, 2008).

Significance of Thermophilic Enzymes

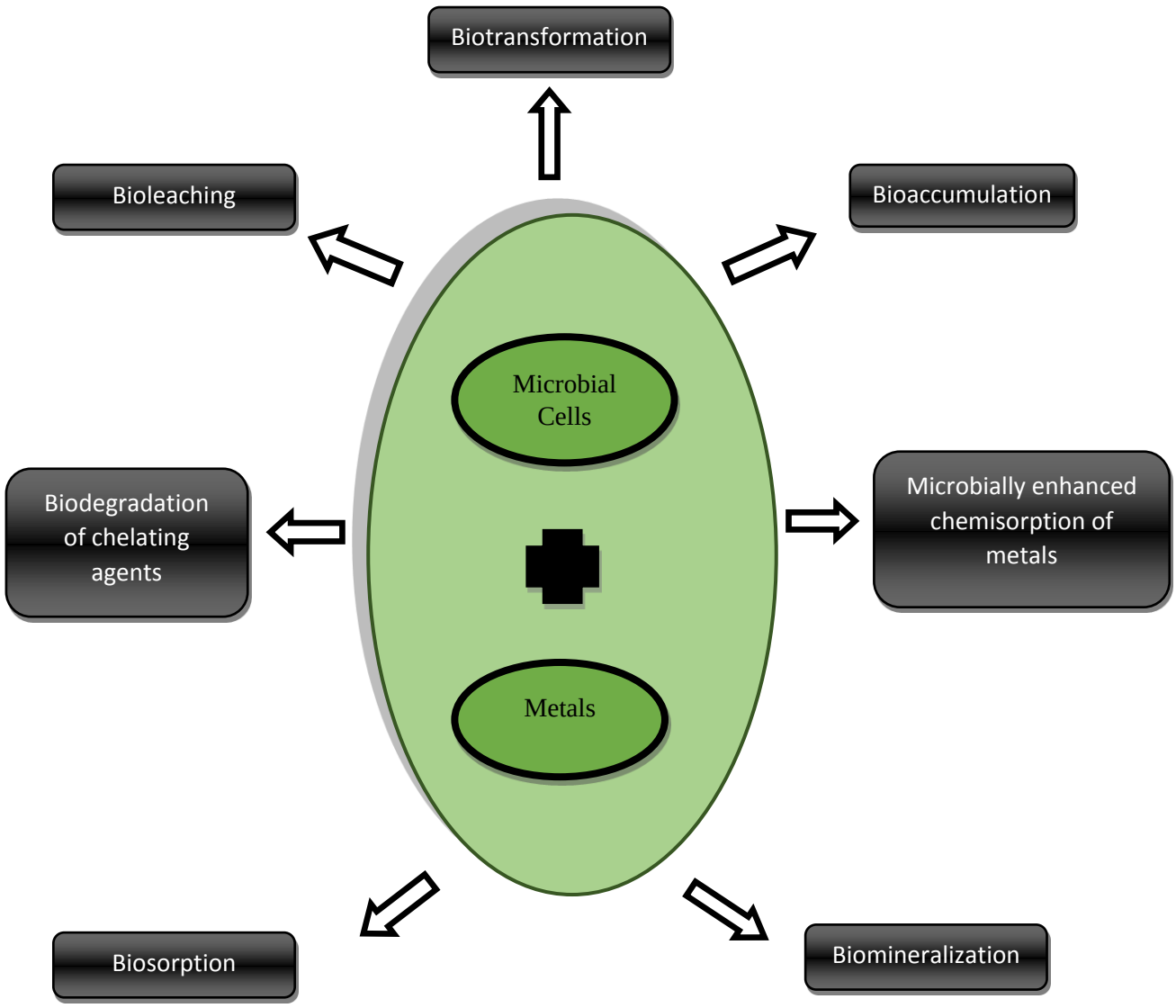
Before long after their revelation of thermophiles, the heat-stable proteins of thermophiles demonstrated to be exceptionally vital to the field of biotechnology. For illustration, two thermophilic species *Thermus aquaticus* and *Thermococcus litoralis* are utilized as sources of the protein DNA polymerase, for the polymerase chain reaction (PCR) in DNA fingerprinting. As thermophiles have ended up progressively vital in biotechnological investigation, numerous groups of analysts are taking an intrigued in thermophiles. Astrobiologists, including researchers from NASA, recommend that hot springs all over the world give a few of the finest "entryways into early Earth." These extremophilic microorganism which have a place to space of Microbes and Archaea, can encourage the enzymatic debasement of polymeric substrates such as starch, cellulose, xylem, pectin, chitin etc. It is very likely that higher living beings are incapable to outlive at extraordinary conditions since of their cellular complexity and compartmentalization (Bertoldo et al., 2002). For the last few decades, researchers are interested in doing investigate in microorganism that develops in extraordinary natural conditions. Their center is to characterize the microorganism and inevitably utilize them in different industrial applications (Tango et al., 2002). Different metabolic forms and different organic functions of these microorganisms are intervened by enzymes and proteins that capacities beneath extraordinary conditions (Niehaus et

al., 1999). As numerous common enzymatic industrial reactions are performed at high temperatures, thermostable proteins have pulled in much consideration in biotechnology and industry in recent time. Their expanded solidness about mesophilic proteins makes them more reasonable for harsh industrial forms. In expansion, their thermo-stability is ordinarily related with the next resistance to chemical denaturants commonly utilized in numerous industrial reactions. Performing enzymatic responses at high temperatures permits higher response rates and prepare yields since of: (i) a diminish in viscosity, (ii) an increment within the diffusion coefficient of substrates, (iii) an increment within the solvency of substrates and products, and (iv) a favorable equilibrium uprooting in endothermal reactions. Proteins from thermophilic living being as a rule display significantly higher inborn warm solidness than other proteins. Proteins including distinctive property have potential significance in gigantic industrial belt. Revelation and utilization of thermostable chemicals in combination with recombinant production and advancement utilizing site-directed and protein advancement technologies, have eradicated a few of the primary recognized hinders (e.g., restricted get to and substrate specificity) for utilize in mechanical bio-catalysis. Nowadays, a few biotechnology companies are persistently prospecting for modern and adjusting existing proteins to responses of higher volumes and more extreme handle conditions (Henry et. al OECD, 2001). Protein prospecting regularly centers on quality recovery straightforwardly from nature by molecular probing methods, taken after by recombinant production in a chosen host. Accessibility of genes encoding steady enzymes, and knowledge on auxiliary highlights within the chemicals, can moreover be utilized in atomic improvement for chemical enhancement. Bio-catalysis, including enzymatic or microbial activities, embrace a double errand within the bio-refinery frameworks, both producing metabolizable building blocks (creating sugars from polymers) for assist transformations, and acting as particular catalysts within

the change of building blocks into wanted products (conversion specificity). A wide extend of response sorts, e.g., oxidations, reductions, carbon-carbon bond formations, and hydrolysis, can be catalyzed utilizing proteins. Thermophilic chemicals are possibly pertinent in a wide run of industrial forms basically due to their exceptional operational solidness at high temperatures and denaturant resilience. Such proteins are utilized within the chemical, food, pharmaceutical, paper, textile, and other businesses (Zaks, 2001; van Beilen et al., 2002; Demirjian et al., 2001 and Gomes et al., 2004).

Thermophilic microorganisms and it's enzymes in Metal Reduction

Thermophilic microorganisms perform an assortment of work, among which one of promising work is metal diminishment. Thermophilic microorganisms can diminish Fe (III), Mn (IV), Cr (VI), U (VI), Tc (VII), Co (III), Mo (VI), Au (I, III) and Hg (II). Ferric iron and Mn (IV) can be utilized as electron acceptors amid development (Slobodkin, 2005). Such microbial Fe (III) reduction is a critical geochemical handle in weathering, the arrangement of minerals and oxidation of organic matter in an assortment of sedimentary situations, counting freshwater oceanic dregs, submerged soils, marine dregs, profound perfect aquifers, and shallow aquifers sullied with natural compounds. Numerous Fe (III)-reducing microorganisms moreover decrease other shapes of oxidized metals, including radio nuclides such as uranium (VI), and technetium (VII) and follow metals counting arsenic (V), chromium (VI), cobalt (III). manganese (IV) and selenium (VI) (Roh et al., 2002). Within the thermophilic microbial community, these microorganisms can satisfy both degradative and beneficial capacities. Such microorganisms likely carry out worldwide diminishment of metals and thought to play a major part within the testimony of minerals within the earth's hull (Slobodkin, 2005 and Frankel et al., 2003).



When different types of micro-organisms are reacting with metals, verity of formations are occurred. Those formations are made up by some process, these are called,

Biotransformation, Bioaccumulation, Microbially enhanced chemisorption of metals, Biosorption, Biodegradation of chelating agents, Bioleaching and Biomineralization.

Fig. 3: Different types of mineralization

Biomineralization

It is a process by which living organisms create minerals and biomineralization could be a common and broad phenomenon happening in different geothermal frameworks.

❖ Importance of Bacteria and it's enzyme in mineralization

Bacteria are exceptionally very tiny in nature (1.5 μm3) but have the immense volume of surface ratio in any form of life. Therefore, they can interact with the environmental molecules by their largest surface area. In both case of Gram-positive and Gram-negative bacteria, the main charged chemical electors, carboxyl, phosphoryl, and amino groups are found at neutral pH. Generally, to give the cell surface of bacteria overall anionic charge, negatively charged groups dominate over positively charged ones (Douglas, 2004). Microbial activity has been observed at least one third of the elements in the periodic table and it's also responsible for the transformation of those charged molecular ions (Belcova, 2005). The microbes produce carbonate, phosphate, oxalate, silica, iron, or sulfur-containing minerals accompany with more exceptional range of biomineralization.

Biomineralization is classified either as extracellular or as intracellular. Extracellular biomineralization includes inorganic, frequently crystalline materials shaping on the external wall of the cell, inside the cell wall or within the immediate encompassing tissue regions. Intracellular biomineralization is mineral development inside the cell such as the calcite deposition for the group of algae the *coccolithophoridae* (Pentecost, 1985 and Borowitzka, 1982).

❖ Different types of mineralization

A. Sulfur biomineralization:

Two large groups of prokaryotic organisms, where some of oxidize and some of reduce sulfur compounds. Few chemolithotrophic bacteria inordinate sulfur from H_2S to SO_4^{2-} whereas some autotrophic thiobacilli oxidise H_2S, S^0 or H_2SO_4 and therefore sulfur mineralization takes place (Ehrlich, 1996; 2002 and Douglas, 2004; 2000).

B. Iron biomineralization:

Bacteria are prime arbitrator in the deposition of iron minerals such as ferrihydrite and goethite, iron oxide magnetite, Fe3O4, or the magnetic iron sulfides, greigite, Fe_3S_4 and pyrrhotite Fe_7S_8 (Hanzlik et al., 1996). Bacterial $FeAS_2$ mineralization was found in Masutomi hot springs, Yamanashi Prefecture, Japan (Tazaki, 2003).

C. Carbonate biomineralization:

The calcium carbonate minerals are the foremost plenteous biogenic minerals. Calcite, aragonite, vaterite etc. are distinctive shapes of calcium carbonate mineralization (Addadi et al., 2003). Bones and shells are self-evident cases of bio-minerals which are made from calcium phosphate. Ordinarily, the external shells are composed of crystals of calcium carbonate whereas the inward parcels (nacre) are composed of aragonite (Mann, 2001).

D. Phosphate biomineralization:

Phosphates include around 25% of the biogenic mineral sorts. The foremost liberally delivered phosphate mineral is carbonated hydroxyapatite, too called dahllite (Lowenstam & Weiner, 1989).

It is the mineral show in vertebrate bones and teeth, as well as within the shells of inarticulate brachiopods.

E. Silica biomineralization:

Silica is the second-most component of the earth's hull and is display in all sorts of diverse compounds. Utilized materials that are composed of silicates are glass, porcelain, enamel, clay products, cement and water glass are exceptionally vital form in a conservative point of view. Other silicon-compounds such as the silicones (siloxanes) have medical importance, such as to produce implants.

- Different Forms of Silica Mineralization

1. **Silicon dioxide:** Silicon dioxide (SiO2) can be found both in crystallized and amorphous form. Quartz, tridymite and cristobalite belong to the different forms of crystalline SiO2 whereas a chat, opal and flint stone represent amorphous silicon dioxide-materials.
2. **Salicylic acids**
3. **Silicones and silicates:** Silicones are classified into:
    a. Linear polysiloxanes (construction type: R3SiO[R2SiO] nSiR.sub.3)
    b. Branched polysiloxanes (with tri-functional or tetra-functional siloxane-units at their branching sites)
    c. Cyclic polysiloxanes (from di-functional siloxane-units)
    d. Cross linked polymers (chain- or ring-form molecules are linked into two- or three-dimensional networks).

❖ Bio-silicification

In nature, gigatons of silica– SiO_2, an oxide shape of silicon – are created annually from silicon, the moment most inexhaustible component on soil, by an assorted gather of eukaryotes (Treguer et al., 1995). In nature (specifically in marine life forms, higher plants, and creatures) bio-silica – the naturally inferred silicon delivered by a few prokaryotes and eukaryotes – is fundamental for the arrangement of a compositionally assorted gather of complex nano-scale structures in shells, spines, filaments, and granules. These nano-scale structures, which are dazzlingly perplexing and are made up of complicated and most frequently symmetrical designs, are species-specific and hereditarily controlled (Pickett-Heaps et al., 1990).

Over the past two decades, critical advance has been made in understanding the organic chemistry of silicon in marine living beings. Organic chemists and molecular scientists have recognized the qualities, proteins and atomic mechanisms included in controlling the natural nanofabrication of silicon-based materials. Later ponders illustrated that, natural particles have a vital part within the arrangement of bio-silica owing to the specificity of intuitive at the organic–inorganic interface. Although numerous living beings from choanoflagellates (Tacke, 1999) and radiolarian (planktons), to higher plants and mollusks (Muller et al., 2003) utilize silica to make these wonderfully controlled species-specific structures, as it were many have been investigated in depth at the atomic level to explain the robotic premise for the method. Silica deposition is additionally seen in plants (Harrison, 1996) and indeed in higher well evolved creatures, which is detailed within the electric organs of the fish *Psammobatis extent* (Figueroa, 2008), yet there is no report on bacterial origin biosilicification.

The ponder of the hydrolysis and condensation responses amid biosilicification is complicated owing to the affectability of silica, silicates and silicic corrosive to pH concentration and

temperature (Iler, 1979). As silicic corrosive antecedents, tetra-alkoxy-silanes are effortlessly hydrolyzed, and the resulting sialons are condensed amid the arrangement of particulate silica. Silicate in (a protein disconnected from the *T. aurantia* marine wipe) catalyze the in vitro poly-condensation of tetraethoxysilane as well as phenyl- and methyl-triethoxysilanes amid the arrangement of siloxane precipitates. By differentiate, polypeptides disconnected from the *C. fusiformis* diatom (i.e., silaffin) and *E. telmateia* plant (i.e., biopolymer) as well as biomimetic analogs (e.g., polyamines) were detailed to catalyze the in vitro hydrolysis (Perry, 2003 and Cha et al., 2000) or condensation responses (Kroger et al.,1996; 2000; 2001 and 2002) amid the formation of silica. Although investigate has advanced in silica biosynthesis, the atomic mechanisms of these intelligent are viably obscure. But to superior get it the part of different proteins within the biosilicification prepare, considers have been conducted to test the capacity of homologous chemicals to catalyze the arrangement of siloxane bonds (Morse, 2001; Tacke, 1999 and Naik, 2002). A few qualities and their individual proteins were recognized from *Suberitesdomuncula* (demo sponge) that were shaped within the nearness of high concentrations of silicon (Schroder et al., 2003). After looking a sequence database, one cDNA was recognized that coded for a chemical that was homologous to carbonic anhydrase. In expansion to testing positive for carbonic anhydrase action with a normal substrate (i.e., ester hydrolysis), the recombinant protein (rSIA_SUBDO-s or silicas) was found to hydrolyze a watery solution of undefined silica (i.e., treated spicules from *S. domuncula*) amid the formation of silicic acid (Brandstadt, 2005). Several studies also reveal that, homologous lipase and protease enzymes catalyzed the formation of siloxane bonds under mild conditions (i.e., pH 7, 25 ˚C) (Brandstadt, 2005).

❖ Industrial Application of Bacterial Bio-mineralization

The request for modern materials and items is still developing and the intrigued in shaped biopolymers and bio-minerals, such as chitin, calcium accelerates, and silica is expanding. Distinctive microorganisms and photosynthesizing micro algae of the family Bacillariophyceae (diatoms), create calcite, silica exoskeletons etc. with a potential to be utilized mechanical or innovative forms (Engel, 1999).

- ✦ Biomineralization has tremendous application in therapeutic and pharmaceutical industry. Biomineralization forms made a difference within the plan of atomic tests (e.g., magnetoferritin), as well as bone substitutes (e.g., a few bio-ceramics composites) (Spontack &Thomann, 2003).

- ✦ Cyanobacteria confined from the Krisuvik hot spring, Iceland, help in viable UV screening through inactive precipitation of iron-enriched silica outsides (Phoenix, 2001 and Phoenix & Bennett, 2006).

- ✦ Expanding natural contamination in urban regions has been imperiling the survival of carbonate stones in landmarks and statuary for numerous decades. Various preservation medicines have been connected for the security and solidification of these works of craftsmanship. Bacterially initiated carbonate mineralization has been proposed as a novel and ecologically neighborly technique for the preservation of weakened fancy stone. In any case, the strategy showed up to show deficiently solidification and stopping of pores. *Myxococcus xanthus* initiated calcium carbonate precipitation effectively secures and solidifies permeable decorative limestone (Carlos Rodriguez-Navarro, 2003).

- Inadequacies of customary surface coatings have drawn the consideration to elective treatments for the change of the durability of concrete. Promising comes about of an inventive biotechnology based on microbial carbonate precipitation have laid to investigate concerning the utilize of microscopic organisms in or on concrete, called Bioconcrete, an inborn self-repairing biomaterial that can remediate the splits and gaps in concrete (De Muyncket al., 2007).

- Achal et al. (2010) investigated a novel approach to restore or remediate breaks and gaps in concrete structures utilizing Bacillus species intervened biomineralization of calcium carbonate. Their ponder displayed that generation of "microbial concrete" by *Bacillus sp.* on developed offices expanded the toughness of building materials. Chunxiang and his colleagues (2009) have effectively accelerated calcium carbonate layer (138-289 μm) on cement-based fabric utilizing *S. pasteurii*. They have drawn a strategy to progress the water entrance resistance and stand up to the assault of the corrosive (pH 1.5) of the development fabric surface.

Recapitulation of Bio-concrete

Normally happening substances, like clay, rocks, sand, and wood, indeed twigs and clears out, have been utilized as building materials from antiquated periods. Different man-made engineered items are moreover utilized into concrete innovation. Employments of these materials are characteristically sectioned into claim to fame exchanges. Those sorts of building materials are utilized for development of carpentry purposes, insulation, plumbing, and roofing works (Building def. 2 and 4, Oxford University Press, 2009).

Concrete is the foremost versatile construction materials accessible in around the world. Due to its excellent durable and low-cost character, it is utilized to create asphalts, architectural structures, establishments, motorways/roads, and bridges/overpasses and stopping structures, brick/block dividers and footings for gates, fences, and posts. The word "concrete" comes from the Latin word "concrētus" which implies "solidified" or "hard" (Concrētus. Latin Look up. October 2012). Concrete is utilized in huge amounts nearly all over mankind includes a require for framework improvement. The sum of concrete utilized around the world, ton for ton, is twice that of steel, wood, plastics, and aluminum combined. Within the cutting-edge civilization, utilize of concrete is surpassed as it were by that of normally happening water (Cement Trust, January 2013).

Mortar (Latin word "mortarium") is an elective building fabric of without aggregates, comprising cement, sand, and water. It is a successful glue, utilized to tie diverse sorts of building pieces such as stones, bricks, and concrete stonework units together. It fills and seals the regular/irregular openings between the concretes, and some of the time includes embellishing colours or designs in brick work dividers. In its largest sense, mortar incorporates pitch, black-top, and delicate mud, or clay, such as utilized between mud bricks (Mortar Wikipedia). The cement mortar gets to be difficult, coming about in an inflexible total structure by curing strategy. The mortar is simpler and less costly to repair the absconds within the building squares. Be that as it may, it is comparatively weaker than other the building materials and the conciliatory component within the brick work. In later times, the foremost common folio viz Portland cement is utilized in mortar while the ancient binder lime mortar is additionally used in a few modern developments. Lime and gypsum within the shape of mortar of Paris, are utilized especially within the repair and repointing of buildings and structures. But these sorts of mortar are not as tough as other mortars in damp conditions (Introduction to Mortars, Cemex Corporation). Generally, building with concrete and mortar

another showed up in Greece. The pozzolanic mortar could be a lime-based mortar which is made with an added substance of volcanic fiery remains and gets to be solidified beneath water. That's why, it is known as pressure driven cement. After that, by moving forward the utilize and strategies of making of mortar, pozzolanic mortar and cement are made (HCIA - 2004).

Pozzolana may be a fine, sandy volcanic fiery remains which was initially found and burrowed at Pozzuoli, adjacent Mount Vesuvius in Italy, and was hence mined at other locales, as well. The Pozzolana is included to lime mortar and permitted it to set generally quick and indeed under water. There are four sorts of Pozzolana. It is found in different colours: e.g., dark, white dark and ruddy. Pozzolana has since ended up a nonexclusive term for any siliceous and/or aluminous added substance to slaked lime to form hydraulic cement (Pozzolana 10th edition, 2014).

There are numerous sorts of concrete accessible in all over. They are fabricated by shifting the extents of their primary ingredients. Substituting the cementitious and aggregate stages, the ultimate item can be custom-made for its different applications with changing quality, thickness, or chemical and thermal resistance properties (Abdou, 1994; Hasan, 1999 & Al-Jabri, 2005).

"Aggregate" comprises of huge chunks of fabric in a concrete blend, by and large coarse rock, or smashed rocks such as limestone, or stone, alongside better materials such as sand. "Cement", most commonly Portland cement is related with the common term "concrete." A extend of materials can be utilized as the cement in concrete. One of the foremost commonplaces of these elective cements is black-top. Other cementitious materials such as fly ash and slag cement are now and then included to Portland cement and gotten to be a portion of the cover for the aggregate. Water is at that point blended with this dry composite, which produces a semi-liquid that can be molded

(ordinarily by pouring it into a frame) into different shapes. The concrete sets and solidifies through a chemical handle called hydration (Wild, 1996).

Portland cement is the fundamental fixing of concrete, mortar and plaster and comprises of a blend of oxides of calcium, silicon, and aluminum. Portland cement and comparative materials are made by warming limestone (a source of calcium) with clay and pounding this item (called clinker) with a source of sulfate (most commonly gypsum). A few sorts of Portland cement are accessible with the foremost common being called ordinary Portland cement (OPC). It is generally grey in colour, but a white Portland cement is additionally accessible. Portland cement is caustic, so it can cause chemical burns, the powder can cause aggravation or with serious exposure lung cancer and contains a few harmful fixings such as silica and chromium (Hills,1993; Hillier, 1999; Duarte, 2005 & Borka, 1980).

- Durability of concrete structure

There are two fundamental properties of concrete viz quality, and strength make it the generally utilized development fabric. Strength is the capability of concrete to stand up to push like compressive, tensile, shear or torsion. Strength of concrete may be a work of numerous variables as well as concrete blend design, curing method, utilizing different sort of strengthening steels etc. On the other hand, toughness is another critical property of concrete structure. The foremost vital characteristic is believed to be influencing the concrete solidness is penetrability of concrete. There's an inexact reverse relationship between concrete penetrability and compressive strength as well as durability.

Penetrability of concrete can be calculated by measuring the rate of liquids like oxygen, water, and chloride particles infiltration into concrete frameworks to realize a certain level. As an illustration,

most of the sorts of weakening of steel bars are impacted by liquid entrance (or development) in concrete. Solidness is the capacity to final a long time without noteworthy disintegration. A strong fabric makes a difference the environment by moderating assets and decreasing squanders and the natural impacts of repair and substitution. Development and annihilation waste contribute to strong squander attending to landfills. The generation of unused building materials depletes normal assets and can create discuss and water contamination. The plan benefit life of most buildings is frequently 30 years, although buildings frequently last 50 to 100 years or longer. Most concrete and brick work buildings are pulverized due to out-of-date quality instead of weakening. A concrete shell can be cleared out in place in case a building utilization or work changes or when a building interior is redesigned.

- ❖ Physical & bonding strength of concrete structure

Bond strength is the state in which molecules are combined in a chemical bond pays to the valance of each other Bond quality is closely related to bond arrange and can be calculated by bond energy, bond-dissociation energy, and force consistent. Bond quality is the subjective connection between the cover of nuclear orbitals of the bonds and bond energies. The more cover of these nuclear orbitals, the more the holding electrons are to be found between the nuclei and consequently bond will end up more grounded. Cover is fundamental for the creation of atomic orbitals. This cover can be measured and is called the cover necessarily. Bond quality is the shearing push created at the steel-concrete interface on account of composite interaction between the two materials, contributing towards ductility viewpoint of basic behavior. The bond between the steel and concrete empowers the two materials to act together without slip at the benefit capacity constrain state and serves to control the split width and diversion. At extreme restrain state, the quality of the laps and moorings depend in bond. The bond stretches in strengthened concrete part emerges

from the jetty of bars and alter in bar drive along its length or due to shifting twisting moment. Mobilization of bond must be guaranteed beneath assortment of stacking circumstances such as pressure, compression, and flexure. The introductory bond quality comes from the week chemical interaction between steel and solidified concrete, but this resistance is broken at low stress. After event in case slip grinding and contributes to bond (Hardjito, 2005). In case of ribbed bars bond is to a great extent contributed due to the mechanical interaction between the ribs on the surface of the bar and the encompassing concrete (Provis, 2009 & Lloyd, 2009). Hence, mechanics of the bond is complex, and activity isn't as it were since of attachment of steel with concrete, but moreover mechanical locking due to projections on the bar. With High Strength Deformed bar (HSD) the component of support mooring is basically due to reason such as (a) attachment of concrete and steel, (b) Shear strength of concrete and (c) Interlocking of ribs with concrete.

- Disadvantages of concrete

Splitting in concrete may be a common wonder due to the moderately destitute malleable quality. High tensile stresses advertised by the outside loads, temperature slopes, restricted shrinkage, and differential settlement, execute distortions of plastic shrinkage, plastic settlement, and broad responses. Concrete is weakened due to fortification erosion soluble base silica response and sulfate assault etc. Without quick and appropriate repair, small scale splits and gaps made interior concrete/mortar tend to extend encourage and inevitably require exorbitant repair.

Solidness and qualities of concrete/mortar is additionally reduced by these micro-fissures since they give simpler entries for the transport of liquids that possibly contain destructive substances. When micro-cracks develop inside the concrete and come in touch of fortifications, it disintegrated the concrete structure as well as the fortification will & eroded with the uncovered to H_2O and O2, and conceivably CO_2, Cl and SO ions (Lin, 1996; Khoury, 2000; Gjorv et al., 1971; Hong, 1999;

Yonezawa, 1988; Mehta, 1983 and Saleem, 1996). The major source for sulfate is soil, ocean water, sewers, and a few chemical operations. The common sulfate shapes which attack concrete are calcium sulfate (Ca_2SO4), sodium sulphate (Na_2SO_4), and magnesium sulphate (Mg_2SO4), and potassium sulphate (K_2SO_2). All cases of sulfate attack, huge ettringite gems (since of the huge number of accessible water particles) are shaped coming about ductile constrain since concrete is solidified at the time of sulfate attack. Creation of splits and absconds are happened inside the concrete as these forces ended up higher than concrete tensile quality. Close to losing strength and loading capacity, split concrete is defenseless to the other forceful outside variables (Win, 2004). Ocean water and saline groundwater are fundamental sources of chlorides as well as. Due to chloride entrance into the fortified concrete, the de-passive layer around the strengthening bars, which ensures the bars from erosion, is annihilated. Steel bars are ordinarily erosion secured when implanted in tall pH concrete. The range which gets CI particle gets to be an anode and other range of the bar which gets oxygen and water gets to be a cathode. Water and oxygen accessibility is vital for erosion of de-passivated bars.

Due to the entry of hydroxide particle through concrete from Cathode to Anode, expanding the electrical resistivity of concrete can break the erosion electrical circuit. Concrete electrical resistivity could be a work of concrete dampness substance, pore stricture network, and ionic substance of pore arrangement Concretes (in dry environment) are erosion safe since of their high electrical resistivity (Monfore, 1968).

It is worth of noticing that due to the dangerous impacts of chloride on strengthened concrete structures, quickening admixtures containing calcium chloride ($CaCl_2$) is not permitted in fortified or pre-stressed concrete structures.

❖ Self-healing activity

Self-healing concrete is for the most part characterized as the capacity of concrete to repair its little splits independently (Ghosh, 2009). The thought of self-healing concrete was propelled from the normal wonder by organisms such as trees or animals. Harmed skin of trees and creatures can be repaired independently (Ghosh, 2009 & Talaiekhozani, 2014). Remediating splits in concrete structure is critical for its benefit toughness and auxiliary security (Zhong, 2008). A few forms are proposed for the plan of self-healing concrete. self-healing concrete advances containing (1) characteristic (2) chemical and (3) natural forms. **Figure 4** appears the comprehensive scientific categorization for self-healing concrete inquire about.

Within the angle of self-healing concrete, a few surveys are found. A most recent survey on self-healing concrete was by Wu et al. (2012) who highlighted on the common and man-made self-healing. Their audit too secured comprehensive clarification on the chemical and natural strategies (Wu et. al., 2012). Siddique and Chahal (2011) point by point the utilize of ureolytic microscopic organisms for the planning of self-healing concrete. Toohey et al. (2007) checked on micro-vascular as self-healing fabric. Jonkers (2007) checked on on natural strategies to plan self-healing concrete based on calcium carbonate precipitation. Al-Thawadi (2011) recognized the instrument of quality improvement of sand utilizing ureolytic microbes and calcium carbonate arrangement.

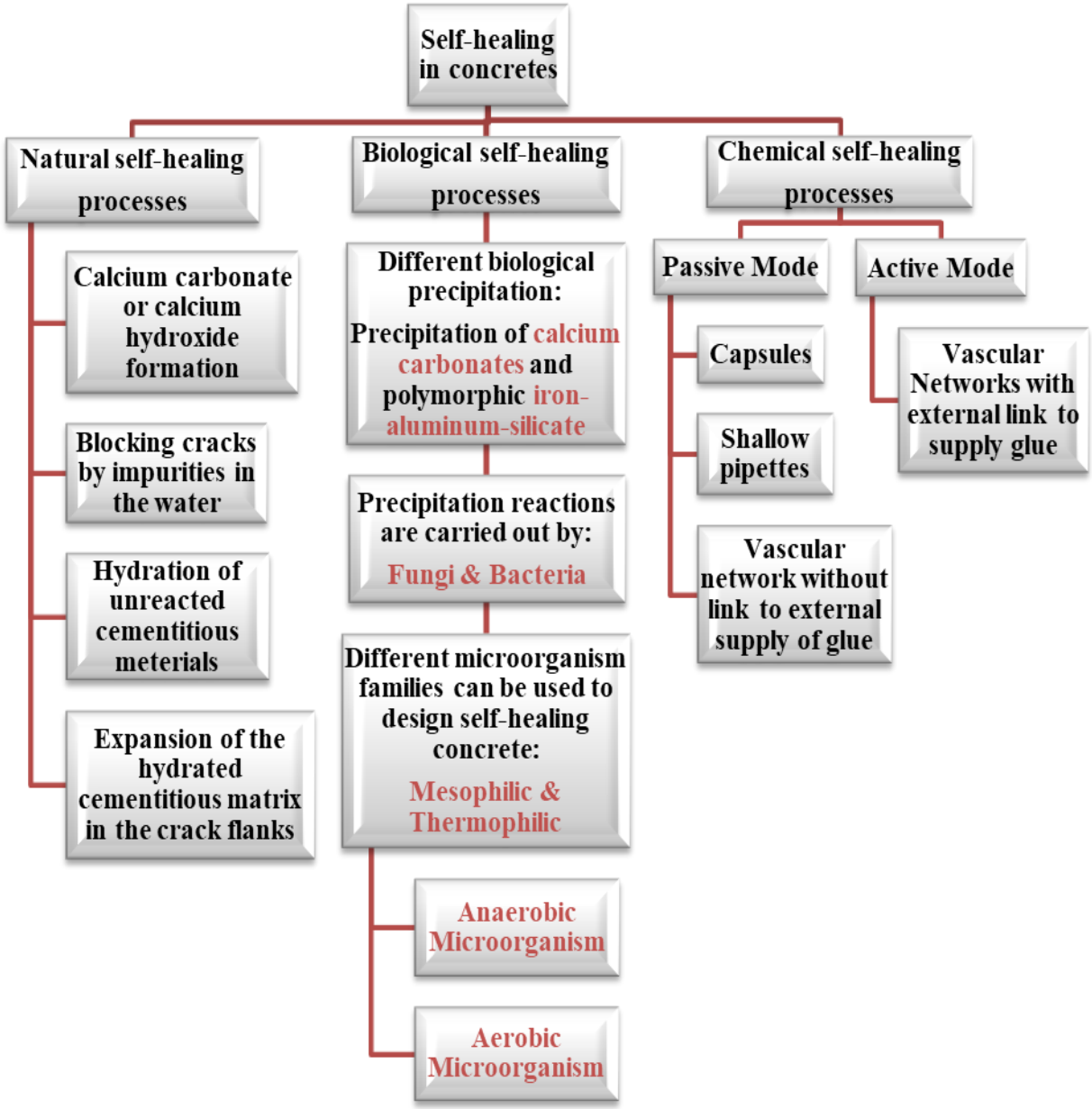


Fig. 4: Flow chart of different types of self-healing process in concrete

Natural Process: Among the proposed self-healing components within the characteristic prepare, arrangement of calcium carbonate and calcium hydroxide are the foremost successful strategies to mend concrete actually (Van Der Zwaag, 2007; Edvardsen, 1999; Yang, 2009; Qian et al., 2009 and Homma et al., 2009). This see is bolstered by the truth that a few white buildup can be found

on the external surface of the concrete splits. This white residue is found to be calcium carbonate and has been widely detailed counting by Wu et al. (2012). The elemental instruments for the arrangement of calcium carbonate and calcium hydroxide (Edvardsen,1999) are spoken to in Eqn. (1) to (3). At the primary step, carbon dioxide is broken down in water.

$$H_2O + CO_2 \leftrightarrow H_2CO_3 \leftrightarrow H^+ + HCO_3^- \leftrightarrow 2H^+ + CO_3^{2-} \qquad \textit{Eqn. (1)}$$

Free calcium particles are discharged results of cement hydration and dissemination through concrete as well as along the breaking surfaces, reacts with CO_3^{2-} and HCO_3^-. As a result, calcium carbonate crystals are formed. Reaction (2) and (3) can as it were happen at pH over 8 or between 7.5 and 8. The crystals develop both at the surface of the splits and at last fills the crevice.

$$Ca_2+ + CO_3^{2-} \leftrightarrow CaCO_3 \qquad \textit{Eqn. (2)}$$

$$Ca_2 + HCO_3^- \leftrightarrow CaCO_3 + H^+ \qquad \textit{Eqn. (3)}$$

Neville (2002) claimed that, advance hydration of anhydrate cementations components is primarily due to the normal self-healing properties in concrete. In any case, this prepare as it were applies to exceptionally youthful concrete and the arrangement of calcium carbonate most likely causes self-healing at afterward ages. Characteristic self-healing can be valuable for cracks with widths up to 0.1–0.2mm (Li et al., 2007).

Chemical process: Chemical recuperating process basically alludes to the fake mending by infusing chemical compounds into the split for mending. Self-healing concrete is outlined by blending chemical fluid officials (i.e. stick) with new concrete in little holders. The chemical self-healing mode for concrete can be partitioned into two categories: **a) active mode b) passive mode**. Active mode employments vessel organize connected with outside supply of glue for the dissemination of glue while the inactive mode employments empty pipettes, capsules or vessel

arrange to disperse glue that's not connected to an outside glue source. Few considers appeared that concrete containing empty pipettes after flexural test and stick discharging into its splits can stand up to 20% more stack beneath a consequent flexural test (Homma et al., 2009). In spite of the fact that a few analysts have detailed empty pipettes and vessel systems containing glue as strategies for planning self-healing concrete, the strategy has to be confirmed for the utilize in genuine projects (Dry, 2000; Joseph, 2007 & Mihashi, 2000).

Biological Process: The utilize of microorganisms to plan self-healing concrete has been categorized as natural technique by a few analysts (jonker, 2007). Microorganisms can develop almost all over such as soil, water and oil supply, acidic hot springs and mechanical wastewater. The utilize of microorganisms to plan self-healing concrete is our essential objective. Gollapudi et al. (1995) presented organic self-healing concrete as environment neighborly process. Microorganisms are for the most part separated into three imperative categories: microbes, organisms, and infections. Among these microorganisms, microbes is my think about zone. In this investigate thermophilic and anaerobic bacterium BKH4 is joined within the cementitious material to create Bioconcrete.

- Bioconcrete:

Bioconcrete has presently gotten to be an imperative region of investigate within the field of concrete innovation to illuminate the issue of self-healing. Particular microbial communities, owing to their biomineralization property, create or filter biomaterials or a few other materials interior the concrete frameworks and alter its chemistry. This wonder is guided by the biochemical activity of a few particular protein(s) inside the microscopic organisms. This leads to a modern measurement in concrete innovation by the improvement of self-healing, eco-friendly and more solid concrete fabric. A novel thermophilic and anaerobic bacterium and one of its particular

protein separated from Bakreshwar hot springs appear that on expansion of this bacterium/protein to the cement-sand mortar or concrete, the in general behavior of the concrete may be progressed (more than 30%) considerably. A silicase (silica leaching) like chemical named as “Bioremediase” is discharged by the bacterium, which plays the crucial part within the improvement of the Bioconcrete fabric. This protein is thermostable and remained dynamic at exceptionally high pH (pH 6.5 to 13.0) and temperature (50°C to 80°C). This may alter the conveyances of the framework composites as well as the mechanical properties of the components of cement paste inside the frameworks. The interaction of the protein with the matrix multiphase components moreover changes the matrix composition which is reflected in micro auxiliary characters of the fabric. The positive alter within the compressive quality, porosity, energetic flexible modulus and hardness of the protein corrected cement paste fabric certainly make strides the quality and strength of the item (Chattopadhyay et al., 2010).

In spite of the fact that, self-healing forms containing common and chemical are well known to plan self-healing concrete, organic prepare may be a youthful promising innovation, which has not been completely caught on however. Till presently, numerous microscopic organisms can be disconnected from nature that are valuable for planning self-healing concrete. Utilizing microscopic organisms have numerous preferences such as (1) Microbes are easy to culture. (2) Separation of microscopic organisms isn't exceptionally complex and (3) numerous strategies have been depict for including microbes to concrete. My investigate work expectation illustrate the awesome potential of organic method,by utilizing the exceedingly alkaliphilic microbes, able of accelerating galhenite (calcium-sodium-silicate), as giving the way forward for creating natural self-healing concrete.

AIMS & OBJECTIVES

Aims & Objectives

Overview of the thesis

The present work deals with the isolation and characterization of profoundly alkaliphilic bacterium BKH4 and the filtration of its excretory protein. The partial characterization of microbial (BKH4) protein in regard of utilitarian space. This protein encompasses a vital part within the application of bio concrete technology. The following aims and objectives of my investigate work:

Chapter 1: Isolation and Characterization of BKH4 Bacteria

Chapter 2: Isolation, Purification and Partial Characterization of (BKH4) Bacterial Protein

Chapter 3: Application of Bacteria & Bacterial (BKH4) Protein in Concrete Technology

Chapter 4: Autonomous Self-Healing Attributes of Bacterial (BKH4) Protein

Consortium of the research work

The display ponder is outlined to isolate and characterize a highly alkaliphilic bacterium BKH4 from the microbial diversity of hot springs at Bakreshwar, West Bengal, India. This bacterium is capable to extend (>50%) compressive strength in concrete/mortar. The bacterium BKH4 secretes few proteins in its growth medium, one of which is responsible for bioremediation of concrete. The protein has silica filtering trait which by catalyzing the biomineralization of certain mineral

inside the pores of concrete causes increase of both compressive and tensile quality when consolidated to it. This proposal comprises four chapters.

In **chapter 1**, the isolation and characterization of bacterium BKH4 is discussed. 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. Through the serial dilution technique and after that the partial r-RNA sequencing of the bacteria, BKH4 is isolated. The isolated BKH4 is coccoid-shape and Gram-positive in nature. Bacterium grows well in a defined semi synthetic iron and magnesium enriched medium at pH 12 and 65°C.

In **Chapter 2**, It deals with the confinement of the protein from the bacterium BKH4 and its refinement through Sephadex G-100 gel filtration chromatography strategy. The filtered protein in this way obtained is affirmed through biosilicification assay. The movement of the protein is additionally checked about temperature, pH, and buffer concentration. Through the partial characterization of this protein, 28 KDa weight is determined by gel electrophoresis (SDS-PAGE) strategy.

In **Chapter 3**, It investigates the independent bioremediation of bacterial protein in concrete innovation. The bacterium BKH4 has special property of biomineralization which, when blended with mortar test can exchange silica from the encompassing environment of CSH (calcium silicate hydrate) gel of mortar framework. Comparative sort of biomineralization is watched with the bioremediase protein that can separate silica from silica containing compounds or stages (anhydrite cement particles) and responds with calcium and aluminum oxides to create calcium-aluminum-silicate, a new phase inside the matrix. Just like the bacterium, the protein when consolidated with

cement paste or mortar too increases the compressive quality. The microstructure investigation of the protein blended cement paste uncovers the reality that the protein increases the hydration of the cement particles and the calcium/silicon proportion inside the matrix. The uniform conveyance of CSH gel inside the framework changes the pore estimate dispersion inside the framework and plays the key part for the enhancement of in general behavior of bacterial concrete.

In **chapter 4,** The self-healing nature of the protein on artificially made micro-cracks within the mortar tests is affirmed with crackscopic examination of the mortar test. The powder crystal X-ray investigation of the mortar tests with or without bacterial protein appeared that there were some additional crests within the XRD spectra of the microbes' treated tests which were missing within the control tests and these modern crests coordinated with the minor crests of calcium-aluminum-silicate stage ($Ca_2Al_2SiO_7$ or Gehlenite) which may be capable for at least 40% of compressive strength increase of concrete.

Hence, it can be concluded that, this hot spring anaerobic bacterium BKH4 separated from Bakreshwar, West Bengal has a silica leaching protein that encompasses a catalytic character for which it can separate silica from silica rich substances and this property has been utilized for biosilicification in cement paste or mortar.

MATERIALS AND METHODS

Materials and Methods

The major experimental studies have been conducted in the Biophysics laboratory of Physics Department and Concrete Laboratory of Civil Engineering Department, Jadavpur University. Some instrumental facilities were taken from the Department of Biotechnology-IPLS, Calcutta University and Indian Association for the Cultivation of Science, Kolkata, India.

MATERIALS & CHEMICALS

Sephadex G-100, Protein marker (Low molecular weight), Tetra-ethyl-orthosilicate (TEOS), Dialysis bag, were purchased from the Sigma Chemicals, USA. Standard silica solution, Tris's buffer, Ferric chloride, Sodium di-hydrogen phosphate, Potassium chloride, Sodium carbonate, Calcium chloride, Magnesium chloride were purchased from the MERCK, Germany. Sodium dodecyl sulphate (SDS), pure grade Sucrose, Sodium hydroxide, Metol, Oxalic acid, Copper sulfate, Sodium potassium tartrate etc. were purchased from the HIMEDIA, India. Acrylamide, Bis-acrylamide, Coomassie Brilliant Blue R-250, Acetone, Glycine, Glacial Acetic acid etc. were purchased from the Spectrochem Pvt. Ltd. India., Protein molecular markers were purchased from the Fermentus, India. TEMED, Yeast extract, Sodium chloride, EDTA, Bromophenol blue, Methyl alcohol, Peptone, ß-mercaptoethanol, silver nitrate, Folin reagent, Bovine serum albumin (BSA), Glutaraldehyde, Ethyl alcohol, Nitric acid, Sulfuric acid, Hydrochloric acid etc. were purchased from the SRL, India. 43 grade ordinary Portland cement (IS 8112, 1989) and natural Ennor sand (IS 650, 1991) were used for concrete/mortar samples preparation.

EXPERIMENTAL PROCEEDINGS

Before jumping into the methodology of my study, first it is essential to know about 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 and Chaudhuri et al., 2016). All the above, the growth of the bacterium BKH4 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.

CHAPTER 1

Chapter 1: Isolation and Characterization of BKH4 Bacteria

Collection and Identification of BKH4

❖ Sample collection

The bacterial strain BKH1 was isolated from Bakreshwar hot springs, West Bengal, India and cultured in the Biophysics Laboratory, Department of Physics, Jadavpur University. There are nine different hot springs in Bakreshwar having different temperature and pH sources. The desired bacterium was obtained from the hot spring having temperature 65℃. Soil specimen along with some water was taken out from that hot spring and cultured in our laboratory by using a specific synthetic medium to obtain a mixed population of culture. The culture was maintained by the process of sub culturing of the mixed population culture on regular basis. Purified BKH1 strain was obtained by growing the mixed population culture through the process of serial dilution technique. 16 S-rRNA sequence of the isolated strain was done and identified the bacterial strain and named as BKH1 strain.

❖ Preparation of culture media

For the growth and maintenance of the isolated bacterial strains, a special type of media was synthesized, which satisfactorily maintained the bacterial population. This water borne hot spring bacteria had some unique requirement of oxidizing agent (Lovely, 2000) needed for their metabolic activities. In artificial growth medium of bacteria +III state iron (0.1) is used which accepts electron and goes into +II state. This special media is formed by combining two separate media – **medium I** and **medium II**.

Media I composition:

Medium I contain 0.1 M $FeCl_3$. At first 0.1 M ferric chloride ($FeCl_3$) reagent was dissolved in 100 ml sterile deionized water and pH of solution was adjusted to 7.5 by adding 5(N) NaOH. Precipitate of ferric hydroxide [$Fe(OH)_3$] was thus formed which was collected by centrifugation at 5000 x *g* for 10 minutes and then washed twice with sterile deionized water to remove chloride ions (Cl^-) from the precipitate. This precipitate was then dissolved in 100 ml sterile deionized water and kept at 4ºC.

$$FeCl_3 + 3\ NaOH \rightarrow Fe(OH)_3 \downarrow + 3NaCl$$

$$FeCl_3 + 3\ NaOH \rightarrow FeO(OH) + H_2O + NaOH$$

Medium I contain iron in +III state as FeO (OH)

Medium II composition:

Sodium dihydrogen phosphate: 0.6 g/litre

Potassium chloride: 0.33 g/litre

Sodium carbonate: 2.5 g/litre

Yeast extract: 0.02% (200 mg / litre)

Peptone: 0.5% (5.0 g / litre)

Magnesium chloride: 4mg/liter

After preparing the two media – **medium I** and **medium II**, they were mixed in the ratio of **1: 9** respectively to obtain the final medium. It was then filtered to remove extra precipitate and then pH of the medium was adjusted to 8 by adding HCL (6 N).

❖ Isolation of pure bacterial strain

1. Serial dilution technique

A **serial dilution** is used to reduce a dense culture of mixed populated bacterial cells to derive pure strain. Through serial dilution process some specific number of bacteria will remove and reduce the concentration of the solution. The mixed culture obtained from the hot spring of 65 ºC temperature was serially diluted with sterile liquid media to obtain a pure strain. This technique was originally developed by Lister.

Procedure

Enrichment growth medium of 40 mL was taken in sealed gas-pressure vials (100 mL capacity) and inside air content of the vial was removed totally by-passing carbon dioxide with the help of syringe-needle system prior to inoculation (Chattopadhyay et al., 1993). From the crude soil sample 1% inoculums was added initially to the growth medium and incubated at 65° C for several days to obtain the sufficient growth of the bacterial population. A mixed population of culture was thus developed and subsequently inoculated in fresh growth medium for subsequent subcultures. Pure culture of the bacterial strain was then obtained from these mixed bacterial populations by serial dilution technique. At first the concentration of the bacteria present in the mixed population of culture was determined as described by Biswas et al. (2010). From mixed population 100 µL of inoculums was diluted with 900 µL of sterilized medium and then from this diluted solution again 100 µL inoculums was added to 900 µL of fresh sterilized medium. This procedure was repeated till the final dilution contains single bacterium/ml of culture. Finally, from this dilution 5 mL of inoculums was added to 35 mL fresh growth medium in 100 mL volumetric sealed anaerobic gas-pressure vial. The vials were kept at 65 ºC for 6-8 days to get enough bacteria in the cultured medium. Sub culturing was done by this method and pure culture was obtained after few subcultures which were confirmed by Scanning Electron

Microscope (SEM) and Atomic Force Microscopy (AFM) analysis of the culture. The bacterium was unable to grow on other nutritionally rich solid media (Tryptic soy agar, nutrient agar, Luria-Bertani agar).

2. Partial rRNA sequencing

Purified BKH4 strain was obtained by growing the mixed population culture through the process of serial dilution technique. 16 S-rRNA sequence of the isolated strain was done and identified the bacterial strain and named as BKH4 strain.

❖ Characterization of the BKH4

1. Morphological study

Scanning Electron Microscopic study

Scanning electron microscopy (SEM) is one of the best suited out method to visualize the external appearance of bacteria. Microscopic organisms live in different situations and their preparation for SEM, hence takes their nature into thought. Although numerous microbes are comparable in morphology but there is exceptional sum of variety due to contrasts in hereditary qualities and ecology.

Procedure:

For Scanning Electron Microscope (SEM), slides were prepared by fixing bacterial cells with 2.5% (v/v) glutaraldehyde in culture medium for approximately 24 hours at room temperature. Samples were dehydrated by incubation at 65°C for 15 minutes in each of a graded aqueous acetone series of 20, 40, 60, 80 and 100% acetone. Samples were air dried and transferred onto SEM alumina bolsters and sputtered with gold by a sputter coater. Slides were observed under SEM (JEOL-JSM 5200) and photomicrographs of bacterial cells were taken.

Study of Gram positive/gram negative nature

The basic principle of gram staining involves the ability of the bacterial cell wall to retain the crystal violet dye during solvent treatment. (Libenson et al., 1955) Gram-positive microorganisms have higher peptidoglycan content, whereas gram-negative organisms have higher lipid content. (Shugar et al., 1954)

Procedure:

The first step in gram staining is the use of crystal violet dye for the slide's initial staining. The next step involves using iodine to form crystal violet- iodine complex to prevent easy removal of dye. Hence, a decolorizer, frequently dissolvable of ethanol and acetone, is utilized to evacuate the color. With the disintegration of the lipid layer, gram negatives lose the primary stain. In differentiate, dissolvable dehydrates the gram-positive cell walls with the closure of pores anticipating dissemination of violet-iodine complex, and hence, microbes stay stained. The extreme step in gram staining is to utilize fundamental fuchsin stain to provide decolorized gram-negative microscopic organisms pink color for simpler distinguishing proof. It is additionally known as counterstain.

Effect of pH and temperature on bacterial growth

The growth curve of the bacterium BKH4 at different pH of the medium (6–13) at 65°C was plotted. Sufficiently grown bacteria culture of 1 ml (107 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 were 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).

2. Amplification and Sequencing of 16S rRNA bacterial Gene for Phylogenetic Analysis

Genomic DNA was isolated from the bacterial cells. All 16S rRNA genes are composed of several variable and conserved regions. Approximate size of the 16S rRNA gene is around ~1.5 kb. We have selected the primer set designed for V3 region (5'-GGCGGATGTGTACAAGGC-3' and 5'-GACTTGCATGTGTTAGGCCTG- 3') for amplifying 16S rRNA gene. The PCR (Veriti® Thermal Cycler, Applied Biosystems, USA) reaction mixture contained bacterial DNA: 1 µl (50 ng) as template, 16S Forward Primer: 400 ng, 16S Reverse Primer: 400 ng, dNTPs (2.5 mm each) 4 µl, high fidelity DNA polymerase 1 µl (3 U/µl) and 10 µl of 10 × Taq DNA polymerase Assay Buffer with a reaction volume of 100 µl. The PCR operating condition was 94℃ for initial denaturation for 5 minutes and subsequently 94℃ for 30 s, 55℃ for 30 s, 72℃ for 30 s (These three temperatures was maintained for 35 consecutive cycles) and finally 72℃ for 7 min. Partial amplification of the 16S rRNA gene was done by performing PCR with bacteria specific universal primers as: 5' - GGCGGATGTGTACAAGGC - 3' and 5' - GACTTGCATGTGTTAGGCCTG - 3'. The genomic DNA was used as template for Ribo-typing and construction of phylogenetic tree. A 1.4 kb band was detected and used for sequencing in PCR. The PCR product was purified using ExoSAP IT (Affymetrix) and then sequenced with capillary sequencer (Applied Biosystems 3500, Genetic Analyser, USA) using the above-mentioned primers. Sequencing reaction was performed using Big Dye Terminator Cycle sequencing Kit V3.1 (Applied Biosystems, USA) following the manufacturer's protocol. The partial 16S rRNA gene sequence of the isolated organism was compared by BLAST search

analysis (http://www.blast.ncbi.nlm.nih.gov) with those available in the public databases. Thereafter, identification of the level of the species was determined by 16S rRNA gene sequence similarity of that of the prototype strain sequence in the GenBank (http://www. ncbi.nlm.nih. gov/genbank). Sequence alignment of the 16S rRNA gene fragment and comparison was performed using the multiple sequence alignment program CLUSTALX (v 1.83) with default parameters and the data converted to PHYLIP (V 3.57c) format. The frequency with which a given branch is found is recorded as the bootstrap proportion. This proportion was used as a measure of the reliability (within limitations) of individual branches in the optimal tree (Felsenstein et al., 1985). Thorough analyses of multiple alignments of several random samples were taken. Minor modifications in the alignment were done using the BIOEDIT (v 7.2.6) sequence editor. Thereafter, uprooted phylogenetic tree was constructed using the neighbour-joining (NJ) method. TREEVIEW (Win 32) programme was used to display the phylogenetic relationship.

CHAPTER 2

Chapter 2: Isolation, Purification and Partial Characterization of Bacterial Protein from BKH4 Strain

Extraction of crude protein from BKH4 culture media

Bacterial cells from five cultural vials (each contain 40 mL of 5-6 days old bacterial culture) were collected by centrifugation at 5000 x *g* for 10 minutes using Eltek Centrifugation Machine (TC 4100D). The precipitated cells obtained through centrifugation were discarded. The supernatant containing the protein of interest named "**bioremediase**" was taken as this protein secreted by the bacterium "**BKH1**" in its growth medium (Biswas et al., 2010). The supernatant contains the desired crude protein was placed in a separate container and stored at -20 ºC for further analysis. Two different methods were adopted for the isolation of crude protein present in the supernatant.

- ### Ammonium Sulphate Precipitation

Ammonium sulfate precipitation may be a strategy utilized to accelerate proteins by changing their solvency. It is commonly utilized as its solvency is so high that salt solutions with high ionic strength are permitted. The solvency of proteins shifts agreeing to the ionic strength of the solution, and subsequently agreeing to the salt concentration. Two impacts are observed – at low salt concentrations, the dissolvability of the protein increments with expanding salt concentration (i.e., expanding ionic quality). This impact is known as salting in. As the salt concentration (ionic strength) is expanded encourage, the solvency of the protein starts to diminish, and the protein will totally be accelerated from the solution. This effect is known as salting out.

Procedure

Ammonium sulfate (90 % ammonium sulfate, 657 g/L) was added to the supernatant and mixed well by shaking and kept at 4 ºC overnight to precipitate all the proteins present in the supernatant solution. The protein was separated from the solution by giving short spin at 15000 x g for 15 min. Precipitated protein was collected and dissolved in minimum volume (200 µL) of sterile distilled water. It was then dialyzed against sterile distilled water for 1hr. using magnetic stirrer to remove the ammonium sulfate salt. After dialyzing, protein solution was wiped out from the dialysis bag and kept at 4 ºC for further analysis.

- ❖ Acetone Precipitation

Acetone precipitation is a common method used for precipitation and concentration of protein. This technique is useful for condensing a protein sample and eliminating components of the substance which are not compatible with desired analysis.

Procedure

The supernatant containing the unrefined protein was taken in an RB flask and lyophilized (Freez dryer FD-1, Rikakikai, Toshiba) to make powder dust. From the dust powder, 20 mg of the lyophilized powder was dissolved in 1 mL sterile deionized water. To remove the fats from the dissolved fraction, 2 mL of ice-cold acetone was added to it and kept the mixture at 4oC for 2 hrs. The protein in the fraction was precipitated and then separated by centrifugation at 10000 x g (Plasto Craft Super Spin R-V/FM) for 15 minutes at 4oC. The precipitate was again washed with

1 mL ice-cold acetone and centrifuged similarly for the complete removal of fats. After air-dried, the precipitated protein was dissolved in 250 μL deionized sterile water for further analysis.

Purification of bacterial protein by column chromatography

From the crude protein as mentioned above, 200 μL was loaded onto a Sephadex G-100 gel filtration column (100 cm x 1 cm) that was pre-equilibrated with 5 mM Tris-HCl buffers. Proteins were eluted through the column at a flow rate of 12 mL/hour and fractions of 1 mL each were collected by using a fraction collector (Eyela DC-1000). The protein containing fractions were detected by measuring their optical densities at 280 nm using Elico SL-196 UV- Visible Spectrophotometer. As proteins were well separated and purified, so each fraction was then used for biosilicification assay to observe the silica releasing property from silica rich substrate tetraethyl orthosilicate (TEOS). The fractions which showed biosilicification activities were pooled and concentrated (250 μL) by lyophilization. The concentrated fraction was again eluted similarly through column chromatography. The eluted fraction was then concentrated (250 μL) through lyophilization and dialyzed in dialysis tubing cellulose membrane against deionized sterile water. Concentration of eluted fractions which gave positive results in biosilicification assay was determined after each step of chromatography.

Determination of protein concentration

There are two methods adopted for checking the concentration of purified protein. These two methods are – **Lowry Method** and **Bradford Method**.

❖ Lowry Method

The Lowry protein assay may be a biochemical test for deciding the entire level of protein in a solution. The whole protein concentration is shown by a color alter of the sample solution in extent to protein concentration, which can at that point be measured utilizing spectrophotometer. It is named for the organic chemist Oliver H. Lowry who created this method (Lowry et al., 1951). This method combines the responses of copper particles with the peptide bonds beneath alkaline conditions with the oxidation of fragrant protein buildups. The Lowry method is best used with protein concentrations of 0.01–1.0 mg/mL and is based on the reaction of Cu+, produced by the oxidation of peptide bonds, with Folin–Ciocalteu reagent (a mixture of phosphotungstic acid and phosphomolybdic acid in the Folin–Ciocalteu reaction). The response component includes the decrease of the Folin-Ciocalteu reagent and oxidation of fragrant buildups (tryptophan, moreover tyrosine).

Chemicals Required

- Reagent A – 2% Na2CO3 in 0.1 N NaOH
- Reagent B – 0.5% CuSO4, 5 H2O in 1% NaK-Tartarate
- Reagent C – Reagent A: Reagent B in 50: 1 ratio
- Reagent D – Reagent C without NaOH
- Reagent E – Folin reagent: Distilled water in 1: 2 ratios
- BSA solution: 1 mg of BSA (Bovine serum albumin) was dissolved in 1 ml of double distilled water
- Bioremediase protein

Procedure

At first 0 mL (0 µg), 0.005 mL (5 µg), 0.01 mL (10 µg), 0.02 mL (20 µg), 0.05 mL (50 µg), 0.1 mL (100 µg) of standard BSA solution were taken in series of test tubes and 1 mL of reagent C was added to these and allowed to stand for 10 min at room temperature. Next 0.1 mL reagent E was mixed with each, and volume of each tube was made up to 1 mL with distilled water. All reagents were mixed rapidly and kept at room temperature for 30 min. Spectrometric reading was taken at 660 nm by using Elico SL-196 UV-visible spectrophotometer and a standard curve was prepared. Then 0.01 mL, 0.02 mL, 0.03 mL, 0.04 mL, 0.05 mL, 0.06 mL, and 0.07 mL of bioremediase protein were taken in series of test tubes and experiment was done according to the above procedure. Optical density of the final solution was measured at 660 nm and concentration was determined from BSA standard curve. Final protein concentration was determined by taking average of all data.

- Bradford Method

The Bradford protein measure could be a spectroscopic explanatory method utilized to measure the concentration of protein in a solution. The Bradford protein measure was created by Marion M. Bradford. It is based on an absorbance shift of the color Coomassie Brilliant Blue G-250 in which beneath acidic conditions the red form of the color is changed over into its bluer form to tie to the protein being tested. Amid the arrangement of this complex, two sorts of bond interaction take place: the red form of Coomassie color to begin with gives its free electron to the ionizable bunches on the protein, which causes a disturbance of the protein's local state, consequently exposing its hydrophobic pockets. These pockets within the protein's tertiary structure tie non-covalently to the non-polar region of the color by means of Van der Waal forces, situating the

positive amine bunches in vicinity with the negative charge of the color. The bond is assist reinforced by the ionic interaction between the two. The binding of the protein in this way stabilizes the blue form of the Coomassie color. In this way the protein concentration can be assessed by utilizing absorbance perusing of the test show in arrangement. The bound form of the color has an assimilation range most extreme at 595 nm. The increment of absorbance at 595 nm is relative to the sum of bound color, and hence to the sum (concentration) of protein display within the test.

Chemicals Required

- Bradford Reagent
- BSA solution: 2 mg of BSA (Bovine serum albumin) was dissolved in 1 ml of double distilled water
- Bioremediase protein

Procedure

Before going to start the experiment, the Bradford reagent bottle was gently mixed and kept at room temperature. Next, different concentration of standard BSA solution (0µg/µL, 0.25µg/µL, 0.5µg/µL, 1.0µg/µL) was prepared in series of Eppendorf from the stock solution (2mg/mL). The assay was performed in a test tube by adding 0.05 mL of the sample in 1.5 mL of Bradford reagent. After adding, the solution was vortexed, and the samples were incubated at room temperature for 5-45 min in dark room. Spectrometric reading was taken at 595 nm by using Elico SL-196 UV-visible spectrophotometer and a standard curve was prepared. Then 0.01 mL, 0.02 mL, 0.03 mL, 0.04 mL, 0.05 mL, 0.06 mL, and 0.07 mL of bioremediase protein were taken in series of test tubes

and experiment was done according to the above procedure. Optical density of the final solution was measured at 595 nm and concentration was determined from BSA standard curve. Final protein concentration was determined by taking average of all data.

Characterization of bacterial (BKH4) protein

❖ Molecular weight determination of purified protein by SDS-PAGE

PAGE (Polyacrylamide Gel Electrophoresis) is an expository method utilized to isolated components of a protein blend based on their measure. In this procedure a charged atom will move in an electric field towards a cathode with inverse sign. The common electrophoresis procedures cannot be utilized to decide the atomic weight of natural particles since the portability of a substance within the gel depends on both charge and estimate. To overcome this, the biological tests must be treated so that they secure uniform charge, at that point the electrophoretic portability depends fundamentally on estimate. For this purpose, different protein molecules with different shapes and sizes, needs to be denatured (done with the aid of SDS) so that the proteins lost their secondary, tertiary, or quaternary structure. The proteins being covered by SDS are negatively charged and when loaded onto a gel and placed in an electric field, it will migrate towards the anode (positively charged electrode) and separated by a molecular sieving effect based on size. Depending on their measure, each biomolecule moves in an unexpected way through the gel lattice: little atoms more effectively fit through the pores within the gel, whereas bigger ones have more trouble. The gel is run often for many hours, though this depends on the voltage connected over the gel; relocation happens more rapidly at higher voltages, but these comes about are regularly less precise than at those at lower voltages. After the set sum of time, the biomolecules have relocated distinctive distances based on their measure. Smaller biomolecules travel more

distant down the gel, whereas bigger ones stay closer to the point of beginning. Biomolecules may hence be isolated concurring to size, which depends on atomic weight beneath denaturing conditions, but moreover depends on higher-order compliance beneath local conditions. After the visualization by a recoloring (protein-specific) strategy, the degree of a protein can be calculated by comparing its migration distance with that of a known nuclear weight ladder (marker).

Procedure

Sodium dodecyl sulfate – polyacrylamide gel electrophoresis (SDS-PAGE) was performed at a constant voltage of 100 volt using 12% acrylamide gel. Protein samples were boiled for 3 min in sample buffer with a reducing agent and loaded onto 12% gel along with the Sigma protein molecular marker. Molecular masses of various protein bands were stained with Coomassie brilliant blue R-250 (Weber, 1969 & Laemmli, 1970) followed by de-staining procedure to visualize the band.

- ❖ Biosilicification Assay

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 response was performed at 65 ºC for 180 min and after that ended by centrifugation at 11000 x g for 20 min. The precipitate hence gotten was washed twice with outright 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 according to the method of Douglas et al. (2004). At first 200 µL of 5 (N)

HNO3 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 response tests were measured at 405 nm against control test (without protein).

❖ Effect of pH on Enzyme Activity

Enzymes are affected by changes in pH values. For each protein, there is a locale of optimal stability in pH. Extremely high or low pH values result in partial or complete loss of enzymatic activities. The pH value is also a factor to control the stability of an enzyme. For each protein, there is a region of optimal steadiness in pH. The optimum pH value will vary from one enzyme to another. The present study was designed to find out the optimum pH range of bioremediase protein (enzyme).

Procedure

The experiment was done under a wide pH range, from pH 6 to 12. In the reaction tube, 100 µL of purified protein along with 20 µL of Tris-HCl buffer (20 mM) and 50 µL TEOS (4.5 µM) was added, and the final volume was made to 1 mL with deionized sterile water. Then pHs of the reaction mixtures were set at pH 6, pH 7, pH 8, pH 9, pH 10, pH 11 and pH 12 respectively in different test tubes. The reaction was performed at 65°C for 180 min and terminated by centrifugation (15000 x g, 20 min) as described earlier. The assay for silicification was done similarly. Based on the absorbance values at 405 nm, the absolute amounts of silicic acid were calculated from a calibration curve prepared earlier by using a silicon standard (MERCK,

Germany). Specific activity was determined as described earlier. Significant level of all data was checked at $P<0.05$ by using ANOVA.

❖ Effect of Buffer on Enzyme Activity

The buffer has crucial role to take part in a reaction. It works best when the concentrations of the acid and base components are approximately equal. This means that a buffer is most effective when the desired pH is near the pKa of the acid-base pair being used. The effectiveness of a buffer also depends on its concentration; a more concentrated buffer is more resistant to acid-base change than a dilute buffer. In increasing a buffer's strength, however, limitations due to having too high salt content or solubility problems may be encountered. The present study was designed to find out optimum buffer concentration of bioremediase protein (enzyme).

Procedure

In the series of reaction tubes, 100 μL of purified protein along with varying concentration of Tris-HCL buffer 5mM, 10mM, 20 mM, 50 mM, 70 mM, 90 mM, and the final volume was made up to 1 mL with deionized sterile water. Then 50 μL of TEOS (4.5 μM) was added and the reaction was performed at 65 ºC for 180 minutes and then terminated by centrifugation (15000 x g, 20min). The precipitate thus obtained was washed twice with absolute ethanol and then air-dried. The precipitate was then dissolved in 1 mL 1(M) NaOH and the released silicic acid was determined by molybdate assay (Douglas et al., 2004). Based on the absorbance values at 405 nm, the absolute amounts of silicic acid were calculated from a calibration curve prepared earlier by using a silicon standard (MERCK, Germany).

❖ Effect of Temperature on Enzyme Activity

Temperature can influence a protein in two ways. One could be a coordinate impact on the response rate consistent, and the other is in thermal denaturization of the protein at raised temperatures. To relate the effect of temperature to the reaction rate constant, the Arrhenius equation is used: where k is the rate constant, R is the gas law constant, A is the frequency factor and EA is the activation energy of the reaction. The temperature ranges over which proteins appear action is restricted between the melting point (0°C) and boiling point (100°C) of water. In case a temperature is as well low, there can be no recognizable response rate since the protein is working at a temperature distant underneath its optimum. If the temperature at which the protein is working at is well over 100°C, at that point thermal deactivation can happen. Each enzyme has a particular range of temperature when it remains at its maximum active state, called optimum temperature. In this study, we tried to find out the optimum temperature of the purified protein (enzyme).

Procedure

The experiment was done under a wide temperature range, from 50 ºC to 80 ºC. In a reaction tube 100 µL of purified protein along with 20 µL of Tris-HCl buffer (20 mM) and 50 µL TEOS (4.5 µM) was added, and the final volume was made to 1 mL with deionized sterile water. Then pH of the reaction mixture was set at pH 8. The reaction was performed at different temperature (ranges 50 °C, to 80 °C) for 180 minutes separately and terminated by centrifugation (15000 x g, 20 min) similarly. The released silicic acid was determined by molybdate assay (Douglas et al., 2004) as described above. Based on the absorbance values at 405 nm, the absolute amount of silicic acid was calculated from a calibration curve prepared earlier by using a silicon standard (MERCK, Germany).

CHAPTER 3

Chapter 3: Application of Bacteria & Bacterial (BKH4) Protein in Concrete Technology

Application of (BKH4) bacterial cells in cement mortar matrix (loaded & unloaded)

Concrete is the world's most broadly utilized structural medium, owing to its mind-blowing flexibility. As a building fabric it incorporates a special capacity to be formed and etched into anything from streets and trails to artisanship figures, private homes, and high rises. The natural processes like weathering, faults, earthquakes, and some human activities create fractures and fissures in concrete structures which act as a source of ingression of water and chemicals, causing deterioration of embedded steel structures and reduce the service life of the structures. The require of an innovative innovation to overcome the deficiencies of concrete has driven to the improvement of an inconceivably extraordinary concrete known as Bacterial Concrete. In this technology, the anaerobic bacteria were used in concrete structure by reducing the pore concentration and increasing the compressive strength and durability. There are no limits to where and how concrete can be utilized, and its inalienable quality and toughness underscores its qualifications as one of the foremost feasible building items.

- ❖ Compressive strength analysis

The compressive strength is the capacity of a fabric or structure to resist loads tending to diminish estimate. It can be measured by plotting connected drive against distortion in a testing machine. Few materials fractures at their compressive strength limit; others deform irreversibly. So, a given sum of distortion may be considered as the constrain for compressive load. The compressive

strength of concrete is particularly critical because it is utilized more frequently in compression than in any other way.

Procedure

Standard mortar cubes (70.6 mm × 70.6 mm× 70.6 mm) were prepared by mixing different bacterial cell concentrations (102–107 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.

After 28 days of water curing, the samples were dried, and typical breaking stack was chosen moreover by taking five samples from each category. Rest of the samples were employed with 50 % average breaking load (respective to their categories) at 0.5 kN/s which produced micro-cracks in the samples. These samples were at that point kept beneath deionized water for advance suitable curing. After fur there different days of curing (0, 3, 7, 28 days), compressive strengths of five samples from each category were measured by a digital compression test machine.

- ❖ Ultrasonic pulse velocity analysis

Ultrasonic pulse velocity (UPV) test is a non-destructive popular test used to examine the homogeneity, quality, cracks, cavities, and defects in concrete. This test is conducted by passing a beat of ultrasonic through concrete to be attempted and measuring the time taken by beat to actuate

through the structure. Higher speeds show predominant quality and coherence of the material, whereas slower speeds may show concrete with numerous breaks or voids.

Procedure:

Similar type of mortar cubes (70.6 mm × 70.6 mm × 70.6 mm) mixed with different bacterial cell concentrations were prepared as mentioned above. At least 6 samples were prepared for each category and ultrasonic pulse velocity was measured by Pundit plus meter (PC1007) according to the standard test method. 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 test

Absorption testing is a popular method of determining the watertightness of concrete. One of the foremost imperative properties of a prevalent quality concrete is low penetrability, particularly one resistant to solidifying and defrosting. A few concretes with low penetrability stand up to ingress of water and is not as helpless to freezing and defrosting. Water enters pores within the cement glue and indeed within the total. For concrete pavers, the test method includes drying a specimen to a consistent weight, weighing it, inundating it in water for indicated amount of time, and weighing it once more. The increment in weight as a rate of the first weight is communicated as its retention (in percent). The average absorption of the test samples shall not be greater than 5% with no individual unit greater than 7%.

Procedure:

After 28 days of water curing, the mortar samples were airdried 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. As mentioned, the 50 % of average breaking load (obtained after the breaking of five samples respective to their category) was applied to some of the samples and treat them accordingly. 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 test

Sulfate attack comprises an arrangement of chemical responses between sulfate particles and the components of solidified concrete. As these responses may lead to splitting, spalling or quality misfortune of concrete structures, fitting test strategies are required to decide the resistance of concrete beneath sulfate presentation. Quickened test strategies are most reasonable since sulfate attack is ordinarily a long-term process.

Procedure:

Masses were registered after 28 days of water curing, from each category of the respective samples. 50 % of average breaking stack (gotten after the breaking of five tests from each category) particular to their category was connected and their masses were enlisted. Then, the samples were immerged in sulphate solution (5% $MgSO_4$, pH 7 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 guideline of ASTMC1012 (Neville, 2011).

❖ RCPT test of bacterial cell-amended mortar

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 104 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. Three samples were tested for each category as per ASTM C1202 (ASTM C1202, 2000). Rest of the six samples from each category were given with 50 % breaking load (as determined from their respective samples). Three samples of each category were tested after applying the breaking load. The RCPT tests of the remaining three samples of each category were done after 28 days of water curing of each category.

❖ 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 (EDX) was also done by using QUANTAX ESPRIT 1.9 software. For X-ray diffraction (XRD) analysis, dry powder samples were sieved (5 lm) 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 2Θ = 20° to 80°. The peaks within the new positions of the range were checked and recognized from the JCPDS information Record (JCPDS ASTM, 1941).

Application of (BKH4) bacterial protein in cement mortar matrix (loaded & unloaded)

❖ Compressive strength analysis

The **compressive strength** is the capacity of a fabric or structure to resist loads tending to decrease measure. It can be measured by plotting applied constrain against distortion in a testing machine. A few materials break at their compressive strength constrain; others distort irreversibly. So, a given sum of distortion may be considered as the constrain for compressive load. The compressive strength of concrete is especially vital because it is utilized more regularly in compression than in any other way.

Procedure

Control and protein amended mortar cubes (70.6 x 70.6 x 70.6 mm3) with varying concentration (1µg, 2µg, 3µg and 4µg per gram of cement) were initially prepared and compressive strength of these mortar cubes were determined by compressive strength testing machine and compared with control mortar cubes (where no protein is given) after 28 days of water curing as described by Ghosh *et al*. (2005). The water–cement weight ratio was taken as 0.4 and cement–sand weight ratio was 1:3. Three different grain-size classes of sand particles (125–250 µm; 250–500 µm; 500–1000 µm with ratios 1:1:1) were used for mortar specimens' preparation. All the test specimens were cured under water as well as in open air after 24 h of casting at room temperature (30±2 ºC) until compression testing were performed. The total experiment was replicated for at least six times

to get an average statistical data. The compressive strengths of the mortar cubes were assessed after 3, 7, 14, 28, 120 days of curing.

❖ Ultrasonic pulse velocity analysis

Enough 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 category samples was applied (at a rate of 0.5 kN/s) to the rest of the corresponding category samples for determining the 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 Pundit plus PC1007 UPV meter was used to determine the ultra-pulse velocity as per ASTM C597-02 (ASTM 2002)

❖ Water absorption test

For water absorption by mortar samples experiment, the samples of each category (5 in number) were immersed in water for 28 days. After the curing period, the sample in the air for 24 hrs at room temperature. Then initial masses were noted. After that, 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.

❖ Sulphate resistance test

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 had performed according to the procedure of ASTM C 1012 (ASTM, 1977).

❖ Sorptivity test

The sorptivity of the control and protein consolidated mortar cubes (five tests for each category) was decided by the estimation of the water assimilation rate of the samples that happened due to capillary activity only of water rise. The samples were to begin with cured beneath water for 28 days. The samples were at that point pull out from the water tank, air dried, subjected to the application of 50% comparing breaking stack, and taken after by heat curing at 65°C in an oven. A while later, plastic paint was coated carefully to all sides of the mortar cubes except for the exposure confront. This coating of paint not only fixed all fringe surfaces but too kept up the unidirectional capillary stream of water through the uncovered surface of the samples. The masses of the paint-sealed samples were measured and recorded as introductory mass values set for water assimilation calculations. The samples were set on a wire-gauge kept in the water bath that made a difference the uncovered surface to contact the water appropriately. Tap water was poured into the holder gradually until the water level come to around 3 mm over the level of the uncovered

surface. The water assimilation rate of the samples was noted in several interims of time. The sorptivity of the control and protein consolidated mortar cubes was decided by the standard method.

CHAPTER 4

Chapter 4: Autonomous Self-Healing Attributes of Bacterial (BKH4) Protein

Micro-structure Analysis of Self-Healing Material

Enough 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 category samples was applied to the rest of the respective category samples for creating microcracks on the samples. The photographs of the crack's 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.

The substance deposited on the induced-crack surfaces of the bacterial protein-impregnated mortars cured for 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.

- Fe-SEM view of self-healing material

A pinch of powder sample was taken and dispersed in absolute ethyl alcohol and dried in vacuum desiccators for 15 min. The dried powdered sample was examined in field emission scanning electron microscope, FESEM (INSPECTF50 SEM, FEI Europe BV, The Netherlands).

- EDX analysis by FE-SEM

a pinch of powder sample was taken and dispersed in absolute ethyl alcohol and dried in vacuum desiccators for 15 min. The dried powdered sample was examined in field emission scanning electron microscope, FESEM (INSPECT F50 SEM, FEI Europe BV, The Netherlands) for EDS ana lysis using QUANTAX ESPRIT 1.9 software.

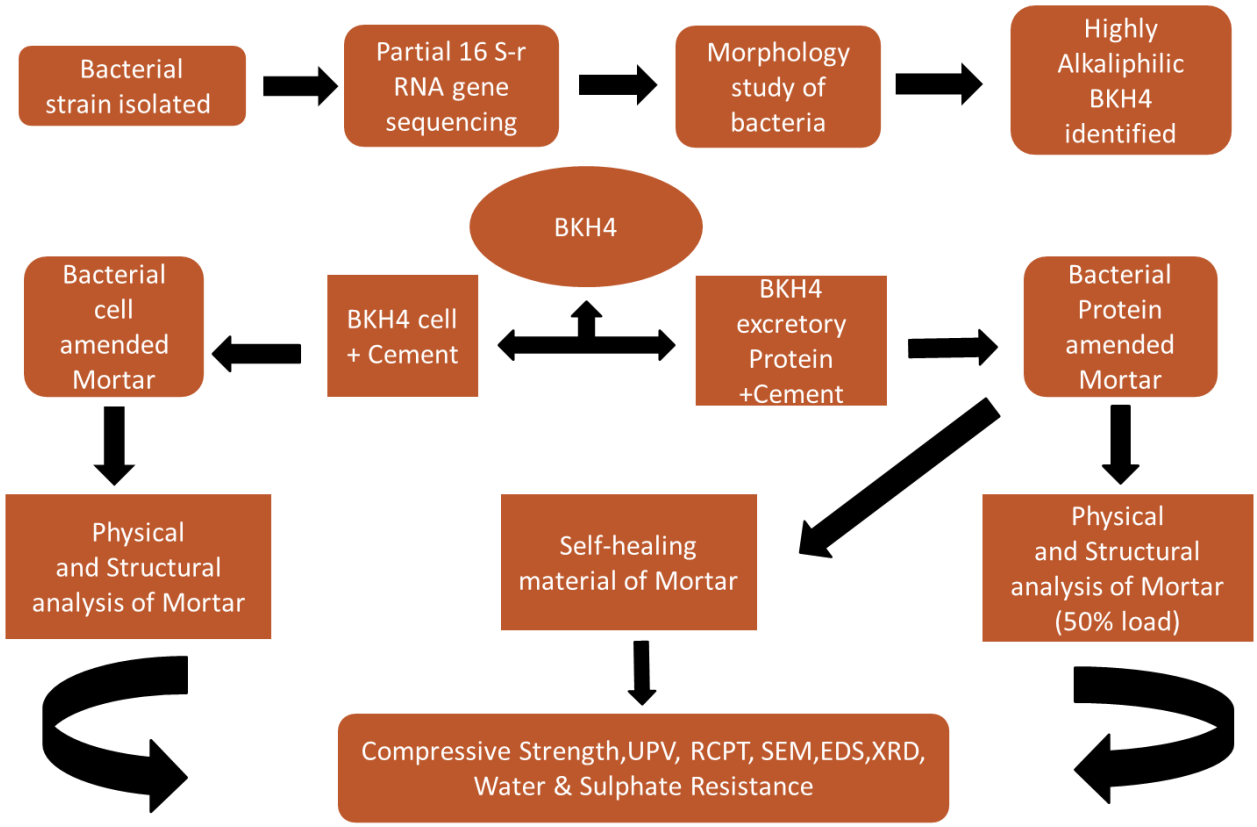
- XRD analysis of the powdered healing material

Dry powder samples were sieved to achieve the uniform particles size (5 lm) for all categories and examined in powder XRD (Bruker AXS, Inc., Model D8, WI, USA) at 40 kV with a scan speed 0.5 s/step. The XRD spectrum was taken from 2h = 10 to 70. The peaks within the new positions of the range were checked, coordinate up to and recognized from the JCPDS data record.

RESULTS

Results

This part is dealing with the finding of results obtained from the experiments as discussed in the "Experimental Part."



Schematic view of BKH4 and it's Protein Application

Chapter 1: Isolation and Characterization of BKH4 Bacteria

BKH4 Bacterium in Its Growth Medium

The growth of BKH4 bacterium in a sealed glass vial was obtained by gassing each vial in presence of CO2. As this bacterium is a facultative anaerobe, thus the anaerobicity of the medium was maintained by removing the oxygen containing air inside the vial by passing through CO2 for the growth of the bacterium. The growth was confirmed by observing the change in colour (blackish) of the medium with a pungent smell. In below, the two pictures depict the well grown culture media (Figure 1A) and the normal media (Figure 1B).

Fig 1: BKH4 content culture media (A) and Normal Culture media (B)

Partial r-RNA Sequencing of BKH4

Before studying the morphology of the bacterium, serial dilution technique was performed to obtain a pure strain. The highest order dilution vial was maintained in our laboratory. The

bacterium grown in this vial was confirmed as BKH4 based on 16S rRNA gene. The Microbe was found to be most like *Lysinibacillus* sp. B-1-43 16S ribosomal RNA gene, partial sequence (Sequence ID: gb|KT583372.1) and the next closest homologue was found to be *Lysinibacillus fusiformis* strain KAR73 16S ribosomal RNA gene, complete sequence (Sequence ID: gb|KR055033.1).

Aligned Sequence Data of Sample -: (1,524bp)

ACGGGTCCCGGGTTTGGCGGCCGCGGCGGATGTGTACAAGGCCCGGGAACGTATTCATCCGCGGCATG
CTGATCCGCGATTACTAGCGATTCCGGCTTCATGTAGGCGAGTTGCAGCCTACAATCCGAACTGAGAA
CGACTTTATCGGATTAGCTCCCTCTCGCGAGTTGGCAACCGTTTGTATCGTCCATTGTAGCACGTGTGT
AGCCCAGGTCATAAGGGGCATGATGATTTGACGTCATCCCCACCTTCCTCCGGTTTGTCACCGGCAGTC
ACCTTAGAGTGCCCAACTAAATGATGGCAACTAAGATCAAGGGTTGCGCTCGTTGCGGGACTTAACCC
AACATCTCACGACACGAGCTGACGACAACCATGCACCACCTGTCACCGTTGCCCCCGAAGGGGAAACT
ATATCTCTACAGTGGTCAACGGGATGTCAAGACCTGGTAAGGTTCTTCGCGTTGCTTCGAATTAAACCA
CATGCTCCACCGCTTGTGCGGGCCCCCGTCAATTCCTTTGAGTTTCAGTCTTGCGACCGTACTCCCCAG
GCGGAGTGCTTAATGCGTTAGCTGCAGCACTAAGGGGCGGAAACCCCCTAACACTTAGCACTCATCGT
TTACGGCGTGGACTACCAGGGTATCTAATCCTGTTTGCTCCCCACGCTTTCGCGCCTCAGCGTCAGTTA
CAGACCAGAAAGTCGCCTTCGCCACTGGTGTTCCTCCAAATCTCTACGCATTTCACCGCTACACTTGGA
ATTCCACTTTCCTCTTCTGCACTCAAGTCCCCCAGTTTCCAATGACCCTCCACGGTTGAGCCGTGGGCTT
TCACATCGGACTTAAAGGACCGCCTGCGCGCGCTTTACGCCCAATAATTCCGGACAACGCTTGCCACC
TACGTATTACCGCGGCTGCTGGCACGTAGTTAGCCGTGGCTTTCTAATAAGGTACCGTCAAGGTACAG
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GGCGTTGCTCCATCAGGCTTTCGCCCATTGTGGAAGATTCCCTACTGCTGCCCCCCGTAGGAGTCTGGG
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TTACCTCACCAACTAGCTAATGCGCCGCGGGCCCATCCTATAGCGACAGCCGAAACCGTCTTTCAGTCT
TTTGCCATGAAGCAAAAGAGATTATTCGGTATTAGCCCCGGTTTCCCGGAGTTATCCCAAACTATAGG
GTAGGTTGCCCACGTGTTACTCACCCGTCCGCCGCTAACGTCAAAGGAGCAAGCTCCTTTTCTGTTCGC
TCGACTTGCATGTGTTAGGCCTGGCGGCCGCTCTAGAACTAGTGGATCCCCCGGGCTGCAGGAATTCG
ATATCAAGCTTATCGATACCGTCGACACTCGGAGGGGGGGCCCGGTACCCAGCTTTTGTTCCCTTTTTA
AGTTGGAAGGGGG

Yellow highlighted: Primer sequence

Blue highlighted: Vector backbone

Fig 2: Partial rRNA Sequence of BKH4 Bacteria

Identification And morphological Study of BKH4 Bacterium

The morphology of the bacterium BKH4 showed round / coccoid shaped in nature as revealed from the Scanning Electron Microscopy (SEM). The dimension of the bacterium was 1.5–3 µm long. Gram staining experiment showed that the bacterium alters the colour of secondary stain (safranin) and appeared blue when observed under microscope, therefore this bacterium was Gram-positive in nature. The bacterium also exhibited fluorescence property when examined under a fluorescence microscope under the DAPI filter.

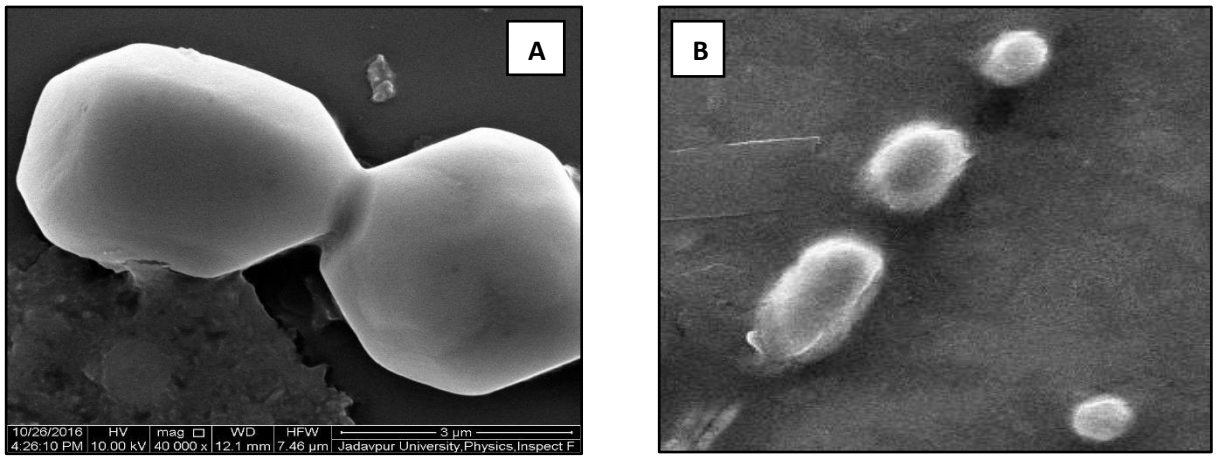


Fig 3: A & B SEM view of BKH4

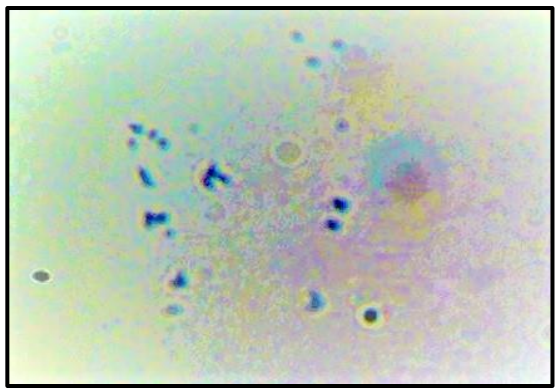


Fig 4: View of Gram positive BKH4

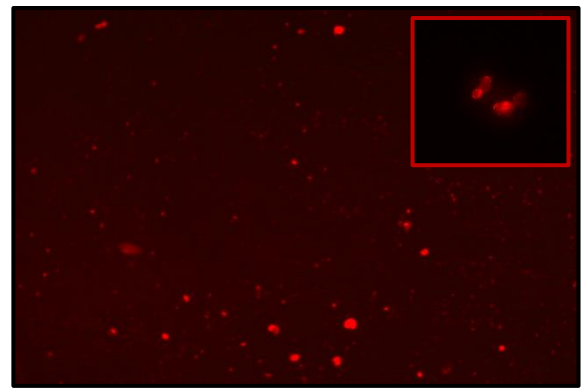


Fig 5: Fluorescence microscopic view of BKH4

Effect of pH and temperature on bacterial growth

The growth curve showed that the bacterium grew well at 65°C (Fig. 6A) and over a wide pH range (Fig. 6B) when grown in the specific semi-synthetic medium. The bacterium was found to be a facultative anaerobic, thermophilic, and highly alkaliphilic, who's optimum growth was obtained at pH 12.0 and 65°C (Fig. 6B).

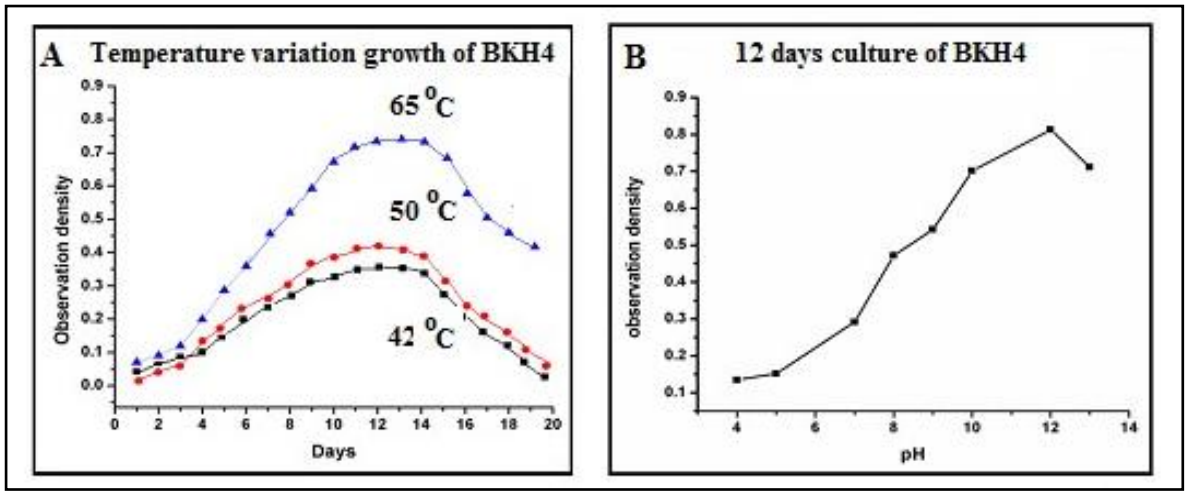


Fig 6: (A) Temperature variation growth curve of BKH4 (n = 3). (B) pH variation growth curve of BKH4 (n=3).

Study of phylogenetic tree of BKH4

The phylogenetic analysis of the isolate was (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.***

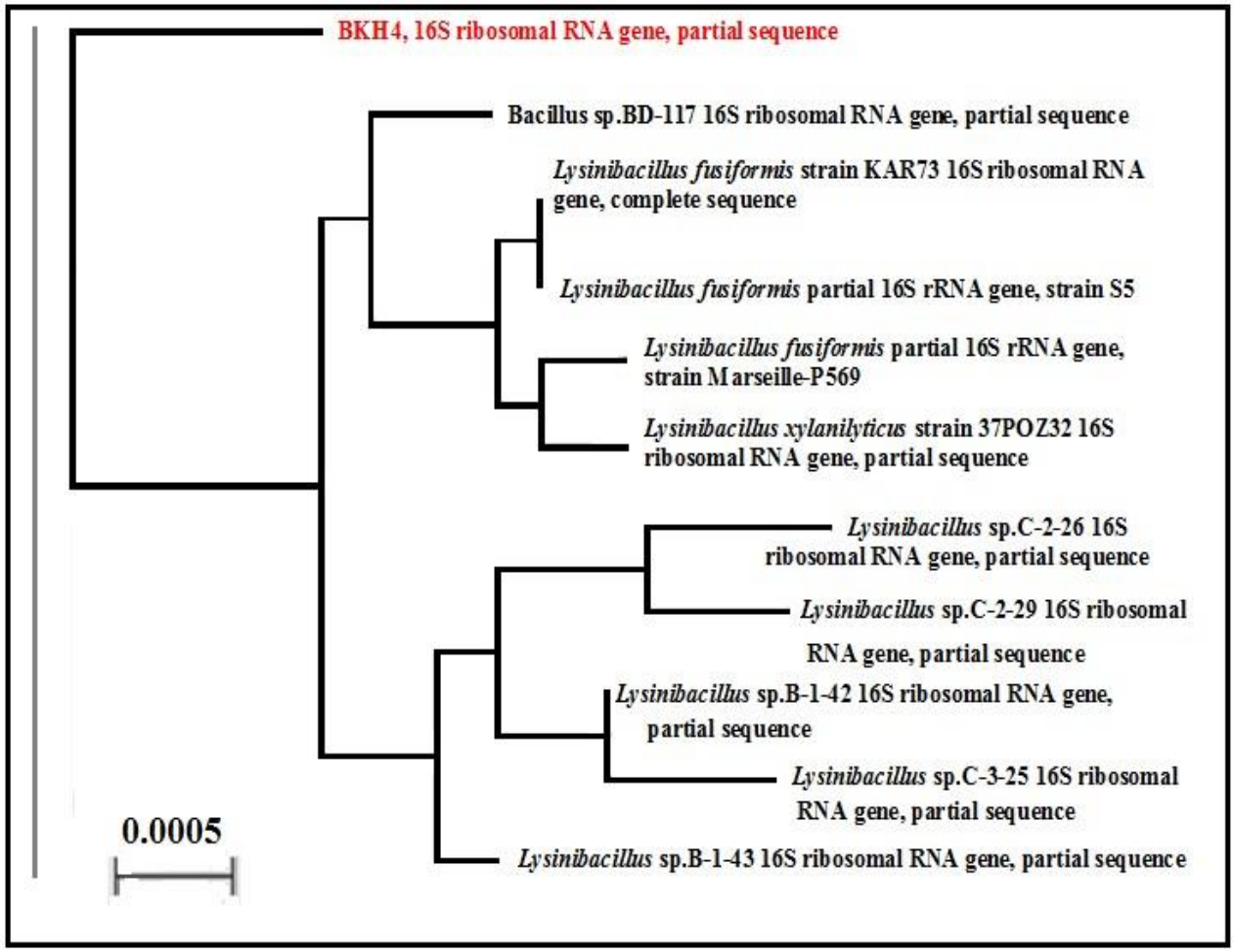


Fig 7: Phylogenetic tree of BKH4 bacterium.

Chapter 2: Isolation, Purification and Characterization of Bacterial Protein from BKH4 Strain

Isolation and Purification of Bacterial Protein from BKH4 Strain

There are several proteins present in BKH4 bacterium. They may be intracellular or extracellular. My goal was to isolate an extracellular protein which was responsible for increasing the compressive strength within the concrete/mortar sample. At first, proteins were isolated from the growth medium of the bacterium and purified through double step Gel Filtration Chromatography using Sephadex G-100 as column packing material. The optical densities of eluted fractions were measured at 280 nm and plotted against their respective fractions.

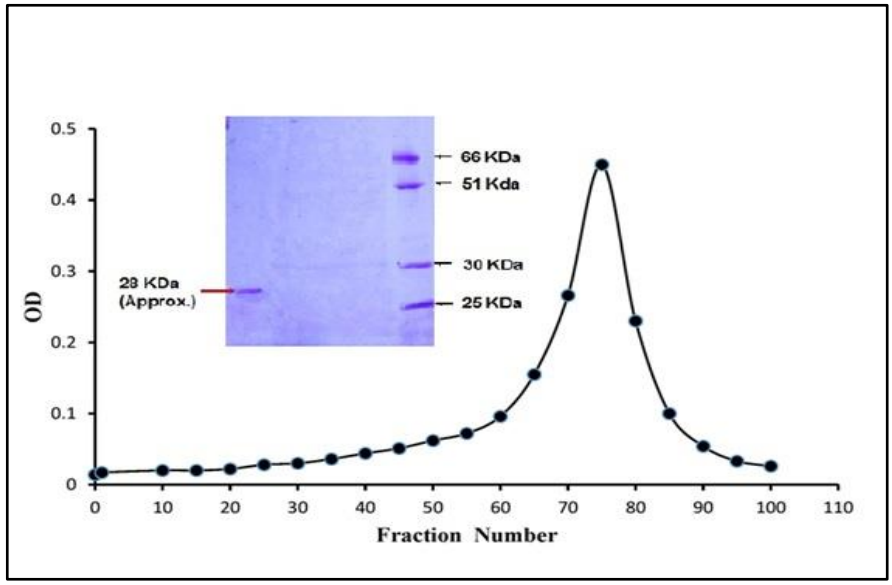


Fig 8: Purification and SDS-PAGE (inset) of secretary protein of BKH4 bacterium.

Determination of Protein Concentration

The protein concentration was determined by Lowry and Bradford method using BSA (2mg/ml stock solution) as a standard curve (Figure 9A and Figure 9B). The protein concentration of bacterial protein with respect to BSA standard was delineated in the Table 1(for Lowry Method) and Table 2 (for Bradford Method).

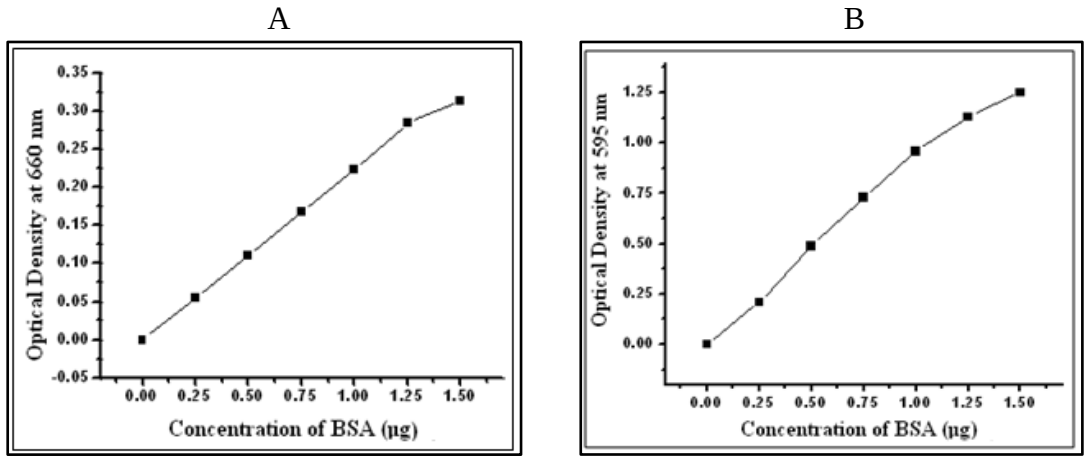


Fig 9: Standard Curve of BSA

Table 1: Determination of Protein Concentration by Lowry Method

Sample (µl)	Optical Density	Amount of BSA from Standard Curve (µg)	Concentration (µg/µl)	Average Concentration (µg/µl)
10	0.087	0.56	0.723	2.317
40	0.262	1.20	1.255	
80	0.423	2.09	2.541	
100	0.508	3.06	4.749	

Table 2: Determination of Protein Concentration by Bradford Method

Sample (µl)	Optical Density	Amount of BSA from Standard Curve (µg)	Concentration (µg/µl)	Average Concentration (µg/µl)
10	0.887	1.20	0.890	3.115
40	1.256	1.56	1.332	
80	3.829	4.29	4.350	
100	4.410	5.06	5.886	

Molecular Weight Determination of Purified Protein by SDS-PAGE

Molecular weight of the purified protein was determined by SDS-PAGE (12%) analysis which showed that the purified protein possess 28-kDa polypeptide and was active as a monomer [Fig 8 (Inset)]

Biosilicification Activity of Protein

The silica leaching activity of bioremediase protein was determined by biosilicification assay. To quantify the amount of silica released, a silicon standard curve was drawn which is given in **Figure 10**. Proteins containing peak fractions eluted through double step Sephadex G-100 column were directly taken for biosilicification assay (molybdate assay) along with the crude protein obtained from the bacterial supernatant. Optical densities were measured at 405 nm and summarized in the **Table 3**

Table 3: Biosilicification activity of isolated microbial protein

Sample	Volume (ml)	Total protein (mg)	Activity (units)	Sp. 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

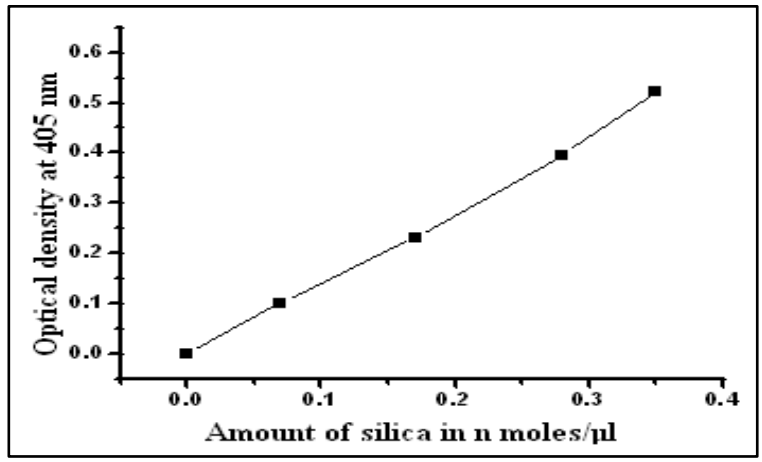


Fig 10: Silica Standard Curve

Effect of pH on Enzyme Activity

Enzyme activity was highly affected by pH and the bioremediase protein attains its maximum activity at optimum pH 12.0. The pH kinetics study showed the activity of this protein at different pH concentration (Figure 11). Data were presented with ± S.D. and were statistically significant at $P<0.05$, where $N = 6$.

Effect of Buffer on Enzyme Activity

Buffer had an essential role on enzyme activity and the activity of bioremediase enzyme start increasing from 20 mM concentration, attains its maximum at 50 mM concentration then falls. The buffer kinetics study showed the activity of this protein at different buffer concentration (Figure 12). Data were presented with ± S.D. and were statistically significant at $P<0.05$, where $N = 6$ (Figure 12).

Effect of Temperature on Enzyme Activity

Temperature also affected the activity of enzyme. This study showed the activity of this protein (enzyme) in terms of product formation from the reaction at different temperatures and showed its optimum temperature (Figure 13). This figure showed that the optimum temperature was 65 °C though this protein could work over a wide range of temperatures. All the values were presented with ± S.D. value and were statistically significant at $P<0.05$, where $N = 6$.

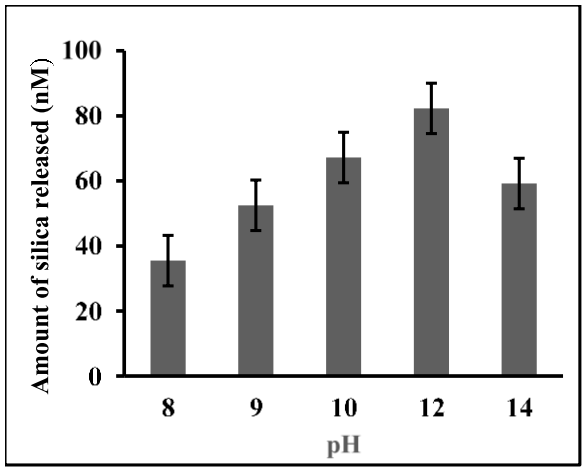


Fig. 11

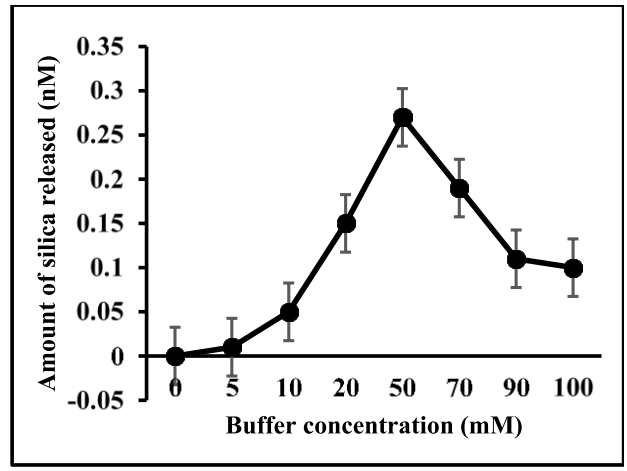


Fig. 12

Fig 11: Effect of pH on silica leaching activity of bacterial protein

Fig 12: Effect of Buffer on silica leaching activity of bacterial protein

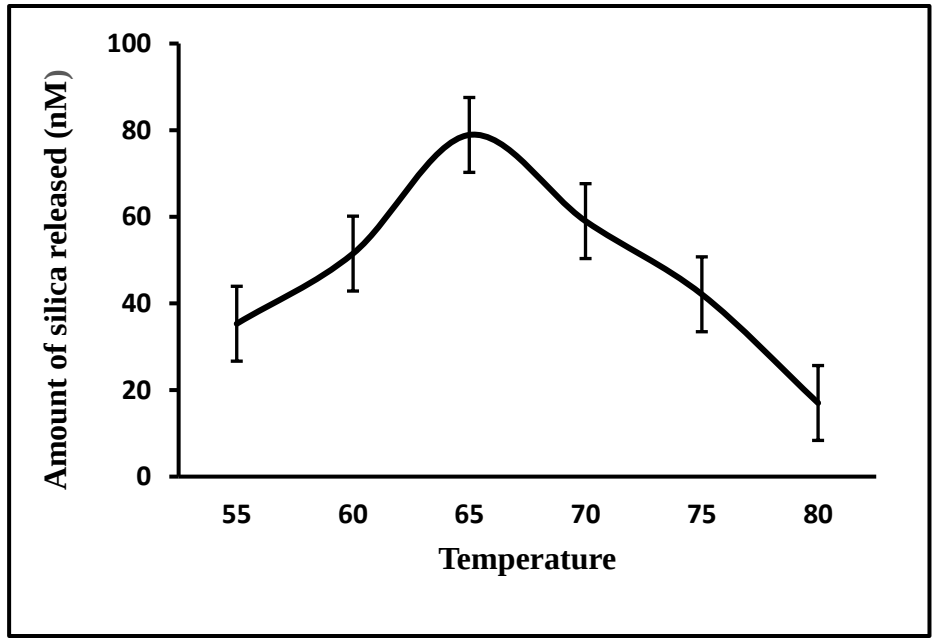


Fig 13: Effect of Temperature on silica leaching activity of bacterial protein

Chapter 3: Application of Bacteria & Bacterial (BKH4) Protein in Concrete Technology

Application of (BKH4) bacterial cells in cement mortar matrix

❖ Compressive Strength Analysis

The bacterium could increase the compressive strength of the cement–sand mortars when incorporated at different cell concentrations as shown in Fig. 14. The maximum strength increment (>50%) was observed at 104 cells per ml of water used under the 28-day water curing period.

❖ UPV Analysis

A more than 25% increment of UPV at 104 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 conditions (Fig. 15).

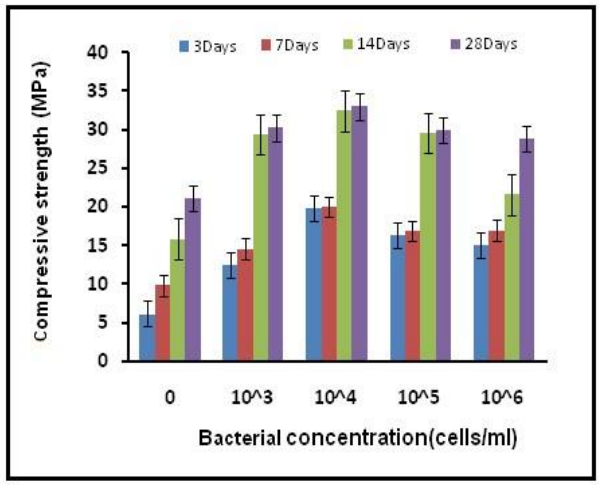


Fig. 14

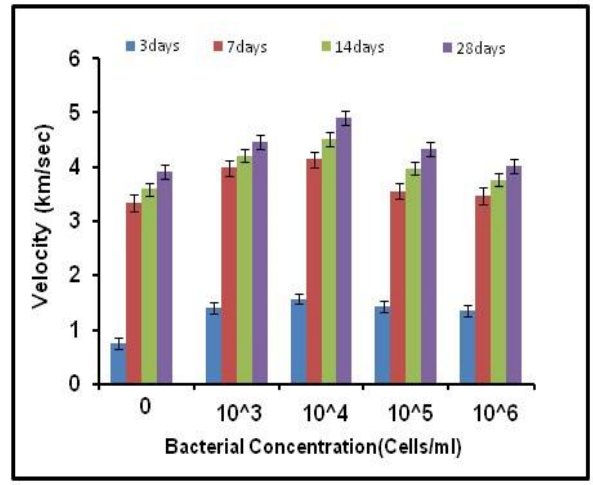


Fig. 15

Fig 14: Compressive strength & Fig 15: Ultrasonic pulse velocity of BKH4 bacterium-amended mortar samples at different days of water curing.

❖ Water Absorption Test Analysis

The bacteria-incorporated mortar samples were seen to be more water resistant compared to control samples.

Table 4: Water Absorption Test Analysis

Sample	Initial mass (g)	Mass after 30 min. (g)	Percentage of increasing	Mass after 24 h. (g)	Percentage of final increment
Control	**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**

❖ Sulphate Resistance Test Analysis

It also showed higher sulphate resistant activity compared to control mortar samples.

Table 5: Sulphate Resistance Test of Mortar Samples

Sample	Initial mass at 0 day (g)	Final mass at 90 days (g)	Percentage of increasing
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**

❖ RCPT Test Analysis

The result of rapid chloride permeability test (RCPT) suggested that bacterium-incorporated mortars were more resistive against chloride ions' attack than control samples' attack.

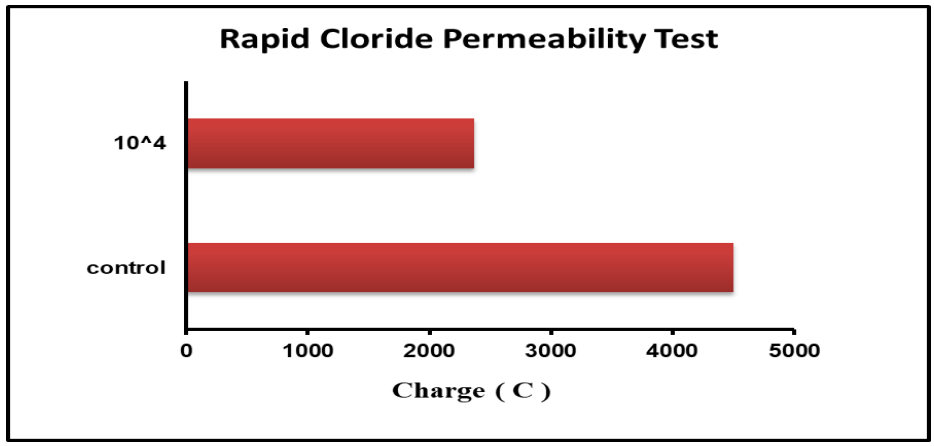


Fig 16: Result of RCPT test of control and bacterium amended mortar samples

❖ Microstructure analysis of bacteria-incorporated mortar samples

FE-SEM view

Microstructure analysis of the samples showed that the needle-like structures appeared in the bacterium-incorporated mortars (Fig. 17A) which were absent in control mortars (B).

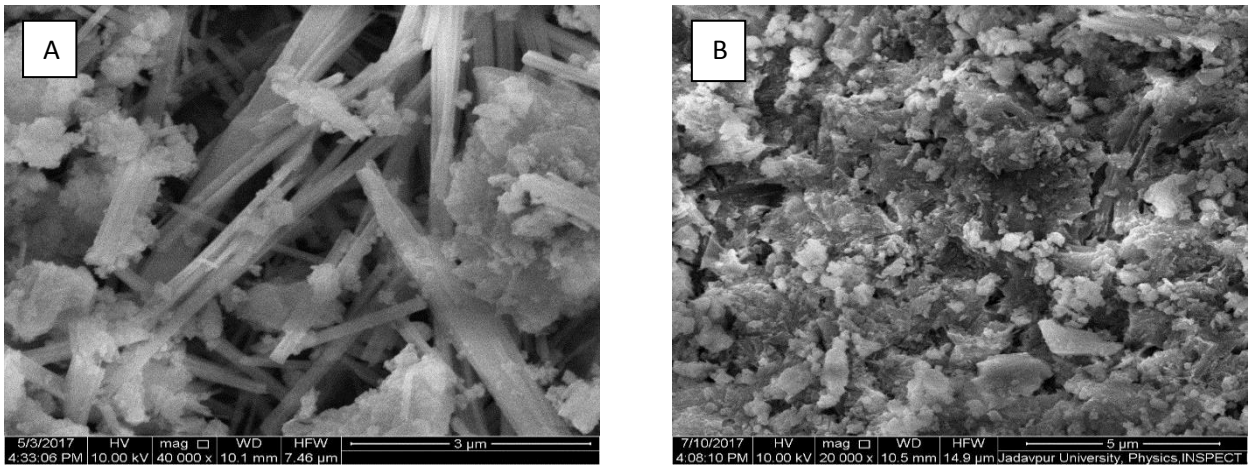
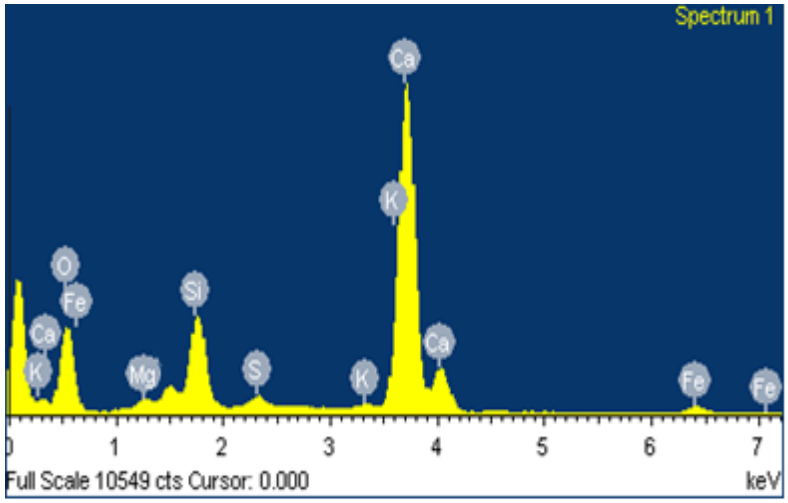


Fig 17: SEM image of (A) BKH4 bacteria-amended mortar matrix and (B) control mortar matrix

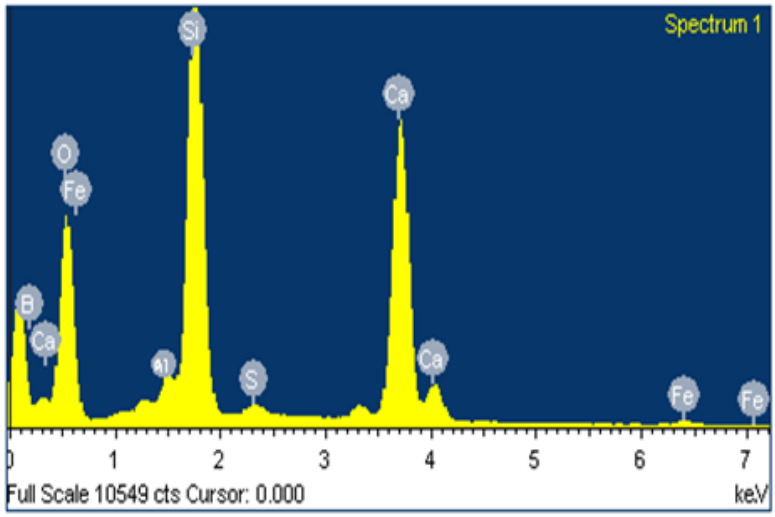
Energy-dispersive spectra analysis (EDX)

The EDX analyses of the mortar matrices also suggested the formation of a new phase inside the bacterium incorporated mortar matrices.



A

Element	Weight%	Atomic%
O K	54.94	73.73
Mg K	0.84	0.75
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	



B

Element	Weight %	Atomic %
O K	42.54	70.38
Si K	29.31	23.73
S K	0.28	0.17
Ca K	23.12	3.78
Fe K	2.11	0.39
Al K	2.64	1.30
Totals	100.00	

Fig 18: EDX analysis of control mortar matrix (A) and BKH4 bacterium-amended mortar matrix (B).

X-Ray Crystallography (XRD) view

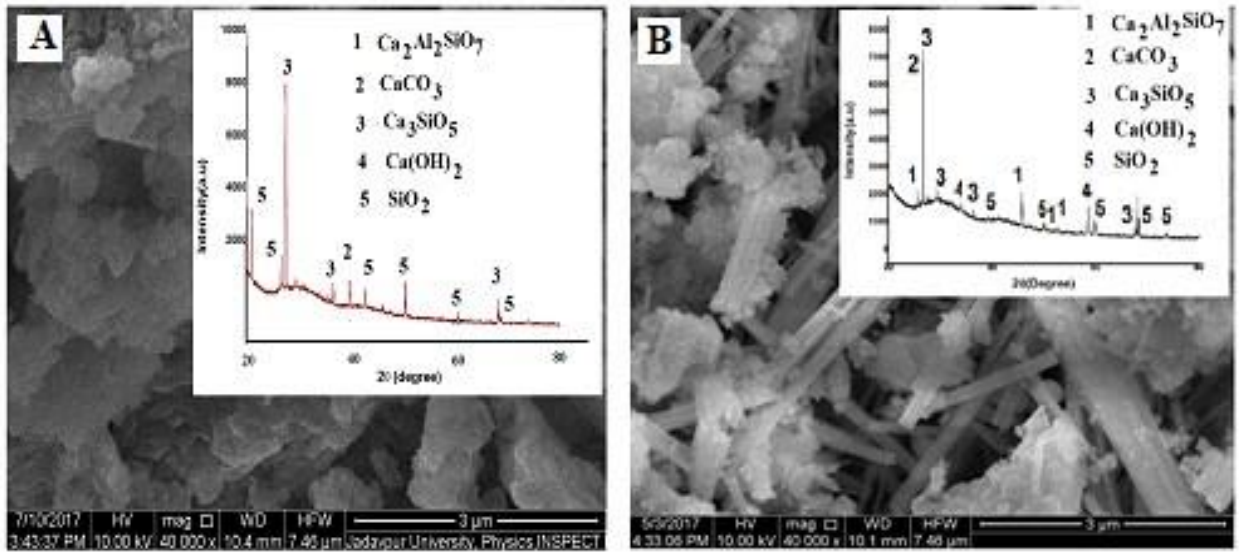


Fig 19: XRD analysis of the [A] control mortar matrix, [B] BKH4 amended mortar matrix

XRD analysis of bacterium-incorporated mortars (inset of Fig. 19 B) showed extra new peaks of Gehlenite (Ca2Al2SiO7) but those were not present in control mortar sample (inset of Fig. 19A)

Application of (BKH4) bacterial excretory protein in cement mortar matrix (loaded & unloaded)

- ❖ Compressive Strength and UPV Analysis

Enough 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 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 (Fig. 20 & Fig. 21), and another set of samples (5 samples) from each category was kept in air for different days of curing (3, 7, 14, 28 days) (Table 6,7,8,9). After the curing periods, a digital compressive strength testing machine was used to measure the compressive strength of the samples, and a Pundit plusnPC1007 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.

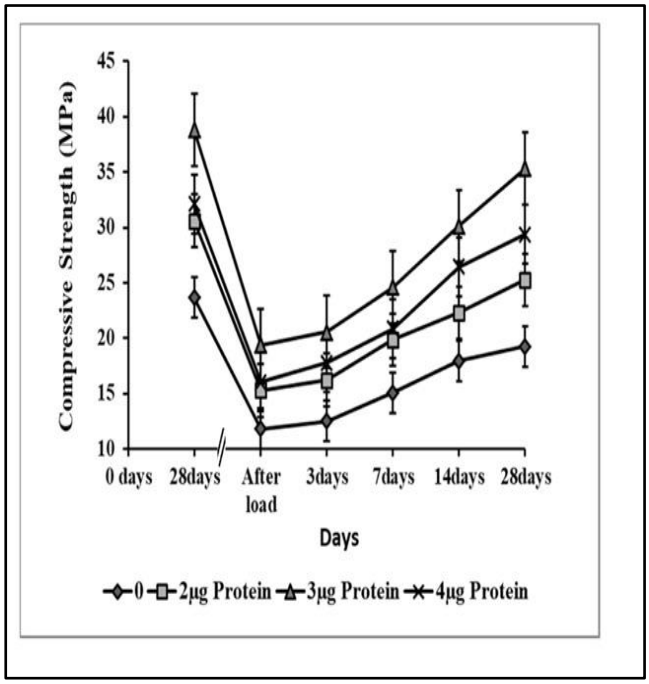


Fig 20

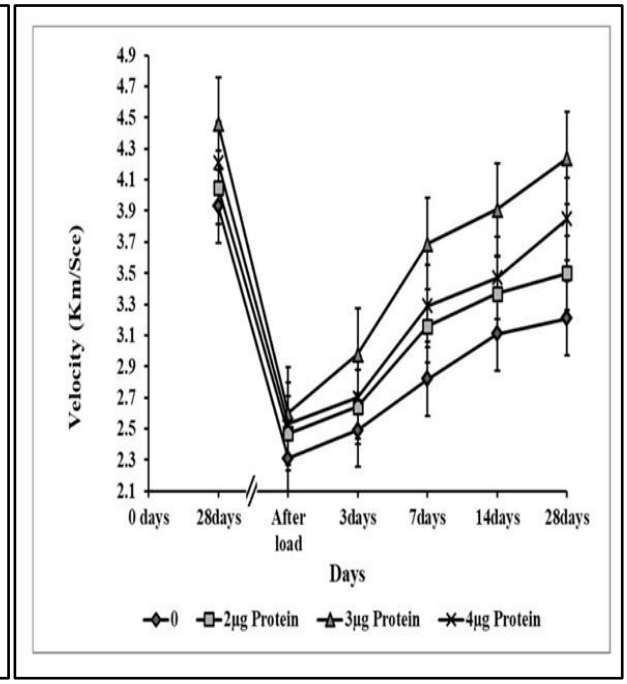


Fig 21

Fig 20: Compressive strength of the loaded mortar samples at different days of curing.
Fig 21: Ultrasonic pulse velocity of the loaded mortar samples at different days of curing.

For air curing different sets of control and samples are used for both loaded and unloaded experiment.

Table 6: Compressive strength of the loaded protein-amended mortar sample under air curing

Mortar samples	28 days (MPa)	After load (MPa)	3 days (MPa)	7 days (MPa)	14 days (MPa)	28 days (MPa)
(Control) 0	23.39±1.09	10.11±0.39	12.24±1.45	14.81±1.08	15.89±1.30	18.63±1.27
2µg protein	29.51±1.22	15.17±0.34	17.42±1.97	20.36±1.76	22.00±2.01	25.82±1.81
3µg protein	42.18±0.54	20.22±1.05	26.83±1.39	29.63±1.46	32.81±1.19	36.92±0.67
4µg protein	32.13±0.34	17.88±0.50	20.81±1.78	23.13±0.97	25.39±1.33	27.54±1.08

Table 7: Compressive strength of the unloaded protein-amended mortar sample under air curing

Mortar samples	3 days (MPa)	7 days (MPa)	14 days (MPa)	28 days (MPa)
(Control) 0	13.32±1.01	20.27±0.39	23.32±2.16	25.10±2.14
2µg protein	15.00±0.51	17.86±0.68	22.65±0.96	29.51±0.92
3µg protein	20.11±0.59	26.64±0.40	35.94±1.05	42.12±1.25
4µg protein	18.23±0.85	21.49±0.51	26.76±0.57	32.18±0.68

Table 8: Ultrasonic pulse velocity of the loaded protein-amended mortar sample under air curing

Mortar samples	28 days (m/sec)	After load (m/sec)	3 days (m/sec)	7 days (m/sec)	14 days (m/sec)	28 days (m/sec)
(Control) 0	3.94±1.59	2.13±1.10	2.39±1.45	2.65±1.78	2.76±2.09	3.23±2.33
2µg protein	4.08±1.43	2.18±0.59	2.42±0.70	2.69±1.89	3.33±2.13	3.57±1.03
3µg protein	4.59±1.02	2.78±0.98	2.98±1.49	3.34±0.48	3.60±1.14	4.15±1.32
4µg protein	4.23±0.98	2.54±0.88	2.89±1.48	3.22±1.89	3.67±1.47	3.77±1.55

Table 9: Ultrasonic pulse velocity of the unloaded protein-amended mortar sample under air curing

Mortar samples	3 days (m/sec)	7 days (m/sec)	14 days (m/sec)	28 days (m/sec)
(Control) 0	2.15±0.74	2.34±1.67	3.23±0.98	3.80±1.23
2µg protein	2.36±2.01	2.77±0.65	3.43±1.09	4.08±0.97
3µg protein	2.89±0.33	3.46±0.76	3.89±1.11	4.59±0.30
4µg protein	2.59±1.43	2.90±1.33	3.63±2.09	4.28±1.13

❖ Water Absorption Test Analysis

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.

Table 10: 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
2µg 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
4µg Protein	717.75±0.25	730.00±0.82	1.70	764.50±1.65	4.72

❖ Sulphate Resistance Test

Table 11: Sulfate resistance test of the 50% loaded Purified protein incorporated Mortar sample

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
2µg Protein	754.50±1.25	774.25±1.65	2.61
3µg Protein	751.75±1.18	761.25±0.95	1.26
4µg Protein	755.25±0.63	768.25±1.03	1.72

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 Analysis

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.

Table 12: Sorptivity test of the purified Protein incorporated 50% loaded Mortar samples

Time (Sec)	Control		2µg Protein		3µg Protein		4µg Protein	
	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
1200	730	1.52	725	1.11	718	0.27	723	0.97
1800	732	1.80	729	1.67	718	0.27	724	1.11
3600	736	2.36	734	2.37	718	0.27	728	1.67
7200	741	3.05	741	3.23	719	0.41	733	2.37
10800	747	3.89	745	3.90	720	0.55	736	2.79
14400	754	4.86	753	5.02	720	0.55	739	3.21
18000	759	5.56	756	5.43	721	0.69	740	3.35
21600	764	6.25	758	5.71	723	0.97	744	3.91
86400	769	6.95	765	6.69	727	1.53	758	5.86
172800	770	7.09	768	7.11	729	1.81	759	6.00
259200	773	7.51	769	7.25	730	1.95	761	6.28
345600	774	7.64	770	7.39	730	1.95	761	6.28
432000	774	7.64	770	7.39	730	1.95	761	6.28
691200	774	7.64	770	7.39	730	1.95	761	6.28

Chapter 4: Autonomous Self-Healing Attributes of bacterial (BKH4) Protein

Bioremediase Protein as Self-Healing Agent

Enough 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 category samples was applied to the rest of the respective category samples for creating microcracks on the samples. The photographs of the crack's 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 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.[1]

[Fig. 22]

[1] [A] & [B] Control mortar cracks were measured 0.05mm. [C] & [D] Test mortar (2μg Protein) cracks were measured 0.02mm. [E] &[F] Test mortar (3μg Protein) cracks were measured 0.01mm. [G] & [H] Test mortar (4μg Protein) cracks were measured 0.01mm. [I] & [J] Control mortar (with and without Protein water immersed) cracks were measured 0.02mm.

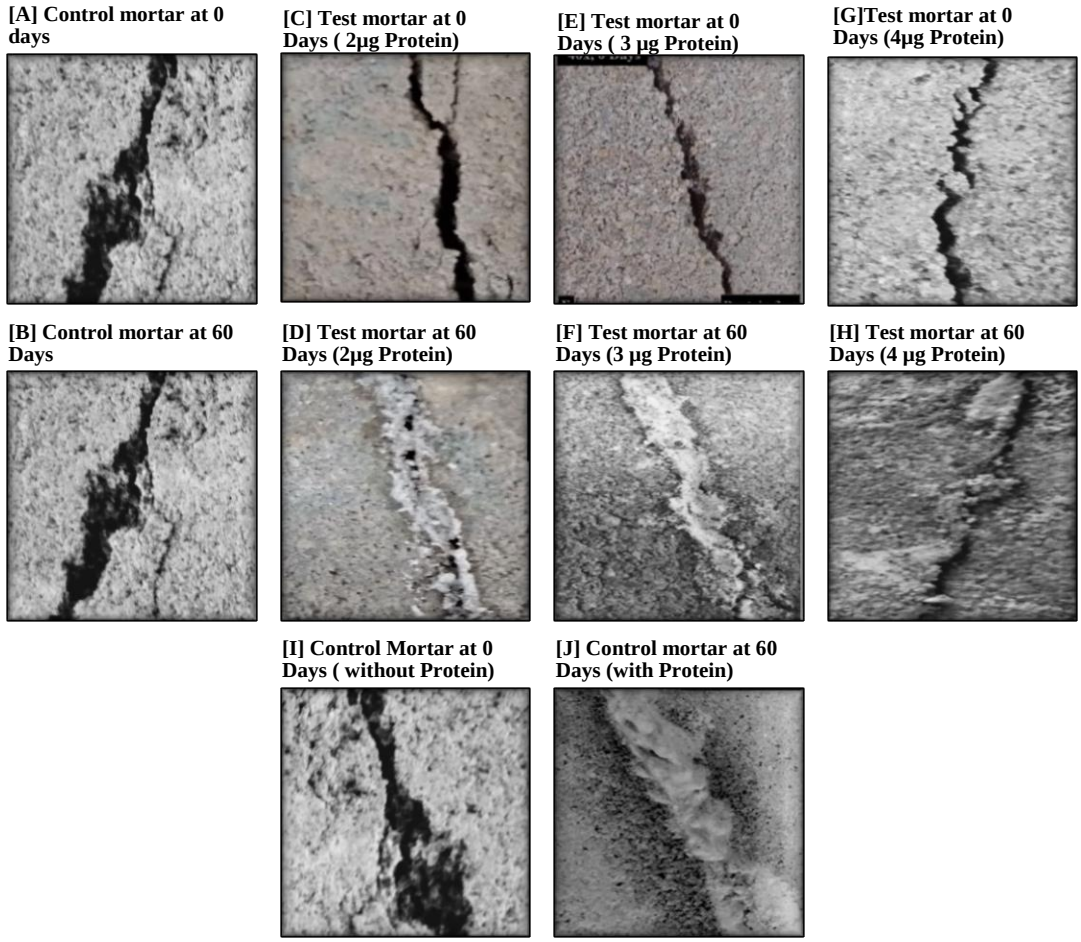


Fig. 22: Microscopic images of the mortar samples viewed by Crackscop. (a) control mortar at 0 days; (b) control mortar at 60 days; (c) test mortar at 0 days when 2 µg protein/g cement was used; (d) test mortar at 60 days when 2 µg protein/g cement was used; (e) test mortar at 0 days when 3 µg protein/g 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.

Fe-SEM View of Self-Healing Agent

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).

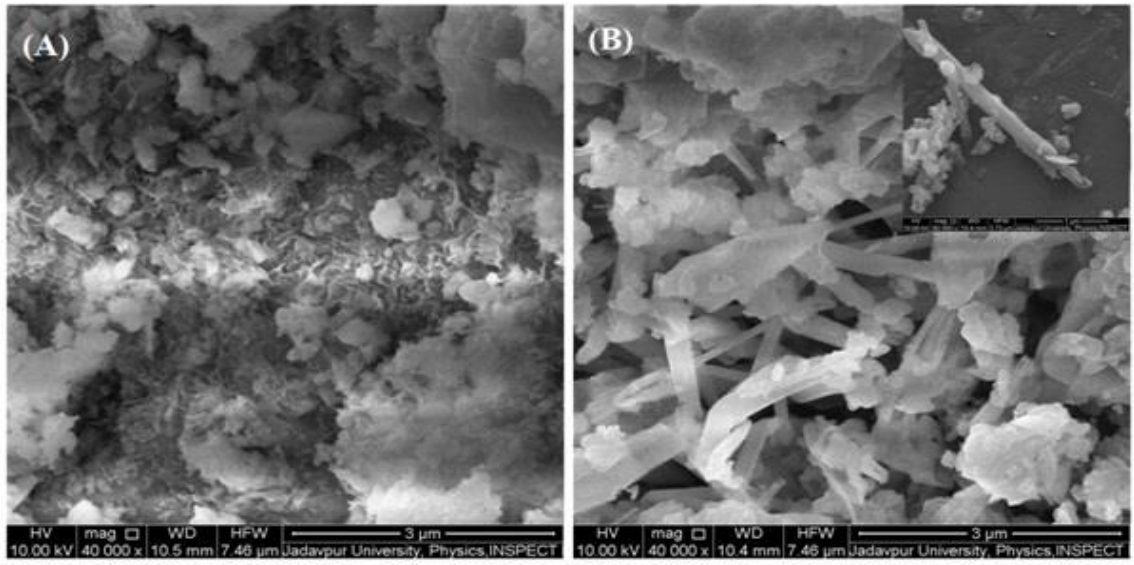


Fig 23: FESEM image of the powder healing materials: (a) control mortar; and (b) test mortar with single rod-shaped structure (inset).

EDX and XRD Analysis of Self-Healing Material

EDS study using the Detector type SDD-LN2 free and QUANTAX ESPRIT 1.9 software. QUANTAX ES-PRIT 1.9 software was used for elemental quantification studies. The EDS elemental study confirmed the presence of Ca = 2.14 %, Al = 7.05 %, Si = 35.68 % and O = 55.13 % (Fig. 24). X-ray spectroscopy analysis (Bruker AXS, Model D8, WI, USA) (Fig.25) 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 files (JCPDS).

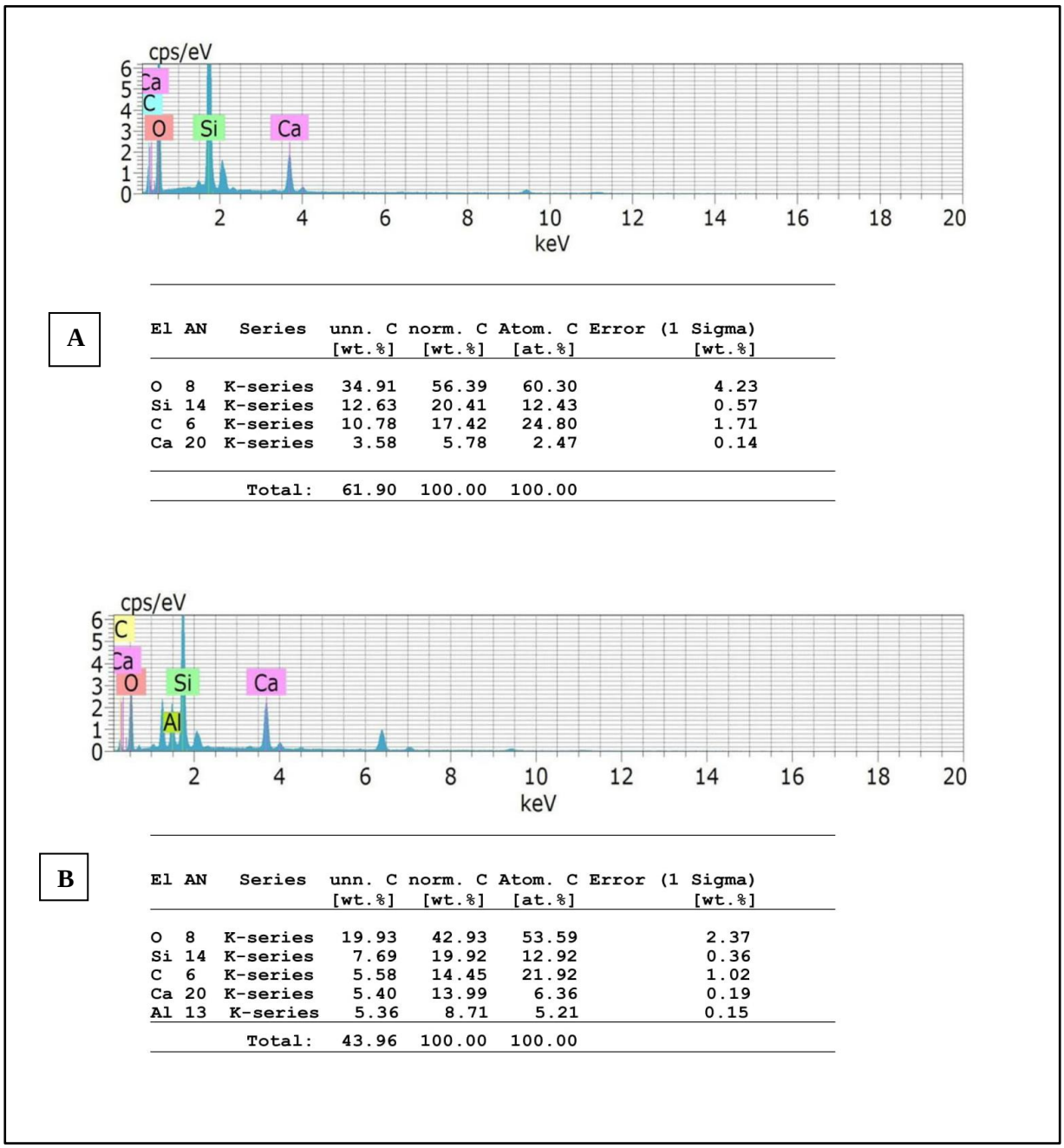


Fig 24: Elemental analysis by FESEM of (A) control; and (B) test sample (healing material) obtained from the mortar samples.

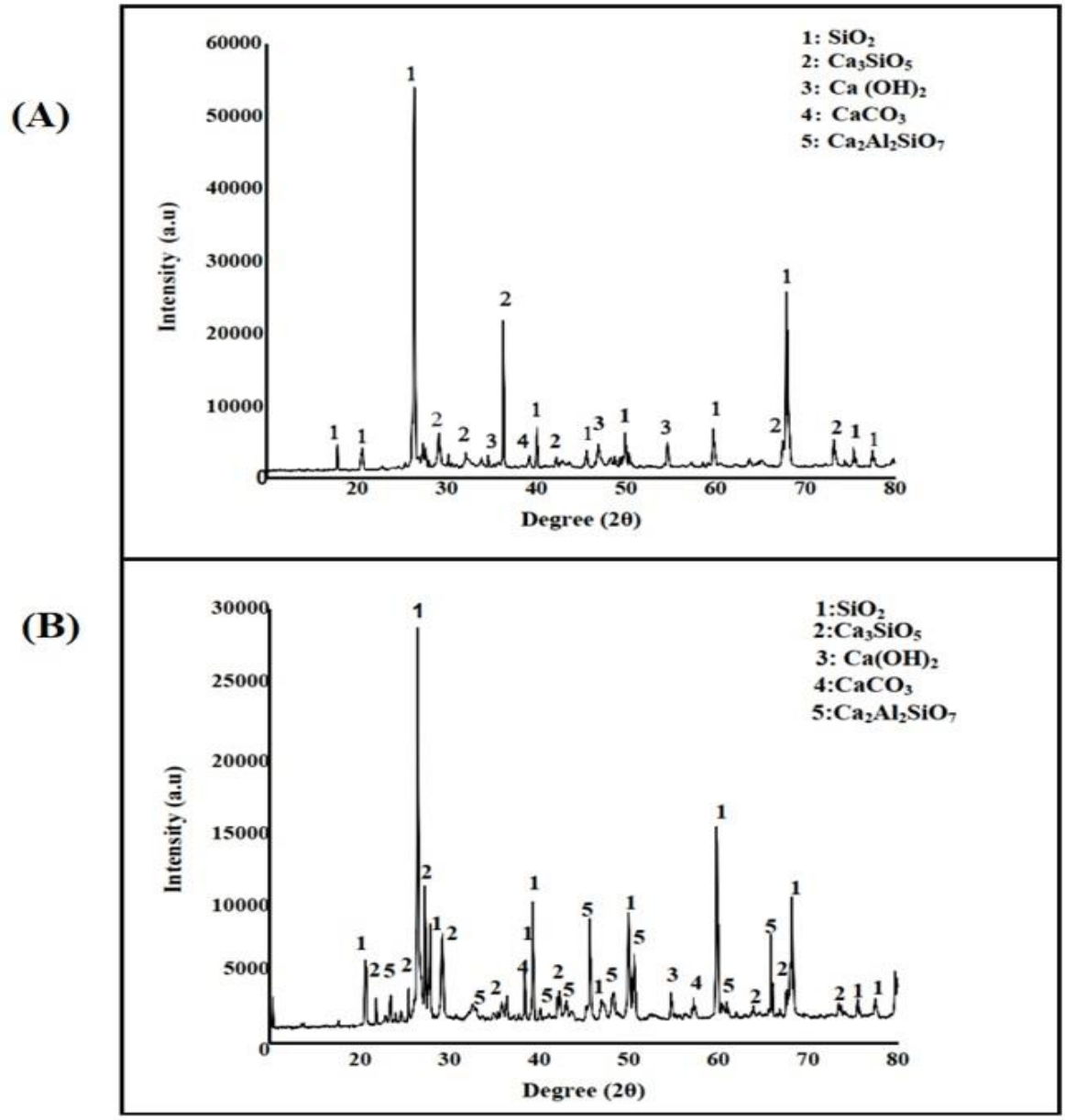


Fig 25: XRD analysis: (A) control mortar powdered healing material; and (B) test mortar powdered healing material.

DISCUSSION

Discussion

Micro-organism-modified mortar/concrete is now a key 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 **[Fig. 3(A & B)]**, Gram positive **[Fig. 4]** and possesses fluorescence property **[Fig. 5]**. 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 **[Fig. 6 (A & B)]**. As reported earlier, various bacterial strains (e.g., BKH1, BKH2, BKH3, etc.) have been isolated from hot spring of Bakreshwar, but none of them were found to be highly alkaliphilic in nature (Sarkar et al., 2014; 2015 and 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 intrametric 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 BKH4 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). **[Fig. 7]** The phylogenetic tree based on partial 16S rRNA gene sequences **[Fig. 2]** of the isolated strains clearly indicates that the bacterium is a novel one which is strongly 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 CO2 due to the presence of urease gene (Benita, 2014). *Lysinibacillus fusiformis* ZC1 strain shows 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 (Sarkar et al., 2022). 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. 14]**. 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 and Sarkar et al., 2014). The increase in the strength of BKH4 bacteria incorporated mortars are due to the development of new filamentous material within the mortar matrices, that is, formation of Gehlenite material as revealed by XRD analysis **[Fig. 19(A & B)]** which supports our earlier findings (Sarkar et al., 2014). **[Fig. 18(A & B)]** EDX analysis similarly shows that there is remarkable 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 and Chowdhuri et al., 2015). A bioremediase protein can leach silica (SiO2) as silica nanoparticles from a silicate compound for it is catalytic activity. 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 (Sarkar et al., 2022). 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. Besides the increment of the compressive strength **[fig. 14]** and compactness of the bacteria-incorporated cementitious samples **[Fig. 15]**, the durability of the sample in another incredibly 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 4]**, sulphate resistance tests **[Table 5]** and RCPT **[Fig. 16]** 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 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.

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 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 bio-concrete mortar. The cells of the BKH4 bacterium release some proteins in the medium during their culture. One of the proteins possesses silica leaching action like the bioremediase protein. This property has been confirmed by the biosilicification reaction of the protein, as shown in **Table 3**. 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 and 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 and De Muynck et al., 2008). Unlikely, all those bacteria usually grow at a normal pH range (pH 7.0 to 8.0) and can 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. 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) **[Fig. 6 A & B]** 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 an extended period. The proteins released by the bacterium BKH4 are separated through the Sephadex G-100 column chromatographic technique **[Fig. 8]**. The protein reveals biosilicification action, molecular weight is approximately 28 KD **[Fig. 8 inset]**. The protein was separated in pure form from BKH4 cultured medium, which concentration was determined by Lowry and Bradford assay **[Table 1& 2]** and

the mechanistic action of silica leaching ability of bioremediase protein (isolated from BKH4) depending on pH, buffer, and temperature **[Fig. 11, 12, 13]** are mentioned here.

It is as of now known that the pH includes a vital part to decide the movement as well as structure of all proteins. Changes within the pH lead to the breaking of the ionic bonds that holds the tertiary structure of the chemical. In this way, the protein starts to lose the useful form of its dynamic location in such a way that the substrate will now not fit into it and the protein is alluded as denatured. With the alter of pH, the charges on amino acids within the dynamic location are influenced for which the protein will not be able to create an enzyme-substrate complex. Most chemicals are useful over a particular pH extend. In common, protein encompasses a pH ideal although it isn't the same for each chemical. It was famous that the bioremediase protein remained dynamic over a wide run of pH (8–14) and the ideal action was watched at pH 12.0 **[Figure 16]**.

In an enzyme-substrate response, buffer plays an imperative part within the component of activity. The viability of the buffer moreover depends on its concentration. The rate of silica filtering action of the bioremediase protein was found to extend with the expanding concentrations of the buffer arrangement **[Figure 17]**. The ideal action of the bioremediase protein was watched at 50 mM Tris–HCl buffer, pH 12.

Essentially, temperature moreover plays a part within the enzyme-substrate response energy. The rate of a protein interceded catalytic response is found to be diminished at an elevated temperature. This is often since protein or substrate gets to be denatured and inactivated at the hoisted temperature environment. Each protein has an ideal temperature extend at which the maximal rate of response is accomplished. The silica filtering action of the bioremediase protein was found to happen over a wide extend of temperature (50 ºC - 80 ºC) and the ideal action of the chemical was

found to be 65 ºC **[Figure 18]**. This result recommended that the bioremediase protein was a thermostable protein.

Likewise, 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 nano silica) from any silicate substance. Thus, nano silica is formed by the interaction between the protein with tetra-ethyl-ortho-silicate (TEOS) as shown in Sarkar et al. (2022). Similar silica leaching proteins have been found in several hot spring bacterial strains (e.g., BKH1 and BKH2). The nano silica 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 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. Those cracks are sealed by some tiny fingers like material after water curing **[Figs. 22 (D, F, and H)]**. No such sealing substance is observed on the cracks of control mortar samples when similarly cured under water **[Fig. 22(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. 22(J)]**. This is unambiguous 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 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 eventually 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 protein-amended mortars **[Fig. 23(b)]**. Whereas no such appearance is seen in the cracked regions of control mortar samples **[Fig. 23(a)]**. The analytical result for elemental identification of the healing material as done by energy-dispersive X-ray analysis is shown in **[Fig. 24]**. It confirms the formation of a novel phase only consisting of calcium, aluminum, oxygen, and silicon atoms. The X-ray crystallographic 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. 25(A & B)]**. The additional new peaks are the correspondent peaks of a pure calcium–aluminum–silicate phase (Ca2Al2–SiO7 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. 20]**, 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. 20]**. Comparable results are obtained for both loaded and unloaded mortar samples' compressive strength in air-curing **[Table 6 & 7]**. 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 and 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 UPV of the protein-incorporated mortar samples when cured in different conditions **[Fig. 21 and Table 8 & 9]**. 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 10]**. It is noted that engrossed water increases the mass of the bacterial protein-amended samples (3 μg protein = 1g 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 11]**. The maximum sulfate resistive action was found at the protein concentration of 3 μg = 1 g cement used. **Table 12** 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 regains, and capillary water absorption. 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. The bacterium BKH4 is a water-grown hot spring bacterium that requires minimal ingredients for its growth (Sarkar et al., 2019). The desired protein which is secreted from bacterial cells 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. It means 3 g of protein can work on 1 ton of cement. Neither the protein nor the bacterium causes any harm to human health. So, this is an eco-efficient and cost-effective methodology (as no additional techniques are required to obtain the protein) which can be used for construction purposes.

To summarize what was discussed previously; firstly, the highly alkaliphilic bacteria BKH4 was isolated from hot spring and characterized morphologically and phylogenetically. After that, the applications of the bacterium BKH4 and its bioremediase protein in the development of Bioconcrete materials have been established. In this study, the applications of BKH4 bacteria incorporated mortar samples characterisation and the use of bioremediase protein in concrete/mortar materials and in self-healing phenomenon were reconfirmed and shown in **Fig. (14-25) & Table (4-12)**. It was noted that the compressive strength of the mortar samples was increased at all ages (up to 28 days) and different curing conditions (both at air curing and water curing) when bioremediase protein was incorporated in different concentrations to the cement-sand mixture to form mortar samples. **Sarkar et al.** (2019) reported that the addition of BKH4 cells (10^4 cells/ml water used) increased the compressive strength of the bacteria incorporated mortar specimens by more than 50% compared to the control mortar specimens at 28 days of curing. The increment of compressive strength was due the formation of calcium-aluminium-silicate (Gehlenite) phase inside the mortar matrices by the biochemical action of bioremediase protein present in the bacterial cell surface. The direct addition of pure bioremediase protein (3 μg/g cement) showed that more than 50% compressive strength was increased at 60 days of curing to the protein amended samples in comparison to the control samples. There were 40% in air curing and 50% in water curing of compressive strength at 28 days of curing noted in the bioremediase protein amended mortar samples as shown in **Table 6** and **Fig. 20**, respectively. This was an exceptionally good example for the application of bioremediase protein against the compressive strength increment of concrete/mortar samples. In the end, the bioremediase protein also showed its potential application against crack repairing ability. The artificial crack generated on the surface of the bio-mortar sample was completely repaired by the self-healing attribute of the bioremediase

protein as seen in **Figure 22**. The crack was repaired due to the formation of a new phase (Gehlenite) on the cracked surface which acted as a self-healing material **[Figure 23]**. The increment in the compressive strength and UPV were best observed with 3 μg of protein for self-healed mortar sample using 50 % breaking load for 28 days water curing **[Table 20 & 21]**. Less water absorption, high sulphate resistance and slower water movement were also best noticed with 3 μg of protein contented self-healing mortars **[Table 10-12]**. The microstructure analysis of self-healing material **[Fig. 24 & 25]** established that formation of some new structure, due to the bacterial protein, healed cracks in damaged concrete/mortars.

CONCLUSION

Conclusion

The recognizable proof of profoundly alkaliphilic thermophile bacterium BKH4 strain is exceptionally much vital since of its biotechnological application in concrete innovation. Not as it were the bacterial cells but too the biosilicification movement of the excretory protein of BKH4 has been set up additionally characterized.

The comes about of the tests clearly appear that the bacterial strain BKH4 increments the compressive quality of the concrete/mortar, which illustrate that thermophilic and exceedingly alkaliphilic BKH4 bacterium would be a perfect and compelling micro-organism which would be utilized for the advancement of higher quality and more strong concrete/mortar fabric within the close future. For illustration, a few microbes require nourishment to develop interior the cementitious networks which diminishes the quality of the test. A few cannot develop inside a tall soluble cementitious environment. Keeping all these realities in intellect, a novel exceedingly antacid, water-grown bacterium (BKH4) has been separated from a hot spring in Bakreshwar which may overcome a few impediments of this think about.

It is additionally found that at a concentration of 3 μg protein/g cement, the compressive quality of the mortar test was maximized. This protein too creates higher quality and more solid bio-concrete fabric due to the arrangement of an unused nano-silicate fiber inside the mortar lattices (Sarkar et al., 2022). The quality self-healing in concrete is conceivable by imbuing the profoundly alkaliphilic bacterial protein (most extreme movement ranges from pH 12.0 to 12.5) within the cementitious fabric that not one or the other requires the supplementation of nourishment nor influences the mechanical properties of the fabric. The modern silicate fiber (Gehlenite or calcium

aluminum silicate) inside the frameworks fills the micro-pores of the mortar that comes about the upgrade of mechanical quality of the materials. This protein is exceptionally much steady over a wide run of pH (6-12) and temperature (60–80◦C). The microbial protein increments the ultrasonic beat speed and compressive quality, increases the sulfate resistance, decreases water porousness, and moderates down water development (sorptivity test) of the protein corrected mortar tests, which uncover that there's generally enhancement in mechanical properties and toughness of the protein-incorporated mortar tests.

So, this ponder concludes that profoundly Alkaliphilic bacterial protein can be utilized for the genuine recuperating reason in green and maintainable development innovation. In this complex setting, the utilize of exceedingly alkaliphilic thermophile microbes can be seen as beam of trust to future economical green cement innovation because it comes from characteristic source. Subsequently it can be utilized without any concern for natural contamination. This strategy is client inviting, does not provide rise to any wellbeing risks and commercially practical in development businesses. This consider will certainly make a modern trust and include an unused heading to concrete innovation and this would be one of the low-cost and compelling measures against concrete weakening for future development innovation.

This novel concept may speak to imaginative pathways for falsely actuating the profoundly alluring self-healing in concrete materials and concrete innovation in close future.

LIMITATIONS & FUTURE STUDY

Limitations of this study

- Double step purification through Sephadex G-100 gel filtration chromatography technique is needed, although the protein is excretory in nature.
- As the column packing material Sephadex G-100 used for purification is very much costly, so some alternative technique or column packing material, for purification may be cost-effective.
- The yield of the bioremediase protein is very low, which required bulk purification to pursue the research goal.

Scopes of future study

- Full length genome sequence of BKH4 bacteria can help to understand more details nature of the bacteria.
- Full amino acid sequencing of the bacterial (BKH4) protein is required to characterize its gene.
- To know the exact structure of the protein, Nuclear Magnetic Resonance (NMR) and X-ray Crystallography studies are required.
- By identifying the gene against this protein, cloning can be done by using suitable vector. Once cloning is possible then by inserting the cloned gene in the appropriate microorganism (E. coli), the bulk production of protein can be done.
- Application in the field of nano technology is still unknown.
- Details study of needle-like structural arrangement of Gehlenite material, in cementitious matrices, is required to calculate the maximum strengths of the mortar/concrete.
- Large scale production of the bioremediase protein for commercially viable concrete industry.

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PUBLICATIONS

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

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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.**

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;

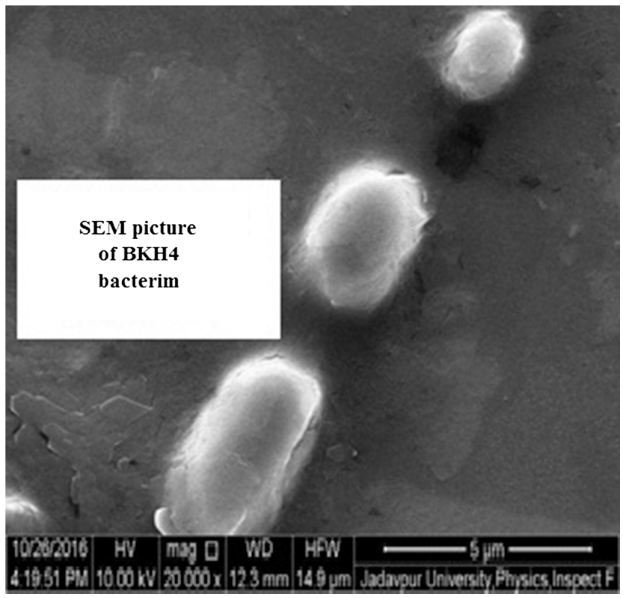


Fig. 1. FESEM image of BKH4 bacterium.

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 $\pm$ 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

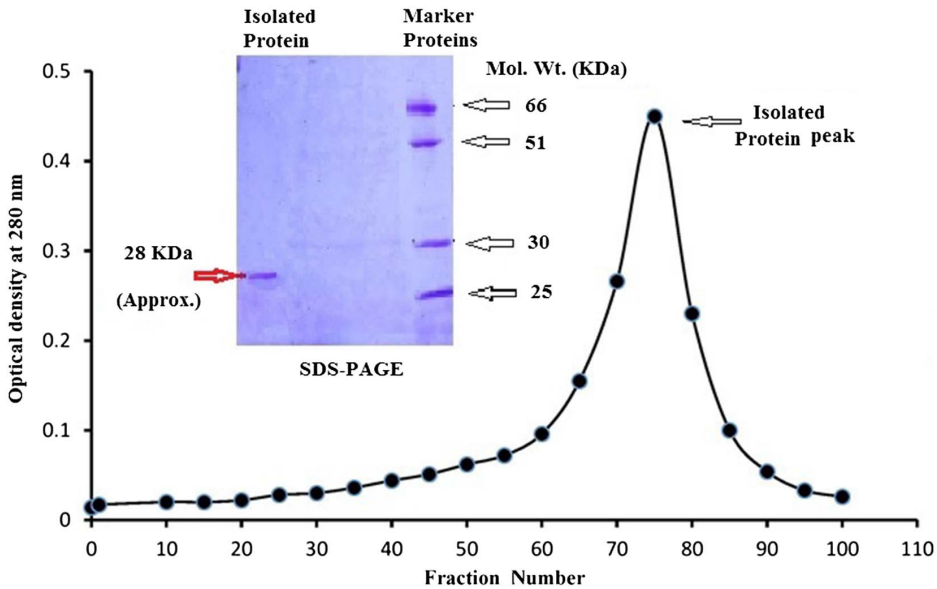


Fig. 2. Purification and SDS-PAGE (inset) of secretory protein of BKH4 bacterium.

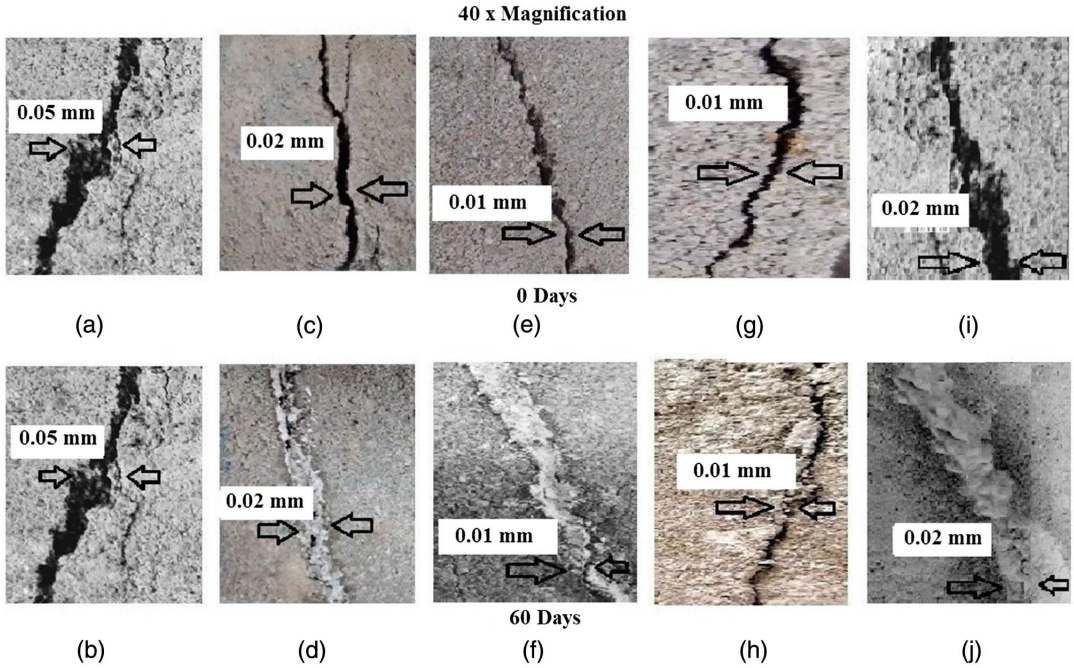


Fig. 3. Images of the cracked mortar samples viewed by Crack scope: (a) control mortar at 0 days; (b) control mortar at 60 days; (c) test mortar at 0 days when 2 μg protein/g cement was used; (d) test mortar at 60 days when 2 μg protein/g cement was used; (e) test mortar at 0 days when 3 μg protein/g 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.

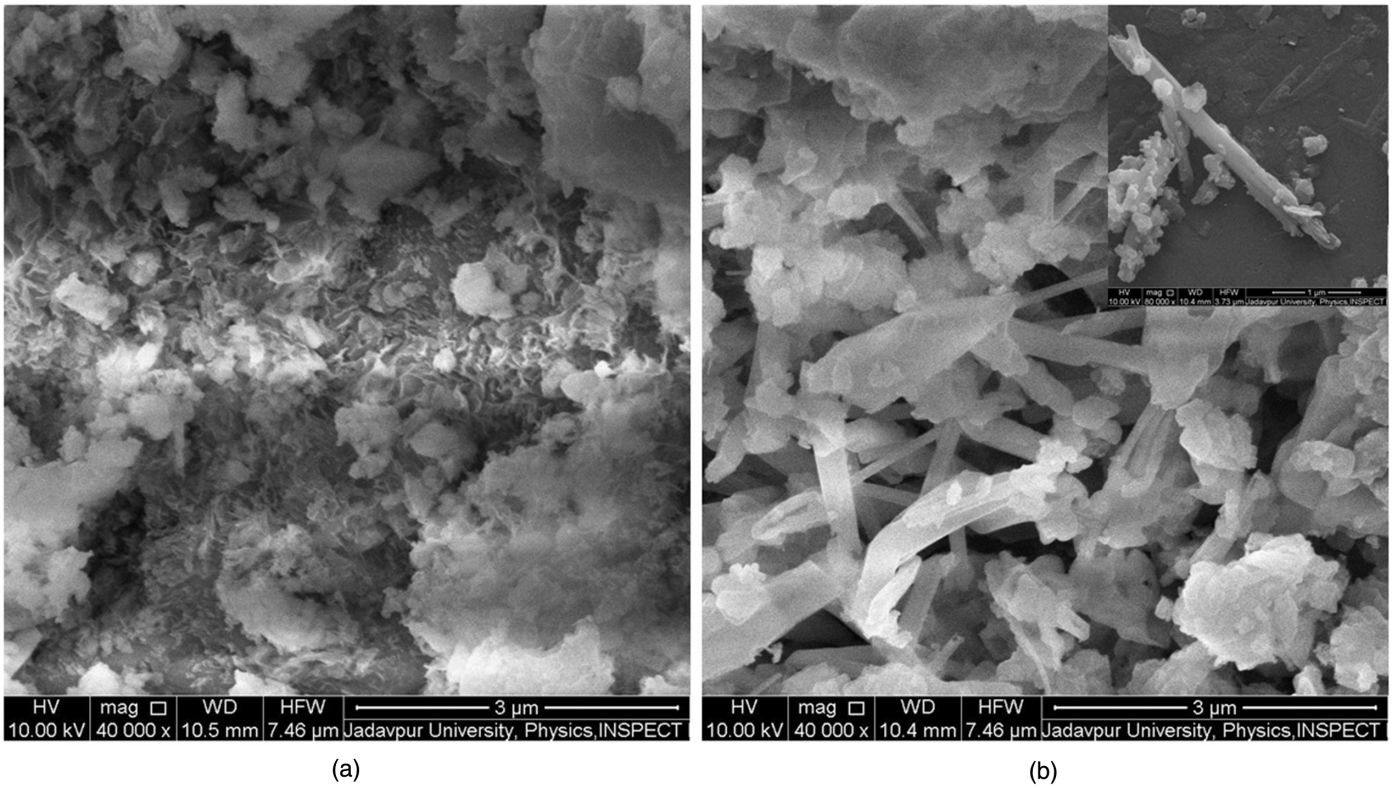
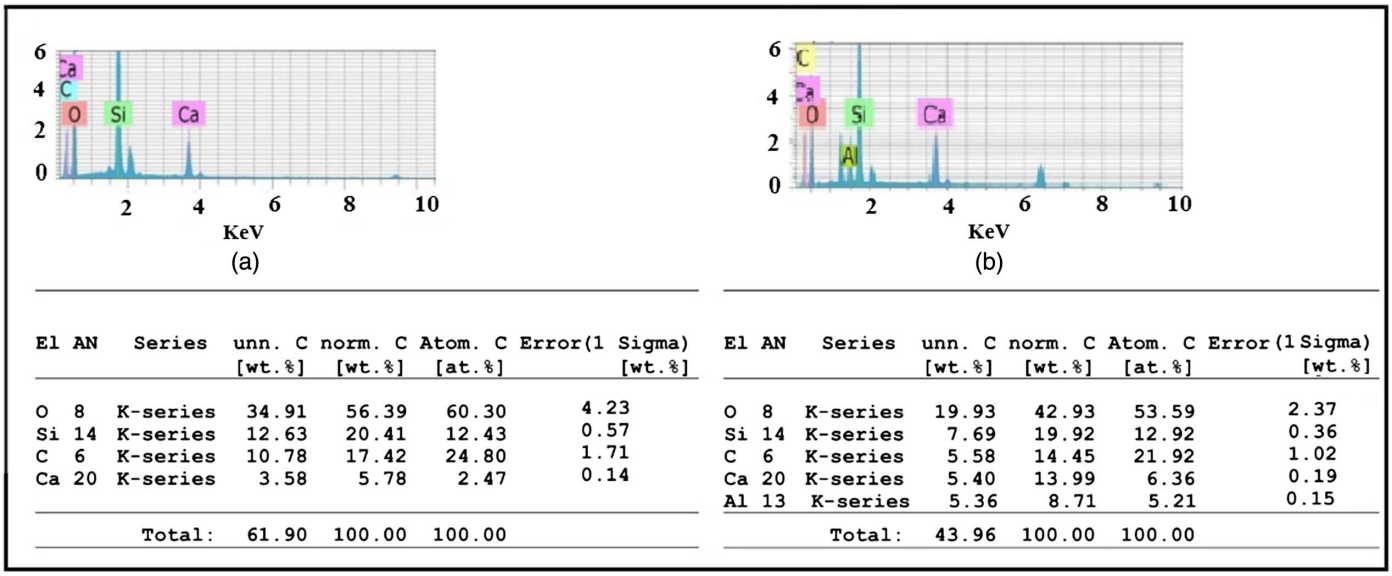


Fig. 4. FESEM image of the powder healing materials: (a) control mortar; and (b) test mortar with single rod-shaped structure (inset).

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.

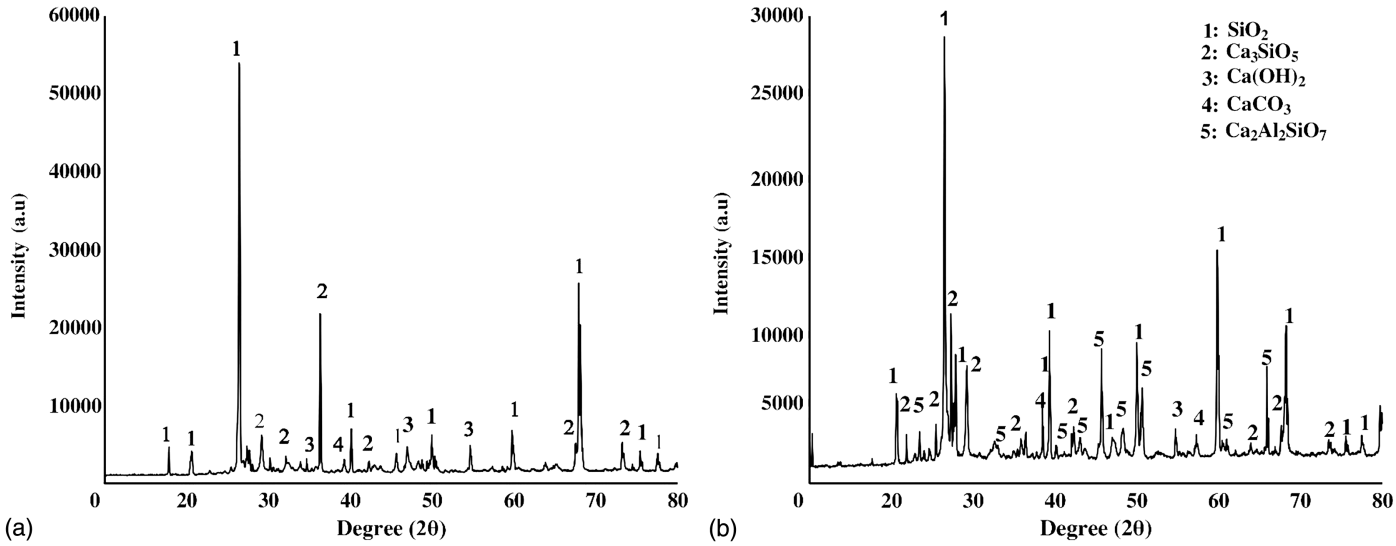


Fig. 6. XRD analysis: (a) control mortar powdered healing material; and (b) test mortar powdered healing material.

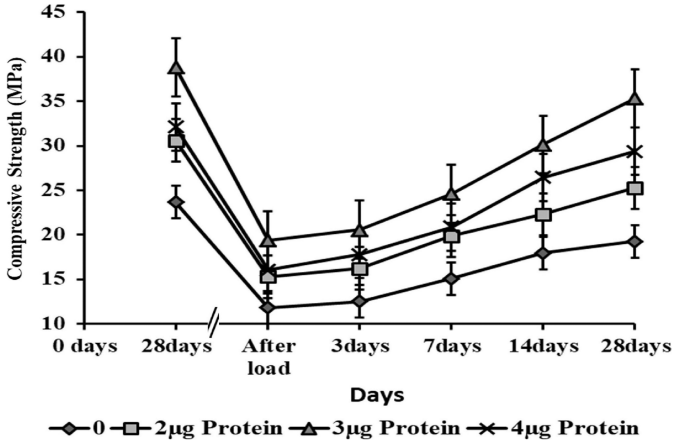


Fig. 7. Compressive strength of the loaded mortar samples at different days of curing.

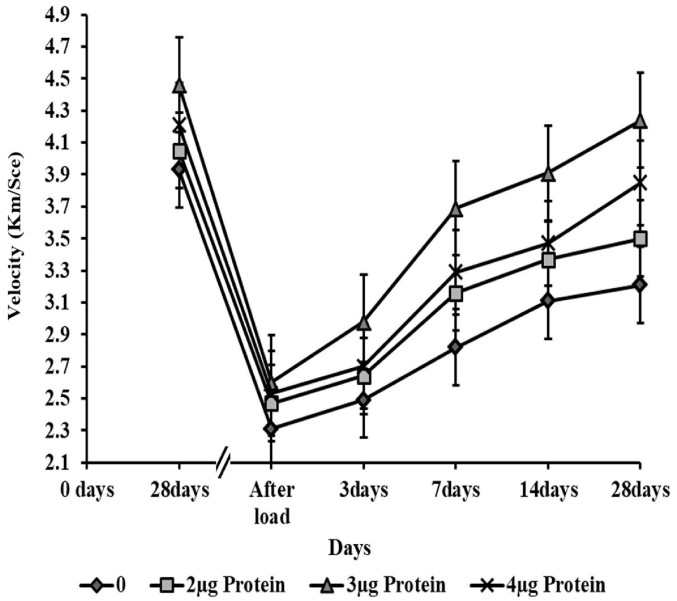


Fig. 8. Ultrasonic pulse velocity of the loaded mortar samples at different days of curing.

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 UPV of 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.

Acknowledgments

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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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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′-GACTTGCATGTGTTAGGCCTG-3′. 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 × 70·6 mm ×70·6 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 × 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 μm) 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 $2\theta = 20°$ 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-μm 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

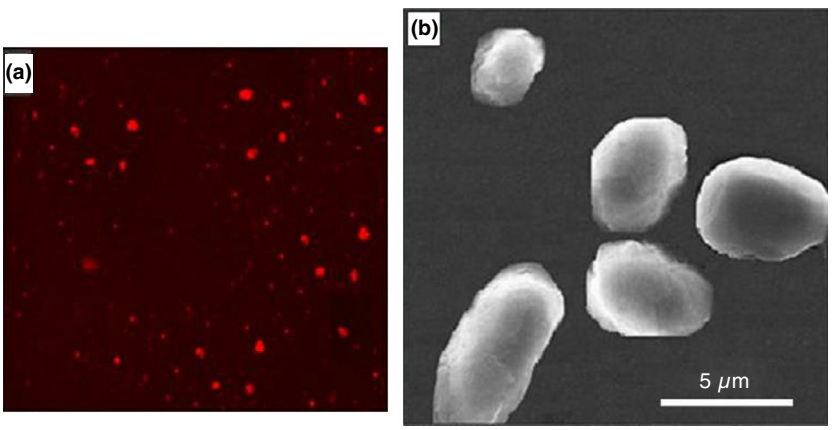


Figure 1 (a) Microscopic view (20X). (b) SEM view of the isolated BKH4 bacterium. [Colour figure can be viewed at wileyonlinelibrary.com]

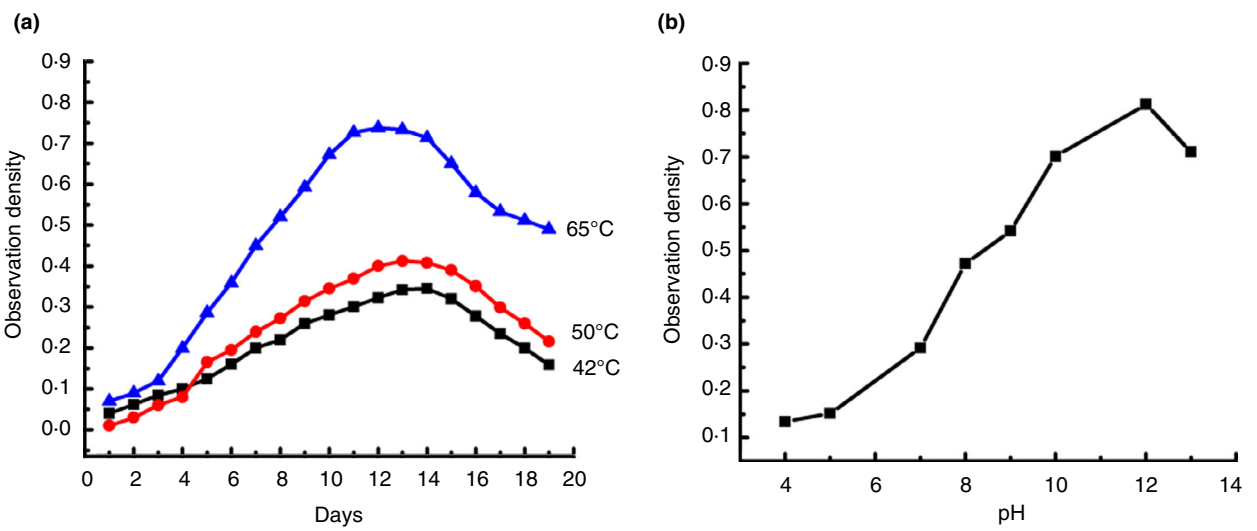


Figure 2 (a) Temperature variation growth curve of BKH4 (n = 3). (b) pH variation growth curve of BKH4 (n = 3). [Colour figure can be viewed at wileyonlinelibrary.com]

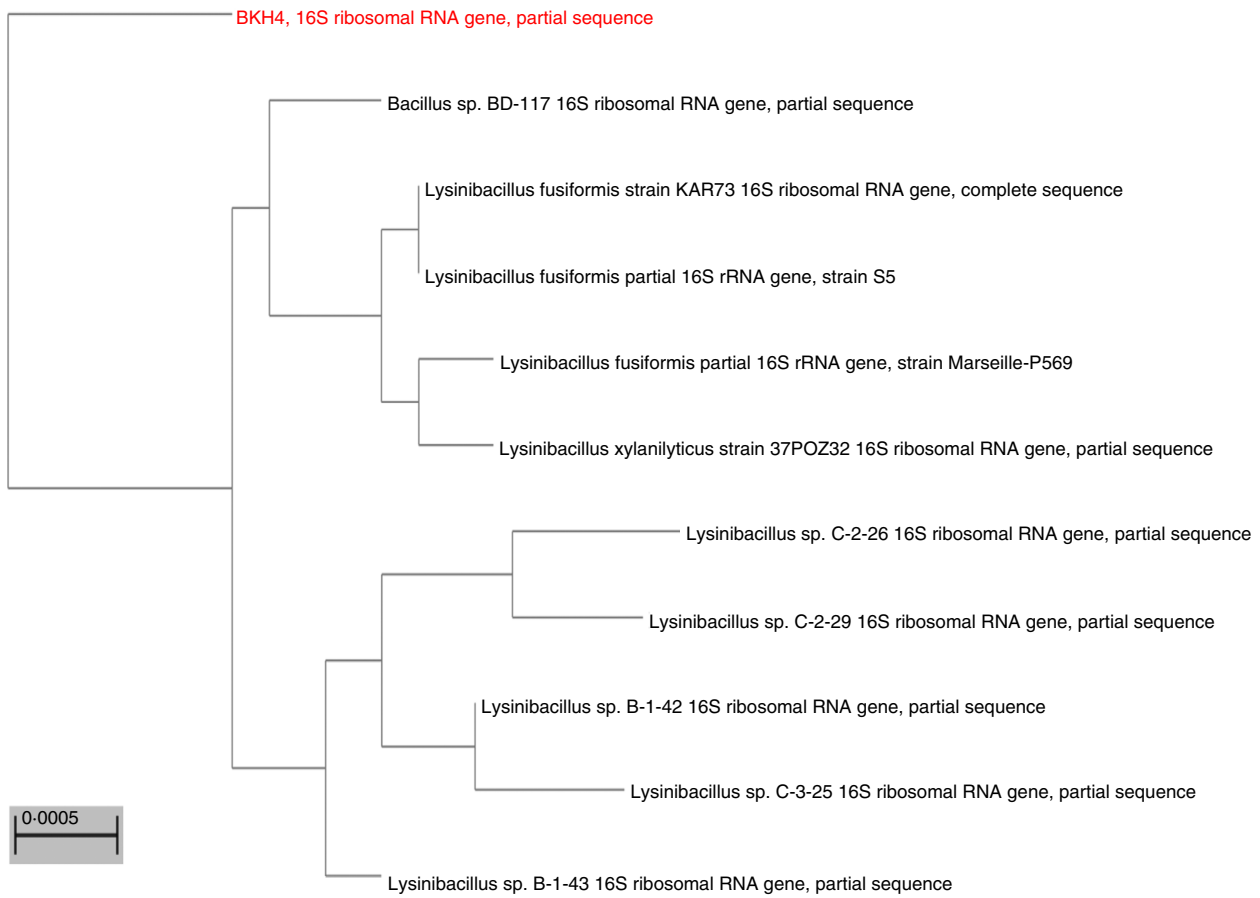


Figure 3 Phylogenetic tree of BKH4 bacterium. [Colour figure can be viewed at wileyonlinelibrary.com]

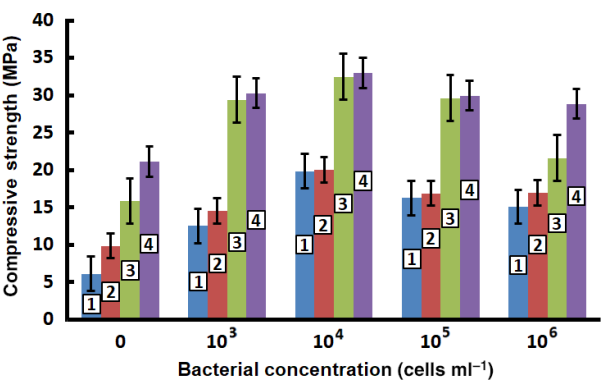


Figure 4 Compressive strength of BKH4 bacterium amended mortar samples at different days of water curing. Where 1: 3 days, 2: 7 days, 3: 14 days and 4: 28 days of curing respectively. [Colour figure can be viewed at wileyonlinelibrary.com]

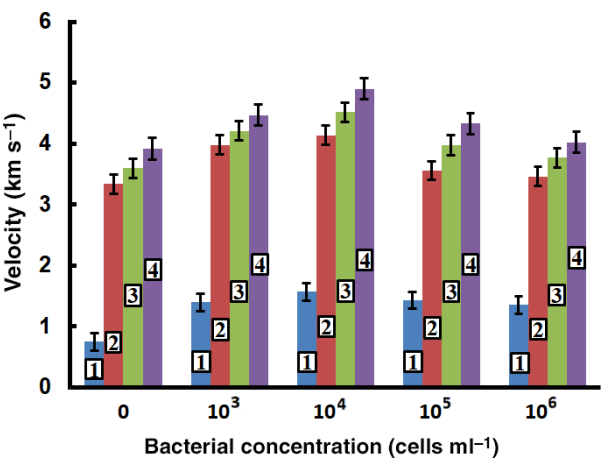


Figure 5 Ultrasonic pulse velocity of BKH4 bacterium-amended mortar samples at different days of water curing. Where 1: 3 days, 2: 7 days, 3: 14 days and 4: 28 days of curing respectively. [Colour figure can be viewed at wileyonlinelibrary.com]

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.

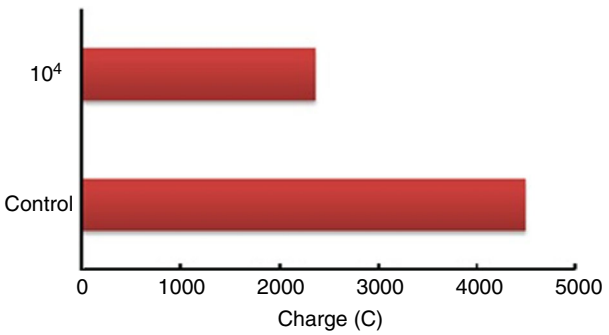


Figure 6 Result of RCPT test of control and bacterium-amended mortar samples. [Colour figure can be viewed at wileyonlinelibrary.com]

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

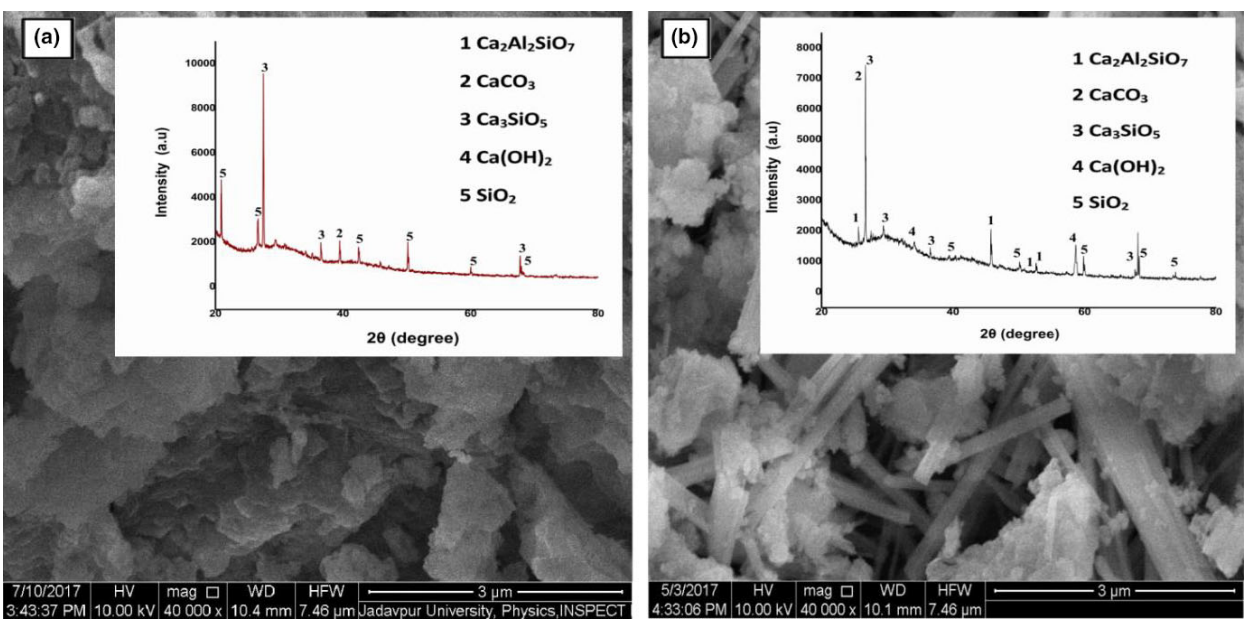
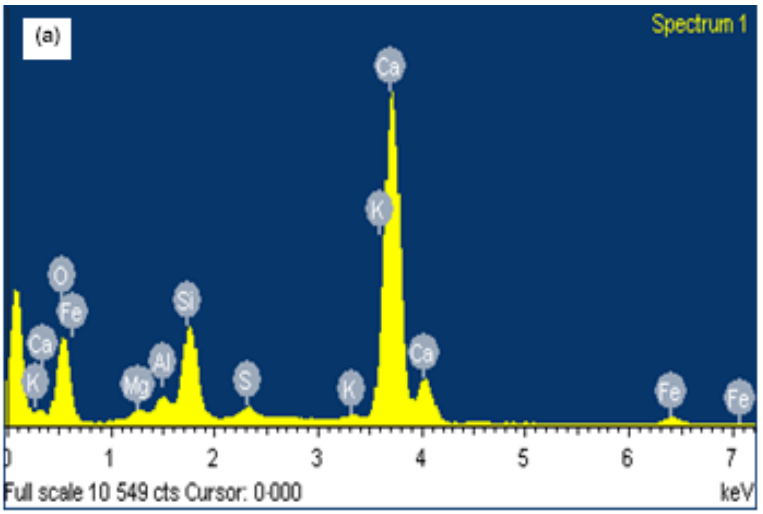
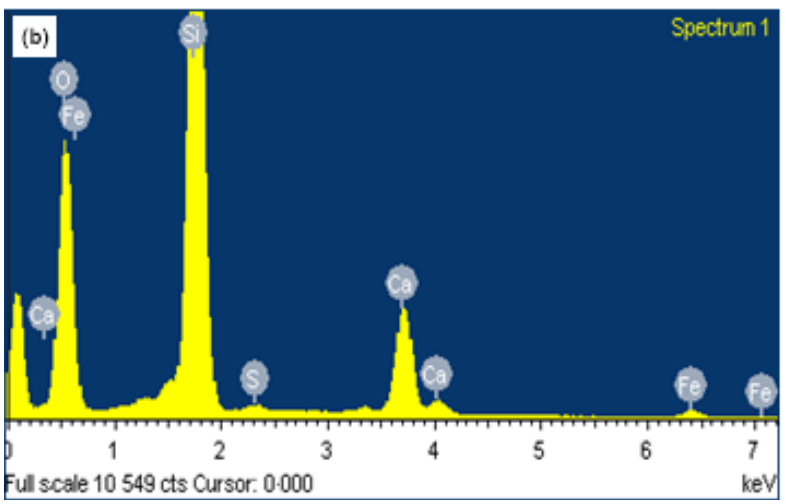


Figure 7 SEM photographs of control mortar matrix (a) and BKH4 bacterium-amended mortar matrix (b); XRD analysis of the matrix are shown in the inset of the corresponding figure. [Colour figure can be viewed at wileyonlinelibrary.com]



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 wileyonlinelibrary.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).

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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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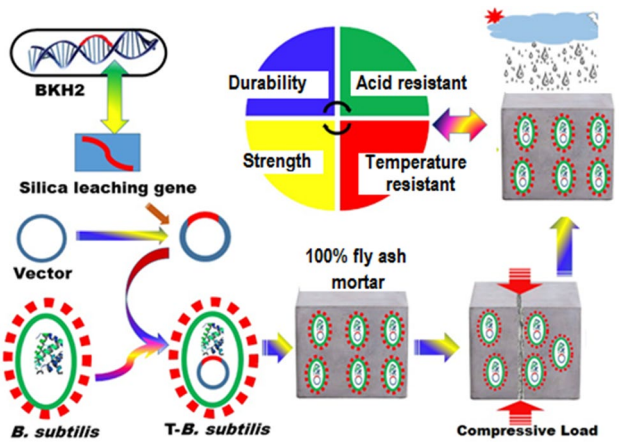
Abstract

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 samples 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 $2\Theta = 10–80$ 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

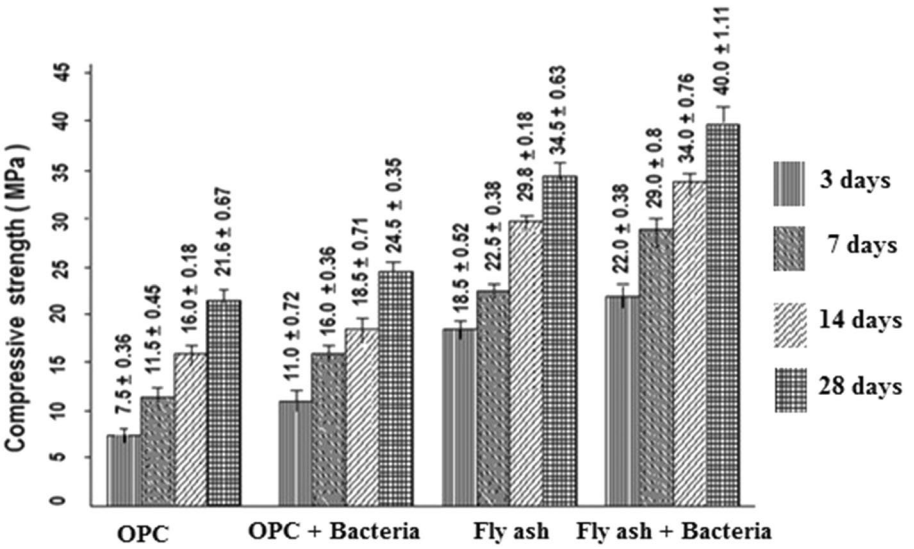

Fig. 1 Compressive strength of different category mortar samples

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

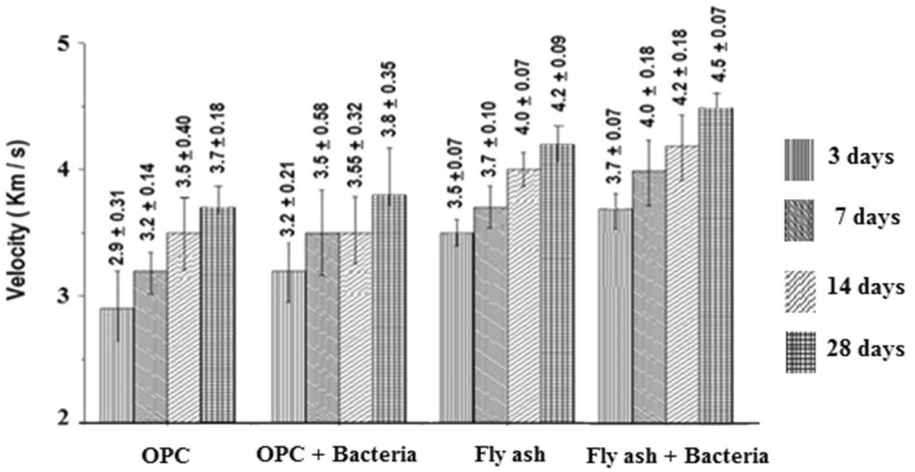

Fig. 2 Ultrasonic pulse velocity of different category mortar samples

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

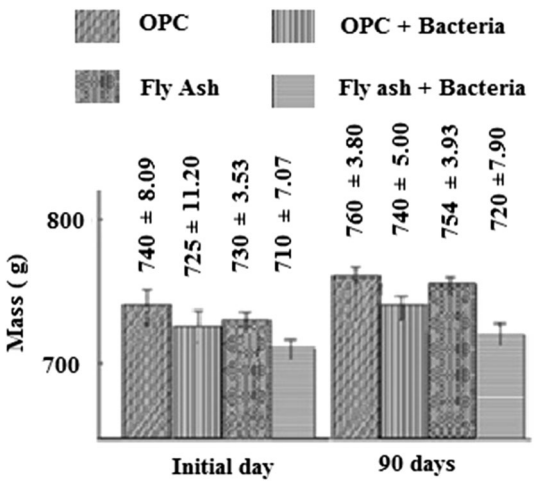

Fig. 3 Sulphate resistance activity of different category mortar samples

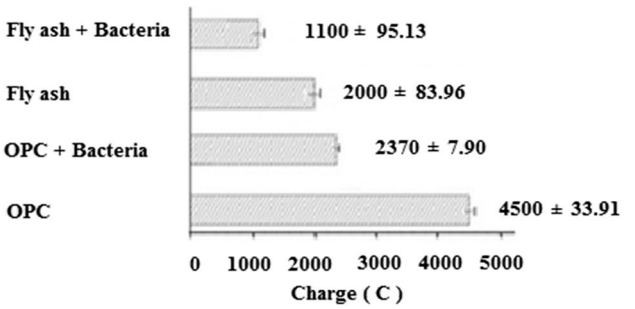

Fig. 4 Rapid chloride permeability of different category mortar samples

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.

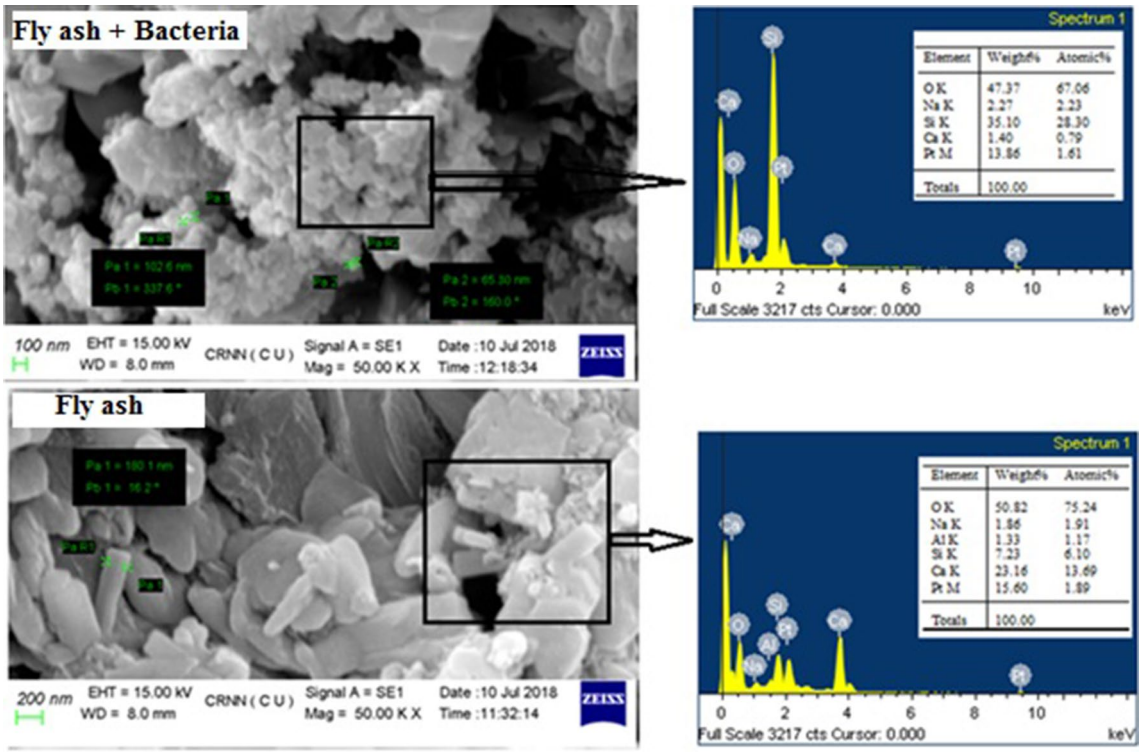

Fig. 5 SEM image with EDX analysis of fly ash geopolymer and fly ash geopolymer + bacteria-incorporated mortar sample

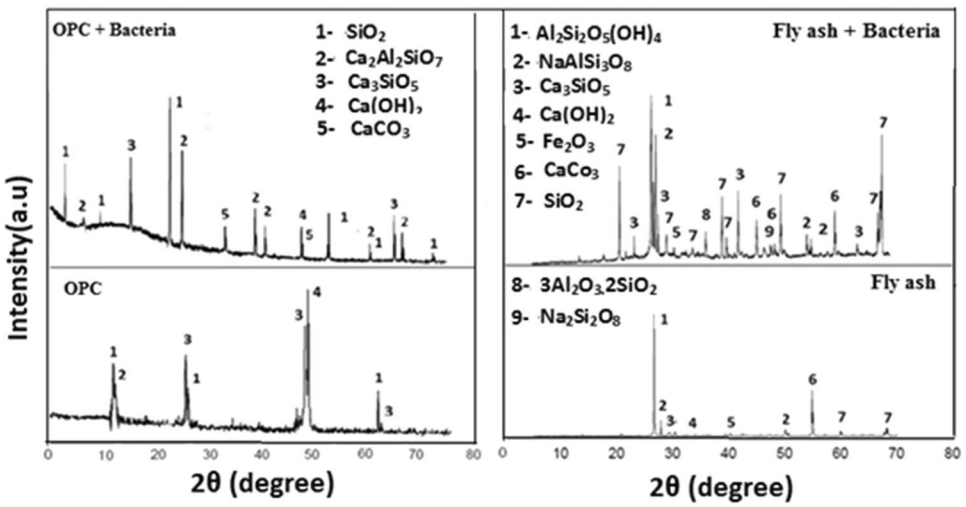

Fig. 6 XRD analyses of different category mortar samples

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

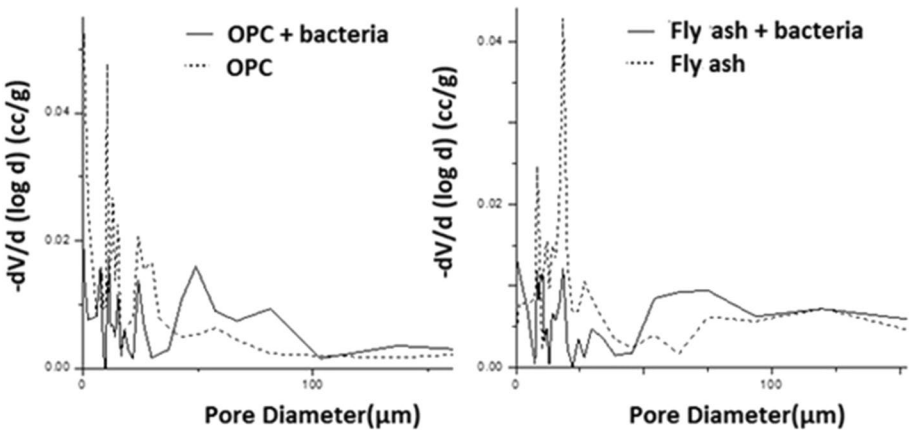

Fig. 7 MIP analysis of different category mortar samples

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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