Influence of microbial activity on mechanical properties & durability of self-healing concrete using indigenous *Rhizopus oryzae* and *Trichoderma longibrachiatum*.

By

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This is to certify that,

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Dedicated

To

Allah Almighty,

(Lord of Multiverse who provided most complex and wonderful mind to Human being and provided Habitat (Earth) despite low probability of 1 in one million million) and my loving mother who always supported me throughout my life.

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Abstract:

For past few years, there has been unceasing upsurge of research to cope with the cracks in extensively used construction material known as concrete. Self-healing of concrete using microbes has shown sustainability and lower environmental impacts. The key aspiration of the study was to examine the compatibility of fungi as self-healing agent and its effects on the mechanical properties of concrete. Two strains *Rhizopus oryzae* & *Trichoderma longibrachiatum* isolated from locally available microflora were obtained. These above-mentioned strains were inoculated in the concrete matrix with and without immobilization in calcium alginate beads which is super absorbent polymer. Field emission electron microscopy confirmed that *Rhizopus oryzae* was able to grow on the concrete as well as able to promote calcite precipitation on the cracks of the concrete. X-Ray diffraction and thermogravimetric analysis revealed the presence of crystalline calcite precipitates confirming the microbially induced calcite precipitation. Despite using super absorbent polymers, the compressive & tensile strength of concrete did not decrease instead it slightly increased. These results show that fungus has potential to be used as suitable Self-Healing agent of concrete in future. Results showed that fungal strain Rhizopus sp. immobilized in calcium alginate beads was more effective in samples pre cracked at 3 and 7 days while bacteria immobilized in light weight aggregates were more efficient in samples pre cracked on 14 and 28 days. In addition, results of compressive strength depict that self-healing concrete made with

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1 Chapter 1 Introduction

1.1 General Introduction

Self-healing concrete is a product which has the ability to fill the cracks by producing chemical products from internally incorporated microbe, admixture or polymers present in it. One of these methods of self-healing, through the production of minerals by using mineral producing bacteria in the concrete, is known as bio-influenced self-healing concrete or bio concrete. Bio concrete is a product which heals the cracks produced in it by producing the mineral compounds to fill the cracks. During earlier studies bacterial solution was sprayed on the cracks formed in a structure which was a less effective and impractical procedure. However, in recent years bacteria along with organic mineral precursor compound are incorporated in the concrete during the mixing phase. Generally, bacteria are incorporated in dormant form along with the nutrient compound which stays inactive until cracks are produced allowing water penetration in the concrete. Bacteria become active when come in contact with water and begin to feed on nutrient compound. Calcium carbonate is produced as the result of metabolic action of bacteria on calcium lactate and seals the crack, restricting the water penetration in concrete hence increasing durability of concrete.

1.2 Objectives

- To study the microbial activity of Fungal strain *Rhizopus oryzae* and *Trichoderma Longibronchiatum*
- To investigate effect of fungal MICP on mechanical properties as well as durability of structural & non-structural concrete.
- To study the crack healing phenomenon due to MICP caused by *Rhizopus oryzae* and *Trichoderma longibrachiatum* on structural and non-structural concrete.

1.3 Organization of the report

The report has been arranged in the following manner.

- Chapter 1 contains general introduction about bio concrete.
- Chapter 2 contains brief Literature review and the work done in the specific field before.
- Chapter 3 contains the detail introduction and specifications of material. Their accessibility is also discussed.
- Chapter 4 contains results and discussion in the brief manner.
- Chapter 5 contains future recommendations and conclusion. Also, the limitations of research are also discussed there.

2 Chapter 2 Literature Review

2.1 Introduction:

Concrete is considered as imperative construction material owing to low cost and accessibility. Cracks are pragmatically unpreventable in concrete due to load conditions. If micro-cracks form nexus it increases the permeability and reduces resistance against antagonistic substances, thereby contrive to reduce the strength and durability of structure[1]. Self-healing concrete is impending practically to lower the maintenance course and environmental impact by eluding the anthropogenic greenhouse gas (GHG) emissions [1–3] To date, self-healing is generally classified as autogenous and autonomous techniques [5]. In autogenous technique, the self-healing is achieved by hydrolysis of left-over anhydrates [6]. This autogenous self-healing is constrained to heal the cracks of 0.2 mm and limited to negligible continual rate [6,7]. However, in autonomous technique, self-healing is attained by addition of microbes, polymers, and admixtures. Encapsulation of polymers in concrete fill crack by forming foam in presence of water but it has compatibility problems with the concrete matrix due to different mechanical behavior which proves inimical for concrete as it engender abominable cracks [9].

The self-healing by microbial approach is preferred owing to sustainability and minimal environmental impacts [9,10]. The word Microbe refers to wide range of micro-organisms but prevalently study on self-healing concrete is limited to bacteria so far and ample research has been carried out. [11–16]. Self-healing with the help of bacteria has been investigated broadly using single component as well as two component system [18][19]. The two-component system involves adding microbes in the presence of calcium source to intensify microbially induced calcite precipitation (MICP) [20].

Fungus which belongs to isolated kingdom of classification is a microbe, many of its strains have been reported to catalyze Microbially induced calcite precipitation (MICP)[21][22][23]. In a previous study, applying the two-component technique urea and calcium chloride were hydrolyzed in the presence of fungus *Penicillium chrysogenum* and resulted MICP were able to stabilize sand column thus increasing the compressive strength [24]. Many strains of fungus have been found to survive in acute environment like high salinity and high acidity and shown mechanical stability making it good alternative of bacterial concrete for application of fungal concrete in saline environment. [25,26] Additionally, fungus has filamentous structure which permits more nucleation sites for MICP [27]. In another study, six fungal strains was tested by Jing et al [28] among them *Trichoderma reesie* was observed to grow in the presence of cement mortar but it was not inoculated in concrete matrix due to lower pore size of cement mortar than spores of specific fungal strain. It suggests that fungus can be potential replacement for the self-healing of concrete if spores of fungi are well immobilized as spores are likely to survive much longer than vegetative parts [29]. The Self-Healing capability of concrete after inoculation of fungal strain has never been investigated to broad extend according to Author's best knowledge. In current study, the self-healing capability and its effect on mechanical properties of two selected fungal strains was investigated. Two non-pathogenic strains *Trichoderma longibrachiatum* and *Rhizopus oryzae* were inoculated in concrete. High concentration of Rhizopus sp. and Trichoderma sp. in indoor walls of concrete buildings exposed to seepage has been reported before, showing their capability to grow in presence of cement environment [30]. *Trichoderma longibrachiatum* and *Rhizopus oryzae* were tested after inoculation in concrete without being immobilized as well as after being immobilized. Self-healing was tested with the help of Ultrasonic Pulse Velocity (UPV) using direct relation of UPV values and density of structure at uncracked state as well as cracked state of 0,7,14 and 28 days of healing. The compressive strength regain was tested to assess the quantitatively

Self-Healing after MICP. Furthermore, these mechanical aspects have never been studied after inoculation of any fungal strain in concrete.

2.2 Significance of Autonomous self-healing

Although autogenous healing has some luxury of healing the cracks without doing any external effort, but it also has some drawbacks of limited healing and minimal cracks. Autogenous on the other hand provide us benefit of healing the cracks which are deeper and act as structural cracks as calcite growth is intensified by adding different admixtures, polymers, or microbes. This autonomous approach has been proved fruitful in healing the bigger and deeper cracks in past.

2.3 Limitations of autogenous healing

Autogenous healing has limitations regarding lower healing rate and healing cracks of lower crack-width smaller cracks. In addition to that the autogenous healing is limited to non-structural cracks which means playing negligible role in the strength of concrete structure.

2.4 Why testing on 7^{th} day of curing is significant.

Compressive strength concrete has shown usually gain of seventy percent of its strength on seventh day of curing. In working fields, it is common practice that laborer can approach upside of slab on seventh day. That's why usually shuttering of concrete structures is recommended at least up to seventh day.

2.5 Why test on 28th day of curing is significant.

Usually, the concrete structures gain their ninety eight percent compressive strength on 28^{th} day. Although concrete continue gaining strength throughout the lifespan up to at least 50 year, 28 days are significant because its behavior quite resembling the with behavior which its shows rest of its life.

2.6 Significance of Research/Research gap:

In this world of new age, many new techniques are being searched to cater complex problems like cracks of concrete structures which are nearly impossible to avoid. New self-healing agents needs to be searched. There is also ample research is carried out using bacteria as self-healing agent. Very little literature is available about fungi in which it is applied as self-healing agent. That's why there is need to study the fungi as self-healing agent. Also, the effect of fungi (as a self-healing agent) on mechanical properties and durability of concrete needs to be investigated. Even there is not enough literature available on the screening of fungal isolates. That is why possible candidate of fungal isolates as self-healing agent needs to investigate in detail. In this research although the screening is not done in detail, but initial screening has been done.

3 Chapter 3 Materials and Methods

The selection of materials was mainly derived by the availability of materials. For past few years the importance of bio inspired self-healing concrete has been increase so much in the field of construction materials. The materials of this research broadly consist of two fields i.e., Biotech and construction materials. In Biotech field the materials belong to isolation, culturing and immobilization of fungal strains. In which the growth medium was primarily PDA (Potato Dextrose Agar) and PDB (Potato Dextrose broth). The immobilization majorly consists on sodium alginate. From the construction field the most important material is concrete which consists of locally available bestway cement. Locally available Lawranspur sand and Margalla crush.

3.1 Screening of Fungal Isolates:

Samples of fungal isolates were acquired from the cement walls and pure PDA plates were prepared. Four isolates were obtained and then their screening was done on the basis of rapid growth. It was observed that a green isolated showing the rapid growth resembled with the aspergillus as well as penicillium morphologically. That's why molecular identification was done. In simultaneous manner two available isolates from the Mycovirus Lab (ASAB) *Rhizopus oryzae* and *Trichoderma longibrachiatum* showed potential candidate to be used as self-healing as Rhizopus sp. and Trichoderma sp. was already reported in the literature to be found in the cement walls and their accessibility show made us interested that we study their effect on the concrete. Moreover, both isolates belonged to least Bio- Safety level.

3.2 Identification screened isolate:

The identification was done according to Sanger method of internally transcribe spacer (ITS). Both forward and reverse primers were applied, and DNA was taken more than 600 Bp.

3.2.1 Extraction

The nucleic acid extraction was done according to Coenen et al., [31]. First the nucleic acid extraction was done in which the PDA plate of respective isolate was prepared on the cellophane for three days so that the isolate can grow well. Then cellophane was removed from the PDA plate and washed with the liquid nitrogen. This cellophane was grinded in the pestle and mortar. Then this grinded nucleic acid was transferred to 20ml microfuge tube. 35µl of extraction buffer was added in this microfuge tube. Vortex was applied. Then incubation for 1 hour at 70°C was applied to the sample. Phenol chloroform was added 35 µl and vortex was applied. It was centrifuged at 13000 rpm for 10 minutes and supernatant was taken. 350µl sevag was added and vortex was applied. Then again centrifuge treatment was done for 10 minutes at 13000 rpm. Then additional 50 µl sevag was added and centrifuge treatment for same duration was applied.

3.2.2 PCR (polymerase chain reaction)

3.2.2.1 Primer sequence

ITS1 Forward TCCGTAGGTGAACCTGCGG

ITS4 Reverse TCCTCCGCTTATTGATATGC

3.2.2.2 Reagents

Sterile distilled water 33 µl Taq Buffer (10X) 5 µl, 2.5 mM MgCl 2 3 µl,2 mM dNTPs 2 µl, Forward primer 2 µl, Reverse primer 2 µl, Template 2 µl, Taq Polymerase 1 µl

3.2.2.3 PCR temperature protocol

Initial-denaturation 94°C (3 min) 1 cycle

Denaturation 94°C (45 sec)

Annealing 58°C (45 sec) 35 cycles

Extension 72°C (45 sec)

Final extension 72°C (7 min) 1 cycle

3.2.3 Purification

The PCR was done using the centrifuges. First the binding buffer was used at ratio 1:1 and through mixed. Then color was maintained to yellow for optimal pH DNA range. Then 800µl solution of solution was transferred to GENE purification column and centrifuged for 60 s and flow-through was discarded. 700µl wash buffer (diluted with ethanol) was added and was centrifuged for 60s and flow-through was discarded. Then additional centrifuge was applied to remove any residual buffer. Then purification column was transferred to 1.5 ml microcentrifuge tube and 50µl of elution buffer was added to the center of purification then centrifuged for 1 min. Purified DNA was stored at -20 °C.

Standard phenol-chloroform purification was applied to protect the RNA from decomposition.

3.3 *Aspergillus fumigatus* Precipitation Capability:

In order to check the precipitation capability of Aspergillus fumigatus it was grown on the PDA plates for 7 days. Incubated cement mortar petri dish was prepared and 10ml PDA was poured in it. After solidification plug from the seven days old aspergillus plate was inoculated on the cement mortar petri dish. Then 1 M solution of urea and calcium chloride was prepared in 5 ml of water and added in this cement mortar plates. This cement mortar plate was incubated at 25°C in for 7 days for the reaction to proceed. In simultaneous manner a control petri dish was also prepared. After 7 days of incubation, it was expected that calcite precipitation could have occurred.

3.3.1 Collection of calcites from *Aspergillus fumigatus* plates:

Calcite was dissolved in the 0.1 M sodium hypochlorite and washed with the 70% methanol at 14000 rpm for 5 minutes in centrifuge. Then sample was placed in the drying oven for overnight [32].

3.4 Pathogenetic nature of *Aspergillus fumigatus*

Aspergillus fumigatus belongs to Bio-safety Level -2 that's why it is essential that according to medical and ethical code it should be worked in bio safety cabinet. This bio safety cabinet was not available in respective lab. that's why no further work was done on *Aspergillus fumigatus.* As, it is also responsible for various human pathogenic diseases [33].

3.5 Trichoderma longibrachiatum

Trichoderma is the pluralistic and dominant component of eco-system [34]. The survival of Trichoderma depends upon varying factors including the microclimate, the substrate as well as complex ecological interlinkage [35].

3.6 Rhizopus oryzae

Rhizopus oryzae is a heterothallic (fungi which need two different sexes for reproduction) and filamentous fungi. It is closely related to *Rhizopus stonolifer* in many cases it becomes very difficult to differentiate between two strains. *Rhizopus oryzae* also acts as saprotroph and found in rotting vegetation, dung and also in soil. It has air dispersed sporangia. *Rhizopus oryzae* is economical for production.

3.7 Preparation of Fungal culture and growth optimization:

Two locally identified fungal samples were acquired from Mycovirus research lab ASAB, NUST. The choice of fungi selected was based on their least biosafety level (Level-1). These two fungal strains *Rhizopus oryzae* (PM3) and *Trichoderma longibrachiatum* (A8) acc.#

KY967258.1 and acc. # KY966045.1 respectively were obtained and inoculated in the concrete. These strains were grown on Potato Dextrose Agar (PDA; Oxoid) consisting of 200gm infused potatoes, 20 gm Dextrose and 20 gm of Agar in 1000 ml of distilled water. The culturing of fungal strains was done in Potato Dextrose Broth (PDB; Oxoid) for seven days through a plug collected from the PDA plates. In order to achieve the homogenous concentration, spore count was kept around $1x10^3$ spores/µl and verified with Hemocytometer.

3.8 Sodium Alginate

Alginate is easily accessible and primordial economic biopolymer made up of polysaccharide found in many species of algae and some strains of bacteria[36]. It forms foam like hydro gel in the presence of water[37]. Hydro gels have been already reported to enhance the bacterial survival and in the harsh environment of concrete [38][39]. Immobilization technique of fungal spores was adopted by M.Yakup et al [40] and alginate concentration was kept 2 percent which was reported ideal in terms of stability and germination rate by Pitaktamrong et al [41].

3.9 Potato Dextrose Agar (PDA)

It is the universal and most important growth medium for fungi. It can be made by 200 grams of infused sliced potatoes boiled in distilled water of 1000 ml for thirty minutes. Then this distilled water is taken, and 20 grams dextrose and 20 grams agar are added. Then autoclaved for 1.5 hours at 121 Celsius.

3.10 Potato Dextrose Broth (PDB)

It is similar to PDA except one thing i.e., agar. Potato dextrose broth consists of only potatoes, dextrose and distilled water in the same ratio as PDA and same procedure is applied.

3.11 Urea

Urea is a well-known organic compound also known as Carbide. It has chemical formula $CO(NH_2)_2$. It serves important role in metabolism if compounds which contain nitrogen by animals. It is also found in the nitrogen containing substance in the animal urine mainly mammals. It is also the widely used fertilizer. When dissolved in water it is neither acidic nor basic nature. In current study it was used to react with calcium chloride in the presence of microbe making possible reaction to produce calcite. It means indirectly it was used as calcium source.

3.12 Calcium Chloride

It is a common inorganic compound also known as salt. It is white in color and readily soluble in water. It acts as crystalline solid in the room temperature. It can easily obtain by the reaction of hydrochloric acid and calcium hydroxide. It is commonly used as deicing and dust control.

3.13 Immobilizing Material & its Viability

The spore suspension of *A8* and *PM3* was prepared from PDA plates and each 20 ml spore suspension containing spores $2x10^7$was mixed with 0.4 gm of Sodium alginate (Sigma Chem. Co. USA) powder and mixed thoroughly until clear gel was formed. To achieve calcium cross linking the sodium alginate gel was induced in 2 % calcium chloride ($CaCl_2$) solution drop by drop with help of syringe to form beads.

After being immobilized, to verify whether the vegetative part of fungal strains able to grow from the immobilized spores if it gets favorable conditions the beads produced were placed on PDA plates. These PDA plates were kept in incubator at 25 °C after 7 days growth of *A8* and *PM3* was observed.

3.14 Swelling Properties of calcium alginate beads:

In order to determine the swelling parameters filtration method by Snoeck et al. was followed[42]. A certain quantity of weighed surface dried calcium alginate beads (CAB) were added in cement filtrate which was prepared by adding the 10 g of cement in 100 ml of deionized distilled water in order to mimic the concrete conditions. After 24 hours saturated beads were filtered using wet filter paper and filtrate was dried using tissue paper carefully and dry weight was recorded. The difference between the weight of surface dried and absorption capacity of saturated beads was calculated in percentage.

3.15 Concrete materials

3.15.1 Cement

Bestway, ordinary portland cement, ASTM C150 Type 1, was used as a binding material in concrete mix. Chemical composition is given in table.

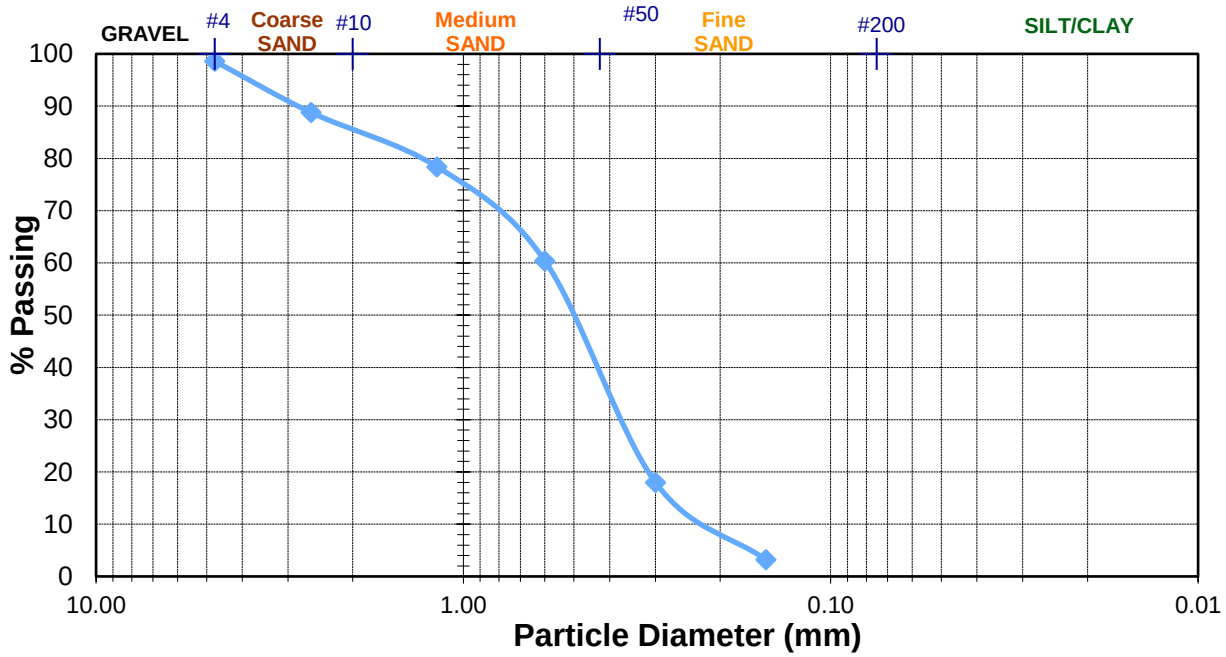
3.15.2 Sand

Lawrencepur sand was used as Fine Aggregates (FA) in concrete mix. Its particle size and finesse modulus (FM) were determined by sieve analysis according to ASTM C33. Then gradation curves were plotted as shown in Figure 3.1. Percentage water absorption of fine aggregates was also determined according to ASTM C128 as given in Table 3.2.

3.15.2.1 Gradation Curve

Weight of Container (g):	400.0	**Weight of Container & Soil (g):**	1000.0
Weight of Dry Sample (g):	1000.0		

Sieve Number	Diameter (mm)	Mass of Sieve (g)	Mass of Sieve & Soil (g)	Soil Retained (g)	Soil Retained (%)	Soil Passing (%)
#4	4.75			14.0	1.4	98.6
#8	2.60			98.0	9.8	88.8
#16	1.18			104.0	10.4	78.4
#30	0.60			180.0	18.0	60.4
#50	0.30			424.0	42.4	18.0
#100	0.150			148.0	14.8	3.2
Pan				24.0	2.4	0.0
			TOTAL:	**992**	**99.2**	



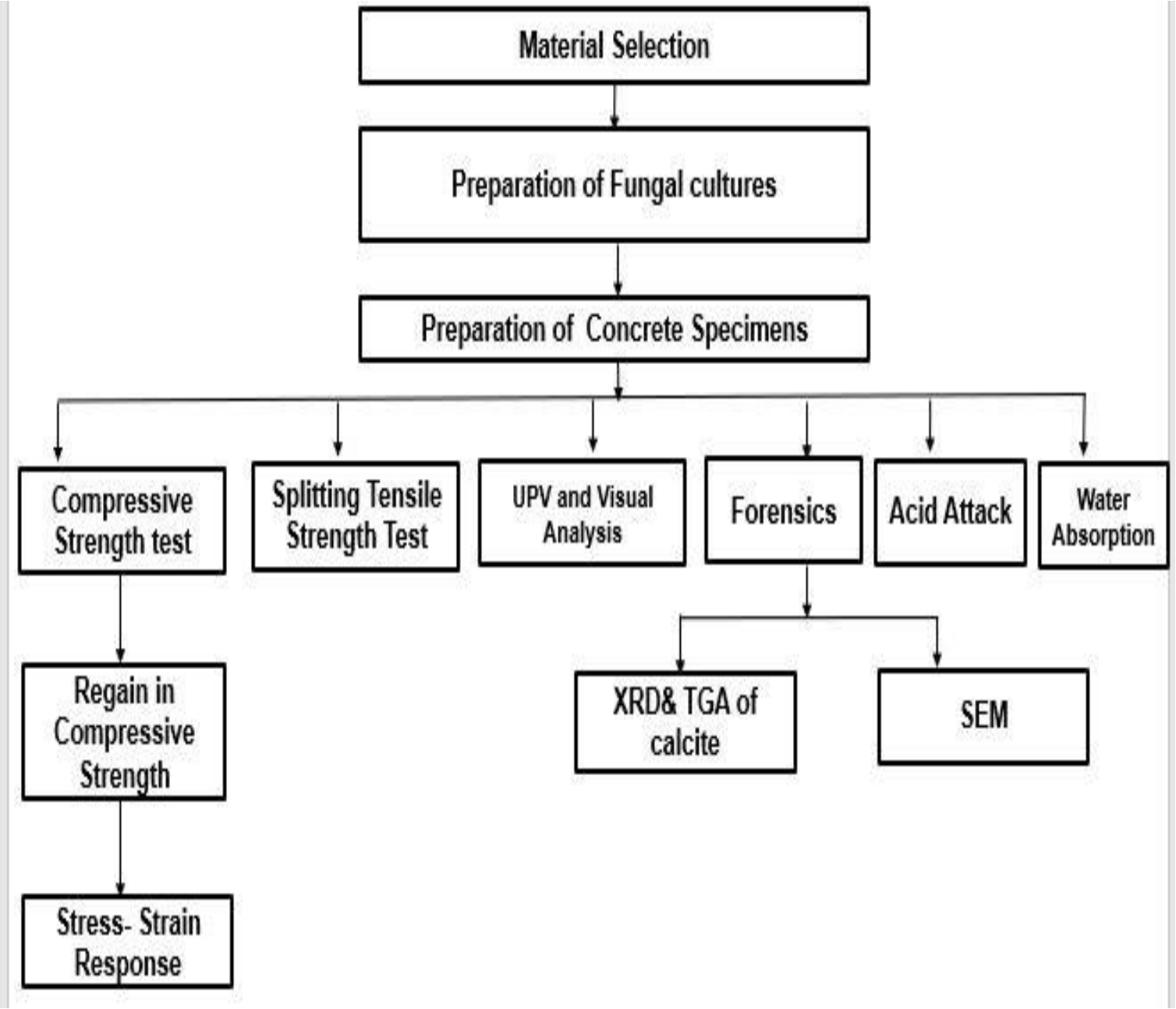
3.15.3 Coarse Aggregate

Marghla crush" was used as Coarse Aggregates (CA) of concrete. Its specific density, water absorption and FM were determined according to ASTM C127 and ASTM C33 respectively, having fineness modulus 6.67 and specific gravity 2.650

Compound	Percentage
CaO	65.00
SiO2	19.19
MgO	2.23
Al2O3	4.97
Fe2O3	3.27
TiO2	0.29
Na2O	0.58
K2O	0.51
P2O5	0.08
MnO	0.04
LOI	3.84

Table 1. *XRF Values Cemen*

3.16 Testing Scheme



3.17 Mixes proportions

Five different types of mix proportions were prepared (Table 3). The concentration of urea & calcium chloride was 2 Mole of available water is used as calcium source to intensify MICP in all formulations. Water cement ratio was maintained constant at 0.40.

Mixes	Cement	Sand	Coarse Aggregate	Fungal culture	Fungal Strain	Calcium source	Immobilizer
	Kg/m^3	Kg/m^3	Kg/m^3	L/m^3		mol of fungal culture	% of weight of Cement
CM	522	783	1096	6.8	----------	2	-------
Mix A	522	783	1096	6.8	*PM3*	2	-------
Mix B	522	783	1096	6.8	*PM3*	2	1
Mix C	522	783	1096	6.8	*A8*	2	------
Mix D	522	783	1096	6.8	*A8*	2	1

Table 3. *Mix Design of different formulations*

3.18 Casting & Testing Regime of Specimen:

In total 18 concrete samples of each mix having cylindrical shape of 100mm diameter and 200mm height were casted. These casted samples were kept at 90% relative humidity for 24 hours and then placed in water container for curing under control environment. The testing was carried out mainly in three phases firstly, mechanical properties and durability. Mechanical properties consist of compressive strength and splitting tensile strength. Durability consists of acid attack and rate of water absorption test. Compressive strength was investigated which include compressive strength test at intervals of 7th day as well as 28thday of curing was done according to ASTM standards C-39 using MCC8 compression testing machine. Tensile strength was tested on 28th day to have brief idea about bonding between concrete nexus at the end of curing following the splitting tensile test procedure ASTM C-496/C-496M. Rate of water absorption of all formulations was carried out following ASTM 1585[43]. In second phase the internal self-healing and densification of structure was assessed by ultrasonic wave pulse velocity (UPV) and regain in compressive strength. For UPV, the samples were placed in the water after being cracked at 85% of ultimate strength as done by Shaheen et al. for creation of wider cracks on 7th days and 28th day of curing [44]. In UPV two types of

transducers are used, the purpose of one is to transmit the wave and the other one to receive the wave by which time taken by the wave to propagate the concrete sample is recorded in microseconds, the time taken in this is related to the velocity which is taken in Km/s which is in a directly proportional to the density of matrix of concrete. In this way relative quantitative idea about density of concrete is calculated according to ASTM C 597–16 [45]. The above UPV procedure was done on uncracked specimen, 0 day (cracked), 7^{th} day & 28^{th} day of immersion in water. Regain in compressive strength was evaluated in order to evaluated self-healing proficiency, samples of all formulations were cracked at 85 percent of ultimate compressive strength at 7 and 28 days of curing then again samples were kept in water to accelerate the healing process. These samples were placed in water for 28 days to regain the compressive strength. The external self-healing in cracks was visually observed under HC-2950 microscope and pictures were taken by holding camera over microscope. Thirdly, the $CaCO_3$ crystals were tested by forensics i.e., X-ray diffraction (XRD), Field Emission Scanning Electron Microscope (FESEM) and Thermogravimetric Analysis (TGA). In XRD, Copper (Cu) was used as x-ray target material as it has ability to cool easily due to high thermal conductivity. XRD scanning was done from angle 2 theta 10° to 80° at the step count 0.02°[46]. In FESEM, samples for the microscopic study were scratched from the internal portion of cracks and dried for 48 hours than gold coating was applied and FESEM was done [47]. TGA was done on the white powder obtained from healed cracks and mass loss vs time was drawn in graph from 0°C to 800 °C.

3.19 Curing

Curing refers to process in the civil engineering to put the concrete samples in the 100 percent relative humidity so that the hydration process continues and concrete gains the strength. In Biotech. this refers to the something else. Kindly do no confuse this term as this term is used for concrete in this draft.

3.19.1 Ultrasonic pulse velocity (UPV):

It the very sensitive technique to indicate the presence of damages of flaws or voids. Interestingly it works in relative manner. It has been used by different researchers to evaluate the self-healing of mortar and concrete before. The ultrasonic waves are propagated with the help of transducer form one end of the concrete sample and received from the other end and time taken by the wave to reach the other is inversely proportional to the density of concrete structure.

4 Chapter 4 Results and Discussion

In this chapter the results obtained from different experimentation programs are briefly described with the help of graphs. The results are arranged in three broad classifications i.e., mechanical testing, self-healing observation and forensics.

4.1 Compressive Strength Analysis

Compression Testing was carried out according to ASTM standards C-39 using MCC8 Compression Testing Machine. The results are summarized in Fig 1. CM showed compressive strength of 23.03 MPa and 32.814 MPa at 7 and 28 days, respectively. The increase in compressive strength on 28 days of curing may be due to continuous hydration process longer than 7 days and are in harmony with Shaheen et al. [48]. Mix A showed compressive strength up-to 22.6 MPa and 30.34 MPa in 7 and 28 days of curing respectively, this shows insignificant difference from CM. This insignificant difference shows the negligible microbial activity and due to direct induction of fungal culture into the concrete nexus did not give promising results. The same phenomenon was observed in Mix C. On the other hand, Mix B showed average compressive strength up-to 23.85 MPa and 34.5 MPa in 7 & 28 days of curing respectively, which is 8.06 % more strength than CM on 7 days of curing and 5.15 % greater than CM on 28 days. Broadly analyzing, Mix B was expected to show slight decrease in compressive strength as in case of CAB used in bacteria Soysal et al. [49] on contrary, it showed slight increase in compressive strength. This anomaly can be credited to net effect of CAB and EPS secreted by fungal mycelia. CAB beads did not contributed in compressive strength of concrete as it acts as hollow pores in the concrete structure [49]. These CAB may have filled with the fungal hyphae. EPS secreted by the fungal hyphae has binding effect which may have contributed in the compressive strength of concrete causing slight increase in compressive strength. Moreover, filamentous fungi has been reported to increase the secretion of EPS to

protect the cell wall from the exogenous material [50] .The same instance 7 % increase in compressive strength than CM was observed in Mix D up to 7 days of curing but negligible difference in compressive strength on 28 days of curing than the CM was observed. So, it may be inferred that after 7 days *Trichoderma longibrachiatum* was not able to survive even after spores being immobilized.

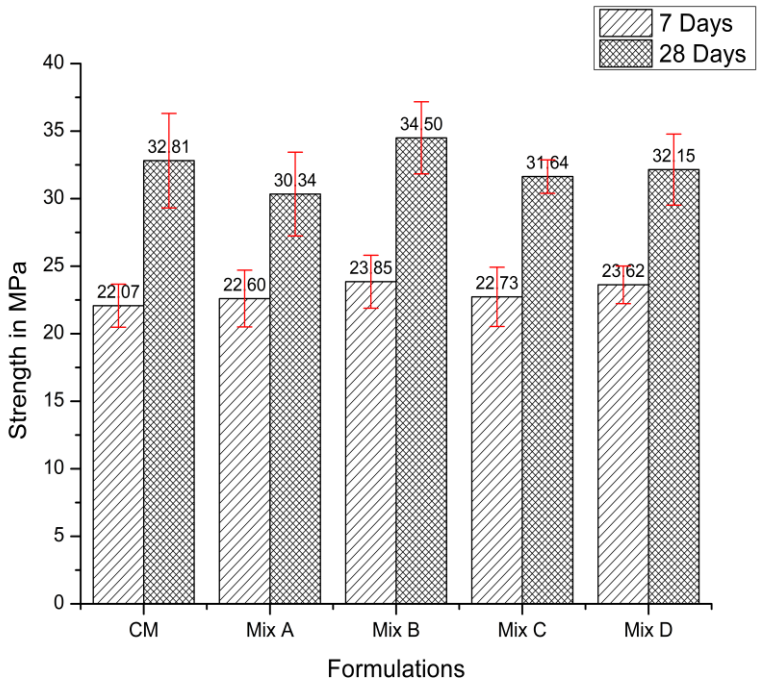


Figure 1 Compressive Strength

4.2 Splitting Tensile Strength:

Splitting tensile strength was used to indirect quantification of bond strength between concrete nexus and growth media. Split tensile test was carried out according to ASTM C-496/C-496M standards. The average split tensile strength of all bio concrete samples was compared with the control sample. CM showed split tensile strength up-to 2.43 MPa. Mix A and Mix C showed negligible difference in strength than CM. Mix B showed 3.06 MPa strength which is approx. 25 % more than CM. Presence of CAB has been reported to accelerate the production of It may be inferred that C-S-H is responsible for increase in split tensile strength

as it has been reported [51] Mix D showed 2.6 MPa split tensile strength which is 15% more than CM.

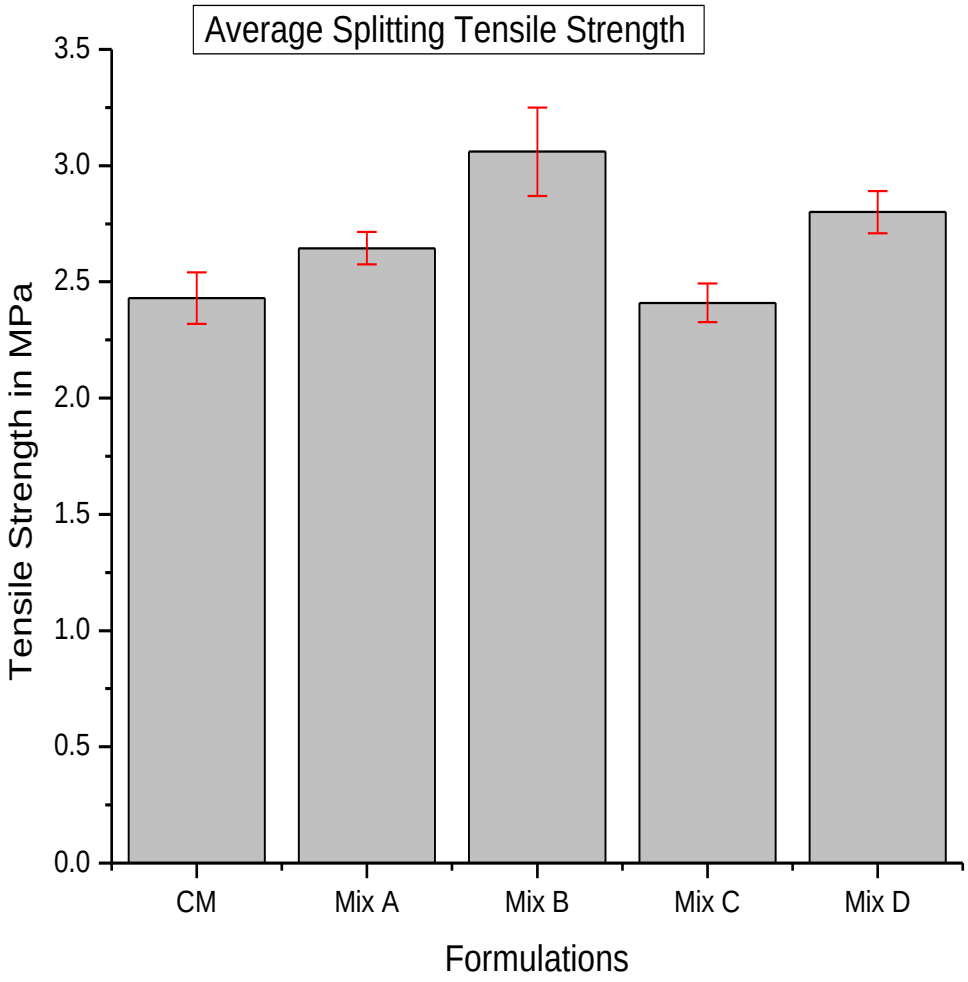


Figure 2 Tensile Strength of different formulations

4.3 Stress Strain Behavior:

Compressive stress strain behavior was studied on compression testing machine with the help of digital strain gauge. The values of digital strain gauge were recorded at regular interval of applied stress and stress-strain relation was drawn on graph in order to have brief idea about the ductility and toughness. It was observed that formulations with alginate beads showed relatively higher strain values as in Mix B and Mix D this may be credited to phenomenon that

SAP have been reported to increase the plastic deformation before[52]. On the other hand, Mix A and Mix C showed slight increase in strain. Ductility and toughness were observed to increase CM.

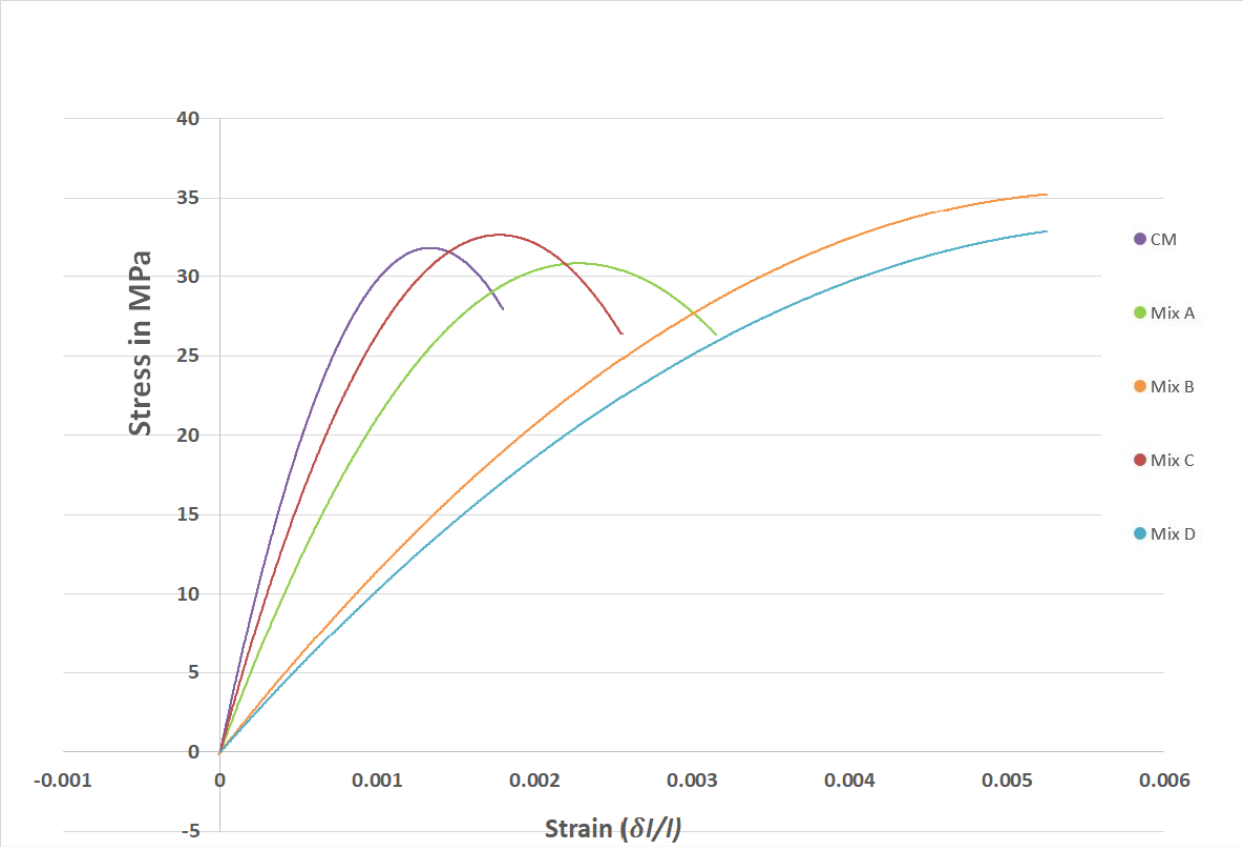


Figure 3 Stress Strain Behavior of all formulations

4.4 Regain in Compressive Strength

In order to evaluated self-healing proficiency, samples of all formulations were cracked at 80 percent of ultimate compressive strength at 7 and 28 days of curing then again samples were kept in water to accelerate the healing process. These samples were placed in water for 28 days to regain the compressive strength. Regain in compressive strength (RC) was calculated as follows:

$$\text{RC}\ (\%) = 1 - \frac{Su - Sr}{Su} \times 100$$

Su= Ultimate compressive strength reported earlier.

Sr= Compressive Strength regain 28 days after pre-cracking

The regain in compressive strength of samples CM pre-cracked after 7 days of curing is 11.78 MPa which is 35.9% of ultimate compressive strength. While samples of CM pre-cracked after 28 days of curing was 10.22 MPa which is 31.16 % of ultimate compressive strength. In Mix A the compressive strength regain was observed about 43.6% and in Mix C it was observed 43.3 % these formulations showed nearly similar trends which may be attributed to survival of fungal strains in very early stages. Mix B & Mix D showed compressive strength regain 59.5 & 53.1, respectively. This higher compressive strength regain can be attributed to the longer survival of fungal strains owing to presence of immobilizer material. This longer survival of respective fungi ensured the continuation of MICP resulting in improved regain in compressive strength.

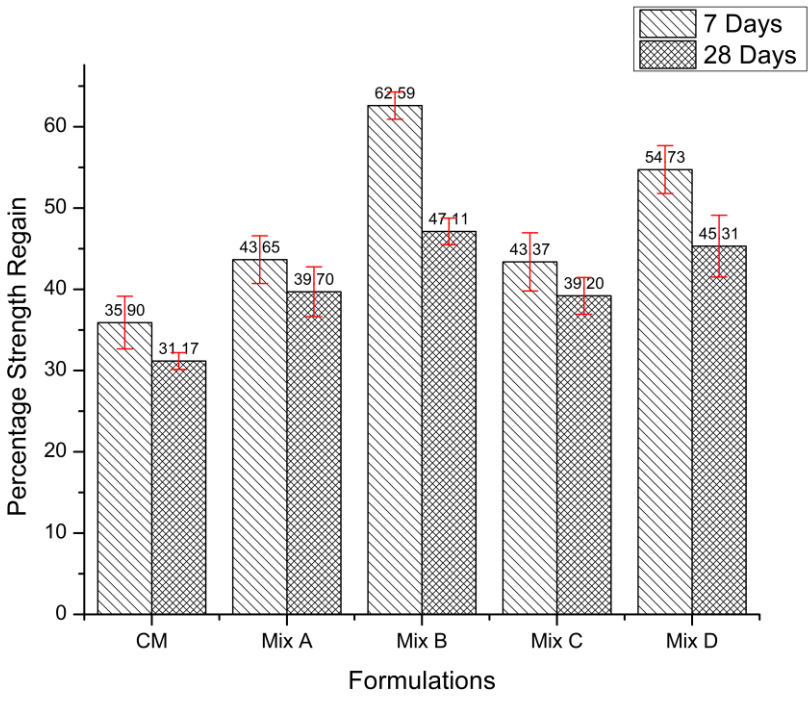


Figure 4 Compressive Strength Regain

4.5 Ultrasonic Pulse Velocity (UPV) Results

UPV is the most proficient test when it comes to ascertaining the cracks and voids present in concrete primarily because of its non-destructive and sensitive nature. This test is carried out as per the code (C597-16). Two types of transducers are present in the UPV equipment, the purpose of one is to transmit the wave and the other one to receive the wave by which time taken by the wave to propagate the concrete sample is recorded in microseconds, the time taken in this is related to the velocity which is taken in Km/s which is in a directly proportional to the density of matrix of concrete. In this way relative quantitative idea about density of concrete is calculated.

In this evaluation, both the transducers were placed on the concrete sample after applying the Coupling gel. Readings were taken on the 7th & 28th days cured samples. Three readings were observed on every sample and from these, the average reading was added in the table with Standard Deviation because of its highly sensitive indicator that detects damage found in the concrete. Higher self-healing rate after 7 days curing than 28 days curing was observed which may be because of reason that as the time pass the quantity of anhydrates available in the concrete reduces. The healing rate was also compared to a variable degree of damage (DD) used by Singh et al. [53]. DD was calculated as:

$$DD = 1 - \frac{Up}{Uo} \qquad (1)$$

Where Up is the UPV in the pre-cracked (uncracked) sample and U° is the UPV value of uncracked sample. It was observed that Higher the DD higher self-healing percentage and vice versa was observed which was due to the fact that bigger cracks allow more oxygen and water causing higher self-healing rate.

Self-healing percentage was calculated in terms of UPV values as follows.

$$SH = \frac{U28 - Up}{Up} \times 100 \quad (2)$$

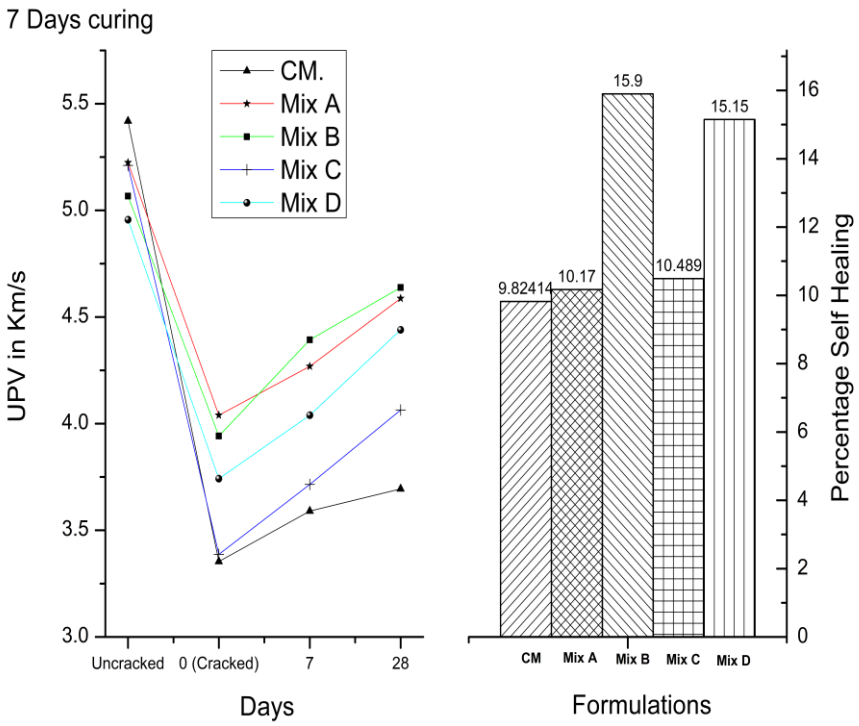


Figure 5 Self-healing on basis of UPV on 7 days cured specimen.

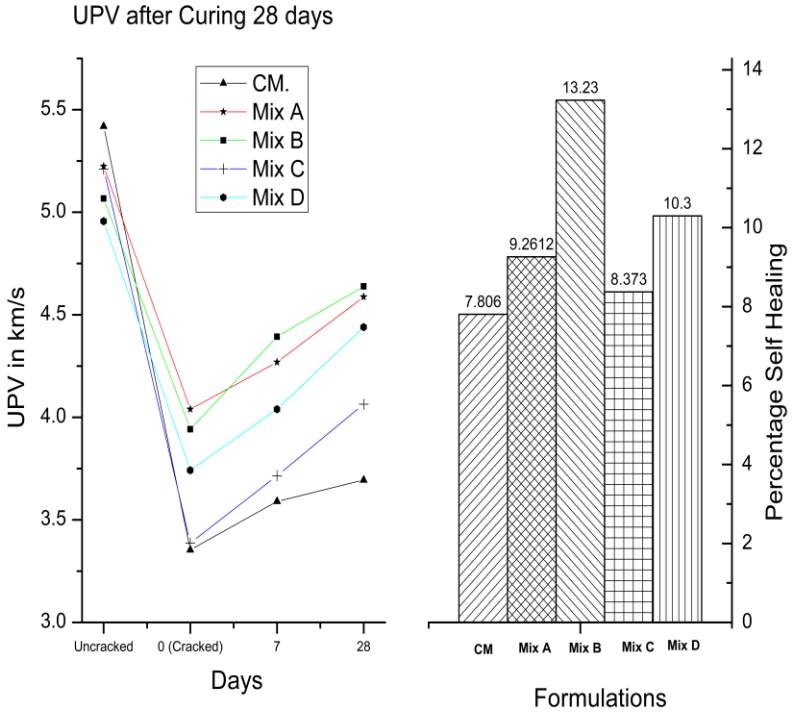


Figure 6 Self-healing on basis of UPV 28 days cured samples.

4.6 Visual Analysis

Cacked samples of each formulation were monitored after curing with the help of crack width measuring microscope. The healing was measured in millimeter(mm) briefed in Table 3 and difference in initial cracks and healed cracks was observed and compared on 0 and 28 days of crack healing. Negligible healing was observed in CM as there was no fungal strain was added to initiate MICP. This negligible healing can be due to autogenous healing is a continuous process which may initiate between hydroxide and carbon in the presence of water producing calcium carbonate [54] and loose particles from concrete matrix clogged in cracks may had also contribute in self-healing. Mix A & Mix C showed healing of cracks up to 0.3mm & 0.25mm respectively. Smaller size of calcite precipitates in these formulations were observed during SEM analysis which may be due to abiotic reason[55]. These smaller calcite precipitates were not observed in CM. Another reason of this phenomena may be, fungus survived in initial days and was not able to survive long enough to heal bigger cracks. In Mix B the cracks up-to 1.3mm were healed which may be credited to the well survival and continuous process of MICP. It is inferred that *Rhizopus oryzae* in the presence of immobilizer performed well. The SEM analysis was done after 28 days of recovery it may be possible that in Mix D *Trichoderma longibrachiatum* survived initially more than Mix C but did not survived up-to 28 days. That is why it healed cracks width greater than in Mix D which is up-to 0.6-mm cracks.

Formulation	Crack Width	Status
CM	0.2 mm	Empty
Mix A	0.6mm	Fully Healed
Mix B	1.4mm	Fully filled
Mix C	0.3mm	Partially filled
Mix D	0.6 mm	Fully healed

Table 2 Crack-Width Healed

4.7 Acid Attack:

In acid attack carried out on concrete the acid attack test was done on 28th day of curing. 5% sulfuric acid solution was prepared and samples of 50mm height and 100mm diameter of each were submerged for 28 days. On 7th day specimens were taken out washed carefully in order to remove loose particles weighed as well as dimensions were calculated then they were kept in fresh acid for up to 28 days. These calculated masses and volume loss were compared in terms of percentage as show in Figure 6. Mix A and Mix C showed insignificant difference as compared to the Control. Mix B & Mix D showed relatively low mass reduction due to relatively more calcite than Mix A and Mix D respectively. As fungus tends to grow well in sub acidic environment on general. it may be possible that microbial activity was continued in the core in Mix B and Mix D.

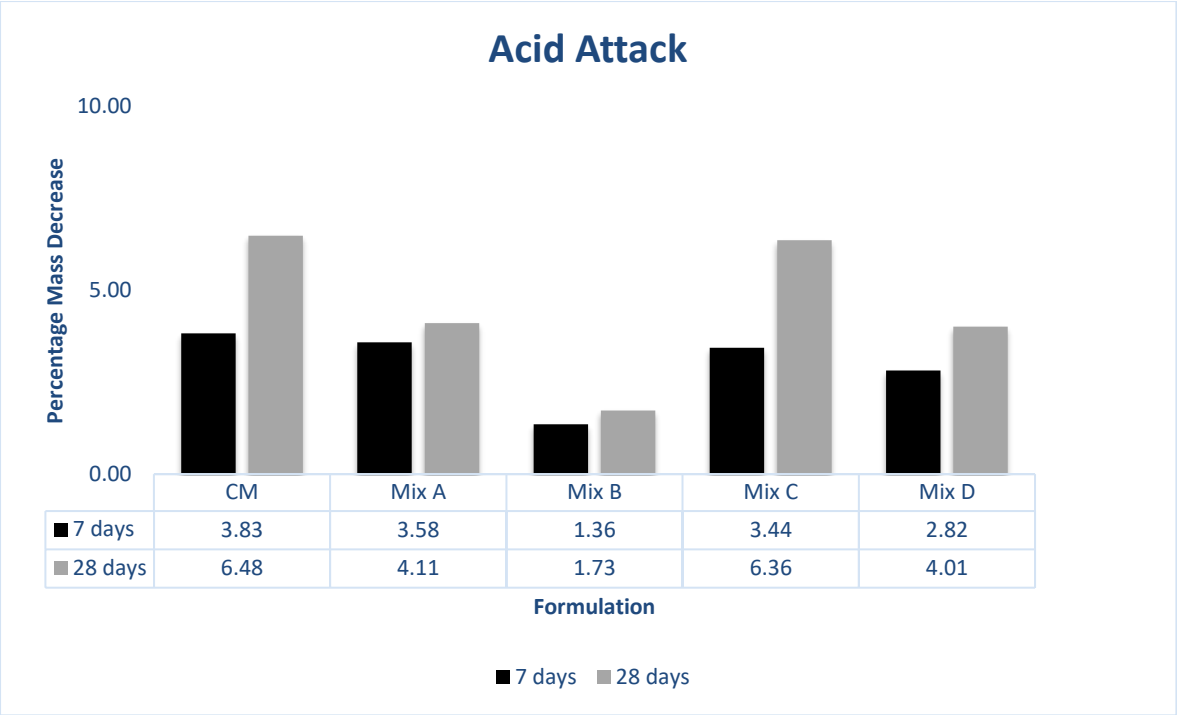


Figure 7 Mass Decreased Percentage due to Acid Attack

4.8 Water absorption

Water absorption was carried out according to ASTM C1585 [43] which is commonly used to determine the rate of absorption of concrete. The size of cylindrical specimen was kept 2" high and 4" in diameter. The preconditioning of specimens consists of the placing in submerged airtight condition for 18 days and then they were kept in the 80 % relative humidity in desiccator for 3 days. After removing from the desiccator each sample was kept in separate sealed containers for at least 15 days at 23°C allowing the distribution of moisture content throughout the specimen. The specimens were kept in a way that only bottom surface of specimen was exposed, and other remaining faces were sealed.

In this absorption test the incremental change in mass at predefined intervals was recorded. The early readings up-to 6hrs represent the primary absorption and reading taken after 6hrs up-to 10 days represent the secondary absorption. It was observed that primary absorption in all formulations was more than secondary absorption it was obvious that at start

relatively more empty pores absorb more water. Mix A and Mix B showed least permeability which was quite interesting because Mix D was expected to perform better than Mix A as in case of mechanical testing. Contrary to this, Mix A performed well and showed less rate of water absorption than Mix D this may be attributed to the relatively larger filamentous structure of *Rhizopus oryzae* than *Trichoderma longibrachiatum*. The fungal mycelia have been reported to reduce the permeability of structure by inducing the hydrophobic layer[56][57]. It may be possible that the larger filamentous fungal structure was able to produce more effective hydrophobic layer even it was able to survive less.

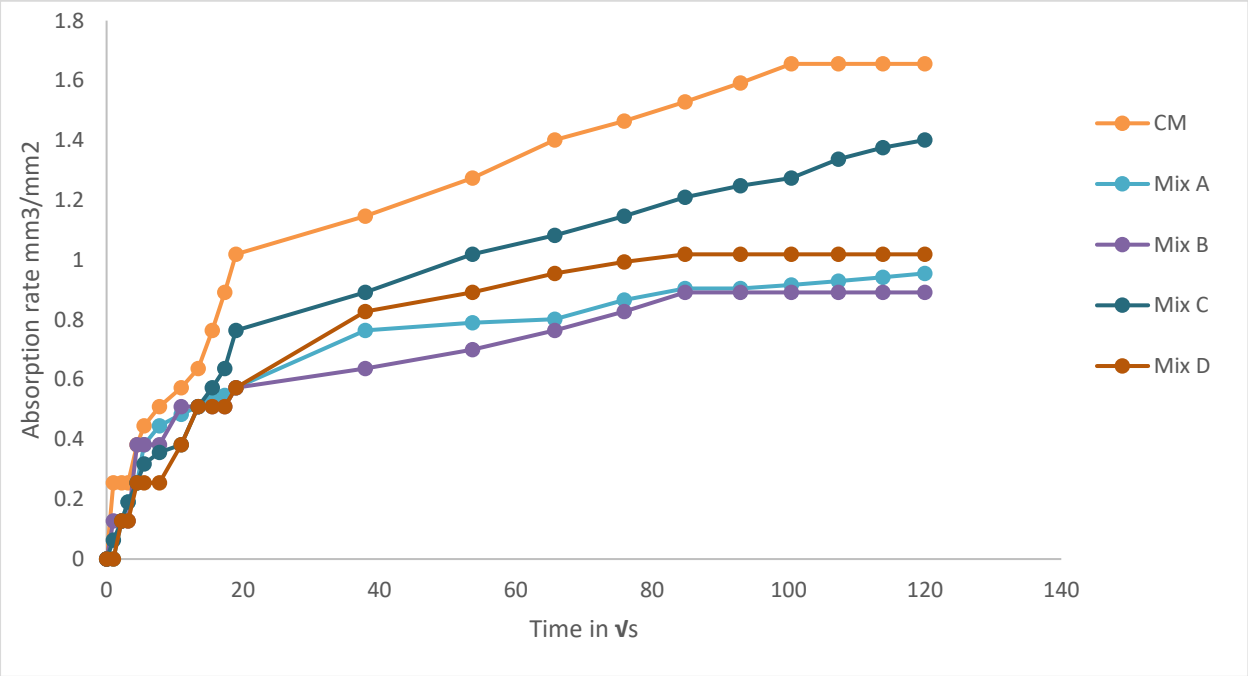


Figure 8 Water Absorption of different formulations

4.9 X-Ray Diffraction (XRD):

The classification of crystals on the basis of their regularity and symmetry was not done until seventeen century A.D. In 17th century a scientist Johannes Kepler hypothesized that snowflake crystals were regularly packed due to their spherical water particles. Then later in

1669 Nicholas Steno started the experimental work and showed the regular pattern for every corresponding crystal shape.

To investigate the morphology as well as phase purity of white powder appeared in the healed cracks, XRD of these particles scraped out of healed cracks was compared with the material scraped out from the empty cracks of CM as shown in Figure 10. The XRD diffractogram of CM showed the presence of silicone and amorphous form of calcite induced due to autogenous healing. On the other hand, the diffractogram of white powder showed the sharp peak between angle 2 theta 29° and 30°[58]. This sharp peak suggested the presence of highly crystalline $CaCO_3$ coexisted due to both autogenous as well as autonomous healing confirming the successful microbial activity. Moreover, this crystalline structure is better owing greater intermolecular forces and dense structure.

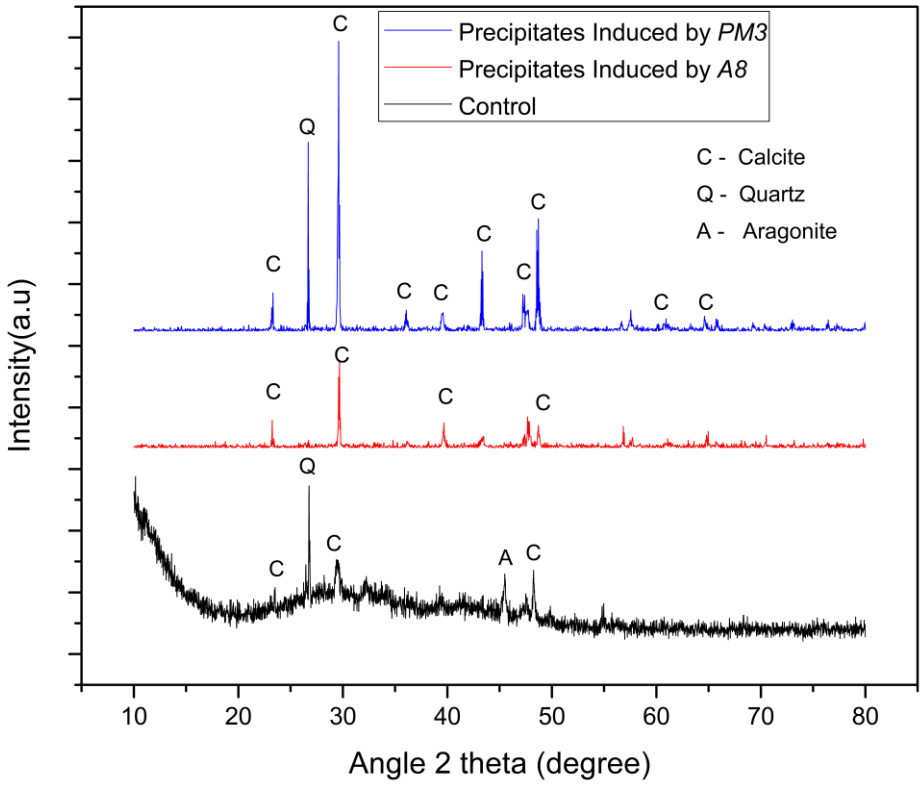


Figure 9 XRD Analysis of extracted calcite from cracks

4.10 Thermo-gravimetric Analysis (TGA)

TGA is a technique used to observe change in mass relating it to change in temperature at constant rate. It also gives information about the physical phenomena of change in phase e.g., vaporization. White powder scratched from the healed cracks was analyzed by TGA. It was observed that major weight around 25 percent occurred between 600°C to 800°C [59]. This sudden loss in mass strongly suggests the presence of crystalline structure.

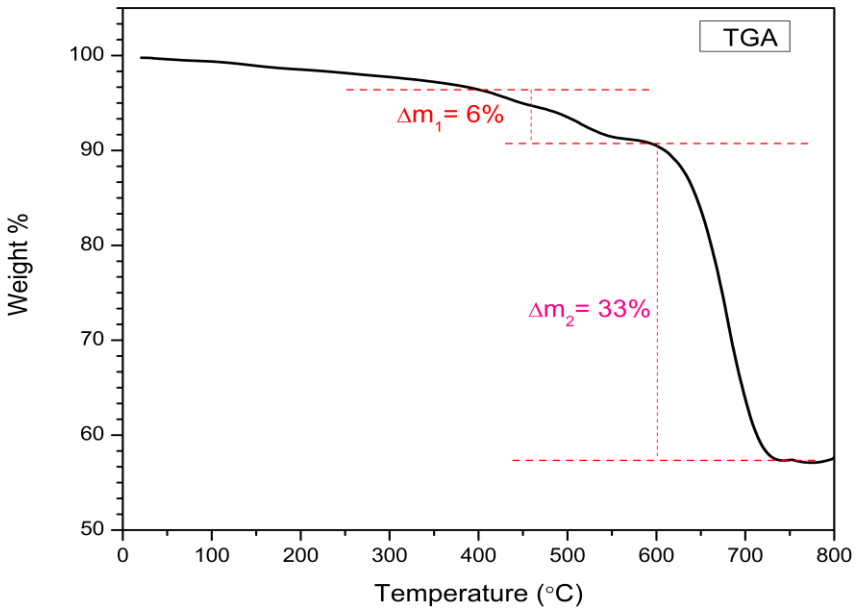


Figure 10 TGA of extracted Calcite powder from Mix B

4.10.1 Significance of TGA

The TGA has an important and significant aspect in which the very small quantity of sample can be useful in identification of any crystalline material which has specific temperature of melting. The sample in placed in the equipment and temperature is increased gradually and change in mass is monitored with the passage of time. The relationship between these two functions is plotted on the graph which then analyzed for crystal identification.

5 Chapter 5 Conclusion and Future Recommendation

Self-healing of concrete has already been achieved before that's why it is not novel aspect of the study. The novel aspect lies in the selection of microbe i.e., fungi which is not studied broadly. MICP capability of microbe depends on length to volume ratio[60]. The fungi have larger filamentous structure i.e., larger length to volume ratio as compared to bacteria than bacteria so it allows more nucleation sites and has potential to heal larger cracks of concrete[61]. In the current study a fungal strain was found having potential of being used as self-healing agent after being immobilized. Four different formulations were tested in the presence of control (no amendments added) and only one i.e., Mix B performed well in terms of healing crack width i.e., up to 1.3mm and compressive strength. Moreover, significant evidence of fungal survival in Mix B was observed. Direct inoculation of *PM3* did not showed positive results this may be due to no longer survival of fungus. As fungus can be seen in FESEM of Mix B it can be inferred that fungus was responsible for the MICP in the cracks. One of the interesting finding of study is that Mix D showed positive results in compressive strength regain and other fields but presence of fungal strain in SEM was not observed this can be attributed to longer survival of *A8* in the concrete matrix than Mix C but was not long enough up to 28^{th} day. *Trichoderma* sp. was expected to show positive results [28] than *PM3* but did not perform as expected this may be due to the reason that on later investigation it was noticed that *A8* was infected dsRNA [Khan et. al unpublished data]. Also, which type of genes are supporting the growth on concrete needs to be investigated. All the research was carried out on the normal strength concrete in future behavior of high strength concrete needs to be investigated after inoculation of immobilized fungal strain. Moreover, permeability aspects need to be

investigated further in detail as fungi has potential to grow mycelia making the concrete structure dense.

- The use of immobilizing media played a key role in the self-healing capability and survival of both fungal strains, i.e., *PM3* and *A8.* In general, mixes in which fungal strains were immobilized showed better results in crack healing, strength, and durability, which can be attributed to longer continuous process of MICP.
- Significant evidence of fungal survival was observed in Mix B enriched with *PM3;* it shows that *PM3* is potent for self-healing agent if well immobilized. However, recipe dosed with the direct incursion of *PM3* did not show promising results in terms of crack healing, strength, and durability.
- Mix C and Mix D both were dosed with *A8*; however, only Mix D regained significant compressive strength on the 7^{th} and 28^{th} days of curing; this may be due to longer survival of *A8* in Mix D than Mix C but not long enough to be observed under FESEM up to 28^{th} day.
- X-ray diffractogram peaks show that precipitates induced by *PM3* are more crystalline than *A8.*
- Compressive strength increases on integration of *PM3* and *A8* fungal species into concrete immobilized on calcium alginate beads. However, there is little or marginal increase noticed with direct induction of fungi in concrete. This is attributed to the better survival of fungi resulting in abundant formation of bio-precipitate on being immobilized through compatible carrier.

- Peak stress and peak strain values along with compressive toughness increase on inducing *PM3* and *A8* fungal strains inside concrete through calcium alginate beads as the potential carrier. The increase relates to the added plastic stiffness provided by the calcium alginate carrier and bio-metabolic calcite precipitate.
- Healing efficiency measured by UPV, and crack width analysis reveal that the specimens with immobilized fungal strains show better crack repairing efficacy than the specimens without immobilization. The maximum crack width of 1.3 mm is effectively healed using *PM3* strain carried through calcium alginate.
- *Rhizopus oryzae* and *Trichoderma longibrachiatum* belong of BSL-1 so it is unlikely for them to cause any infection in healthy adult as long as properly handled. As *Rhizopus oryzae* is already being used in food industry for fermentation of cheese and production of enzymes for many years which are certified safe as food additive for human consumption by Department of health and human services US. ; on proper handling, it can be implied in large scale concrete production. It is recommended, the behavior of high-strength concrete needs to be investigated in the future after inoculation of immobilized fungal strains. Moreover, permeability aspects need to be investigated further in detail as fungi has the potential to induce a hydrophobic layer making the concrete structure dense.

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