**Fungi-Mediated Self-Healing Concrete for Sustainable Infrastructure**

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**Abstract**

Cracking in concrete is very common owing to drying shrinkage, freeze-thaw cycles, delayed ettringite formation, reinforcement corrosion, creep and fatigue, etc. Since maintenance and inspection of concrete infrastructure require onerous labor and high costs, self-healing of harmful cracks without human interference or intervention could be of great attraction. The goal of this study is to explore a new self-healing concept in which fungi are used as a self-healing agent to promote calcium carbonate precipitation to fill the cracks in concrete infrastructure. Recent research results in the field of geomycology have shown that many species of fungi could play an important role in promoting calcium carbonate mineralization, but their application in self-healing concrete has not been reported. Therefore, a screening of different species of fungi has been conducted in this study. Our results showed that, despite the drastic pH increase owing to the leaching of calcium hydroxide from concrete, the spores of *Aspergillus nidulans* (MAD1445), a pH regulatory mutant, germinated into hyphal mycelium on concrete plates with growth only slightly weaker. The precipitation of crystalline calcite on the fungal hyphae is confirmed by X-ray diffraction as well as electron microscopy that reveals micron sized precipitate particles with well-defined multi-faceted crystals.

1. **Introduction**

Sustainable infrastructure is the key to creating a sustainable community, due to its significant impact on energy consumption, land use, and global economy. However, many countries are facing the downfall of progressively aging infrastructure that needs rehabilitation. In particular, concrete infrastructure suffers from serious deterioration owing to the effect of various physical and chemical phenomena, such as drying shrinkage, freeze-thaw cycles, reinforcement corrosion, creep and fatigue, and delayed ettringite formation, all of which could lead to concrete cracking. Cracks themselves may not significantly reduce the load-carrying capacity of concrete in the short run, but they considerably weaken the durability of concrete structures, as they provide easy paths for the ingress of liquids and gasses, which potentially contain corrosive agents for the steel reinforcement. Moreover, cracking may promote severe degradation of the non-mechanical properties of concrete, such as the radiation-shielding properties of concrete elements used in nuclear applications. Nowadays, concrete has been the key construction material for
reactor containment and biological shielding structures, which are essential components of the nuclear reactors in service worldwide for power generation. In addition, cementitious grouts, mortars, and concrete are also often used to provide shielding and encapsulation of various radioactive waste materials from military, research, and power generation applications. Some waste isotopes as well as their decay products will become a serious radiation hazard for hundreds of thousands of years, which requests exceptionally durable storage.

In view of the remarkable social significance of concrete infrastructures and their exceptional service demands, the maintenance and inspection for concrete structures have come into focus. However, continuous inspection and maintenance usually require onerous labor and high investments. Under such circumstances, self-healing of harmful cracks without human interference or intervention could be of great attraction. This idea was originally inspired by the amazing ability of the human body to heal itself of broken bones through mineralization. As for how to equip cementitious materials with self-healing properties, numerous investigations have been conducted during the past decade and generated many innovative solutions1-23.

Self-healing of concrete cracks can be achieved by three different mechanisms: autogenous healing, embedment of polymeric material, and bacteria-mediated CaCO$_3$ precipitation, which have been summarized in a comprehensive review provided by Seifan et al.20 Autogenous healing is the natural process of concrete crack repair in the presence of water or humidity, but it is limited to small cracks with crack widths less than 0.2 mm thanks to the limited expansive potential of the small non-hydrated cement particles, making autogenous healing quite problematic for practical applications. Using encapsulation of polymeric material approach, chemicals released from hollow fibers embedded inside the concrete can effectively heal cracks, but these materials are not compatible with concrete compositions, e.g., they may have different mechanical properties, and thus they may cause existing cracks to propagate further. Moreover, the capsules need to be able to preserve the healing agent for a long time without affecting the mechanical properties of concrete, which makes this approach a difficult practice for commercial concrete product.

Therefore, the biotechnological approach by using mineral-producing microorganisms becomes highly desirable. It has been demonstrated that when a calcium source is present, CaCO$_3$, as one of the most suitable fillers for concrete cracks owing to its high compatibility with concrete compositions, can be precipitated by bacteria through various biologically induced mineralization processes3-21. Bacteria can precipitate CaCO$_3$ mainly through three pathways, as summarized below. (1) Some ureolytic bacteria, such as Bacillus sphaericus, B. pasteurii, Sporosarcina pasteurii, and S. ureae have been explored based on their ability to indirectly cause the formation of calcium precipitates by increasing the local pH via catalyzing the hydrolysis of urea to ammonium1-5. However, the metabolic activity of ureolytic bacteria is often severely hindered at pH values higher than 9, making the embedded bacteria unable to survive under the highly alkaline conditions of concrete with pH approximately equal to 13 over a prolonged period of time. (2) Microbiologists at Delft University of Technology in the Netherlands11-13 tested the second pathway, in which aerobic oxidation of organic acids results in production of CO$_2$, leading to carbonate production in an alkaline environment. During the process, the bacteria are activated as they come into contact with water and then use the calcium lactate as a food source, producing CaCO$_3$ that, as a result, fixes the cracks. This pathway is more sustainable thanks to the nonappearance of ammonium. However, calcite production demands high concentrations of calcium sources, which may lead to
accumulation of high level of salts in concrete matrix. Moreover, in the case of low concentration in oxygen, such as for most underground structures, the efficiency of this approach can be limited. (3) The third pathway to produce minerals is known as dissimilatory nitrate reduction. Minerals are precipitated by means of oxidation of organic compounds by the reduction of nitrate through denitrifying bacteria. This approach can be applied in anaerobic zones. However, it has been shown that the efficacy of denitrification approach is much lower than the ureolysis approach regarding the production of calcium carbonate.

2 Fungi-Mediated Self-Healing Concrete

While the term “microbe” refers to a wide range of microorganisms, studies on self-healing concrete have so far been limited to bacteria. Admittedly, using bacteria has many advantages. For example, bacteria are easy to culture and handle in a laboratory setting, and collection and isolation of bacteria are not complex, as during the years numerous selective media have been developed for direct isolation of bacteria. On the other hand, however, bacteria possess limited ability to survive and promote calcium carbonate precipitation in the deleterious environment of concrete. Therefore, to date there has been limited success in respect to the long-term healing efficacy and in-depth consolidation. So far, the viability of bacterial spores embedded in the concrete matrix was restricted to about six months, which is far from being practical considering the fact that the lifetime of concrete infrastructure can easily be fifty years or even a century. In addition, the healing is limited to small cracks with crack widths less than 0.8 mm owing to the limited calcinogenic ability of bacteria.

The harsh environment of concrete, such as very high pH values (approximately 13), tiny pore sizes (less than 0.1 μm), severe moisture deficit, varied temperatures (high temperature in summer and low temperature in winter), limited nutrient availability, and possible exposure to intense ultraviolet light, dramatically influences the microbial metabolic activities and makes bacteria vulnerable to death. In addition, from the economical point of view, the production of bacteria-based self-healing concrete currently leads to considerable costs owing to the need of aseptic conditions to produce the microbial spores and the use of expensive growth media, making this technique impractical for commercial applications.

To address the above-mentioned problems, further investigation on alternative microorganisms for the application of self-healing concrete becomes potentially essential. In fact, microorganisms are very diverse, and besides bacteria, they also include archaea and most protozoa. Protozoa include fungi, microscopic algae, and some micro-animals, such as rotifers. Among them, fungi are the most species-rich group of eukaryotic organisms after insects, which exhibits vast biodiversity with more than 70 K known species and approximately 1.5 M unknown species. Common fungi include yeasts, lichens, molds, and mushrooms, etc. Although recent studies in the field of geomycology, i.e., the study of the role of fungi in biogeochemical cycles, demonstrated that fungi can play a central role in CaCO₃ precipitation, none of them have been studied for the application of self-healing concrete. According to the existing literature, the investigation of fungi has been mostly focusing on their importance in organic matter degradation, and their connection with inorganic constituents has been mainly limited to mineral nutrition through mycorrhizal symbiosis as well as mineral weathering of lichens.
The cells of most fungi grow as tubular, elongated, and filamentous structures called hyphae, which contain multiple nuclei and grow apically with new apices emerging from the formation of lateral branches, creating a 3D network. In fact, it is widely believed that filamentous fungi possess distinctive advantages over other microbial groups to be used as biosorbent materials to attract and hold metal ions because of their superior wall-binding capacity and extraordinary metal-uptake capability.  

Although the peculiar mechanisms leading to calcium carbonate mineralization by fungi are still not completely understood, it has been concluded that there are several different mechanisms involved in the calcification of fungal filaments. Fungi are known to be involved in both calcium carbonate bioweathering and biomineralization. To precipitate calcium carbonate, two factors are critical, i.e., carbonate alkalinity and Ca$^{2+}$ concentration, and fungal metabolic activities can influence both, potentially leading to induced calcium carbonate biomineralization.

Fungal metabolic activities that can decrease alkalinity usually include heterotrophic respiration leading to an increase in pCO$_2$, production of organic acids, and excretion of H+ during fungal thigmotropism. Fungal metabolic activities that can increase alkalinity typically include water consumption, physicochemical degassing of fungal respired CO$_2$, organic acid oxidation, nitrate assimilation, and urea mineralization.

Ca$^{2+}$ concentration within metabolically active fungal cells should be strictly controlled, i.e., Ca$^{2+}$ must be concentrated at the apex for proper apical growth and instantly decreased in subapical regions. To keep this sharp gradient, fungi need to effectively regulate Ca$^{2+}$. Ca$^{2+}$ concentration in the cytoplasm is maintained at low levels by actively pumping it out of the cell or by binding it onto cytoplasmic proteins.

In addition, fungi may also be involved in CaCO$_3$ biomineralization through organomineralization. An important characteristic that places fungi in a different kingdom from bacteria is the chitin in their cell walls, which is a modified polysaccharide that contains nitrogen. According to Manoli et al., chitin is a substrate that significantly reduces the required activation energy for nucleus formation so that the interfacial energy between the fungi and the mineral crystal becomes lower than the one between the mineral crystal and the solution. Thus, cation binding by fungi can occur by means of metabolism-independent binding of ions onto cell walls, which is an important passive property of both living and dead fungal biomass, resulting in nucleation and deposition of mineral phases. Bound calcium cations can interact with soluble carbonate, leading to calcium carbonate deposition on the fungal hyphae.

Fungi growing in calcium-rich environments are exposed to high concentrations of Ca$^{2+}$ and CO$_3^{2-}$, which probably pose a stress for fungal cells owing to subsequent osmotic pressure and Ca$^{2+}$ cytotoxicity. The formation of calcium oxalates has been suggested as a method to immobilize excessive Ca$^{2+}$. Precipitation of calcium carbonate may be due to a similar passive mechanism to decrease their internal Ca$^{2+}$ concentration. Excessive alkalinity could also pose a source of stress and precipitation of calcium carbonate may be due to intracellular protection.

Therefore, we can conclude that fungi could be used in the application of biogenic crack repair for concrete infrastructure due to its extraordinary ability to both directly and indirectly promote calcium carbonate precipitation. Since fungi have never been investigated in the application of self-healing...
concrete, the current work presents a pilot study to investigate the feasibility of using fungi for self-healing of concrete cracks, in which a wide screening of different species of fungi has been conducted.

3. Materials and Methods

Since many different species of fungi have been reported to be able to promote calcium mineralization, the following criteria were used to select the candidates: they should pose no risk to human health and are appropriate to be used in concrete infrastructure; they should have the ability to form alkali-resistant spores, which can tolerate a wide range of natural environments, so that they can better adapt to the harsh environment of concrete; and it is preferred if the genomes of the fungi have been sequenced and are publicly available so that they can be genetically manipulated to enhance their performance in crack repair.

The fungal spores, along with their nutrients, will be placed into the concrete matrix during the mixing process. When cracks occur and water finds its way in, the dormant fungal spores will germinate, grow, consume nutrients, and precipitate calcium carbonate to heal the cracks in situ. When the cracks are completely healed and ultimately no more water can enter inside, the fungi will again form spores. As the environmental conditions become favorable in later stages, the spores could be wakened again. For existing concrete infrastructures with cracks, the fungal spores and their nutrients can be injected or sprayed into the cracks.

For genetically engineered fungi, since the extremely high pH of the concrete environment can be handled by only a few fungi, pH regulatory mutants were the focus here. It is well known that many microorganisms can tailor gene expression to the pH of the growth environment if they are able to grow over a wide pH range\textsuperscript{37,38}. Fungi respond to ambient pH levels by means of activation of a dedicated transcription factor, PacC\textsuperscript{39-43}. This response is initiated by a signaling cascade which starts with a cell surface sensor, PalH. PalH probes the extracellular pH level. PalF, an arrestin-like protein which interacts with PalH, is ubiquitinated in response to alkaline pH\textsuperscript{44}. The cascade results in the activation of PacC by proteolytic cleavage and, eventually, to transcriptional regulation\textsuperscript{40}. Mutants in the pacC regulatory gene are extremely heterogeneous in phenotype\textsuperscript{37}. A major class of mutations are gain-of-function pacC\textsuperscript{c} created by truncating the C-terminal region\textsuperscript{38}. The pacC\textsuperscript{c} mutations bypassing the need for the ambient pH signal lead to permanent activation of alkaline genes and superrepression of acidic genes, resulting in alkalinity mimicry\textsuperscript{41}. Irrespective of ambient pH, alkalinity-mimicking mutations believe that they are always at alkaline pH and trigger a pattern of gene expression similar to that in the wild type grown under alkaline conditions, which is exactly what is needed for self-healing concrete. The first member of the eukaryotic organism whose gene regulation by ambient pH has been most extensively studied is Aspergillus nidulans\textsuperscript{38}. Thus, in this study, three different types of alkalinity-mimicking mutants of A. nidulans, i.e., MAD1445, MAD0305, and MAD0306, will be tested for the application of self-healing concrete.

We also purchased the following wild type strains from American Type Culture Collection (ATCC): Rhizopus oryzae (ATCC22961), Phanerochaete chrysosporium (ATCC24725), A. nidulans (ATCC38163), A. terreus (ATCC1012), and A. oryzae (ATCC1011). The genomes of these fungi strains have been well-understood and are publicly available\textsuperscript{45-49}. R. oryzae, able to mediate calcium mineral precipitation\textsuperscript{50}, is often used in the food industry, such as in the production of tempeh, and its products are
generally recognized as safe. *A. oryzae*, reported to be able to promote calcium carbonate biomineralization\(^{51}\), is often used in Chinese cuisines to ferment soybeans to make soy sauce. *A. terreus*, cellulolytic in nature, is spread primarily in soil and it is associated with biogenic formation of calcium oxalate crystals\(^{52}\). *P. chrysosporium*, one of the oxalic acid-producing fungi\(^{53}\), has specialized ability to degrade the abundant aromatic polymer lignin. To understand its mechanism to enhance the bioremediation of a diverse range of pollutants, *P. chrysosporium* is the first member of the basidiomycetes to have its genome sequenced\(^{54}\).

The above-mentioned fungi are all filamentous fungi. In addition to filamentous fungi, there are also single-celled fungi, i.e., yeasts, that do not form hyphae. One of the most well-known species of yeast is known as *Saccharomyces cerevisiae*, which is also called brewer’s yeast, or baker’s yeast, as it has been instrumental to winemaking, baking, and brewing since ancient times. Recently, a series of publications were released on the success of designing synthetic set of *S. cerevisiae* chromosomes, which could allow *S. cerevisiae* to rapidly evolve and rearrange its genome, optimizing itself for certain applications\(^{55-57}\). Thus, in this study, *S. cerevisiae* will also be tested for the application of self-healing concrete.

To investigate whether and how fungal hyphae could promote calcium carbonate precipitation in concrete, we here will employ material characterization techniques including X-ray diffraction (XRD), scanning electron microscope (SEM), and transmission electron microscope (TEM). XRD, as an established technique to study mineral crystal structures, has been extensively used to identify biominerals at fungi-mineral interfaces\(^{58-59}\). SEM has been used for the surface visualization of fungal precipitates\(^{60-61}\), and TEM has been used to study and fungal-biotite interfaces and weathered alkali feldspars\(^{62-63}\). In this study, SEM will be used to characterize the morphology and composition of the solid precipitates, which will complement the very local characterization by TEM.

### 3.1 Gene Replacement Procedure to Obtain Alkalinity-Mimicking Mutations

The full genotypes of the strains used in this work are given in Table 1. The details about the parental strains and selection procedures of isolating the classical *pacC* mutations used in this work were previously published\(^{64-68}\).

| Strain     | Genotype     | Reference\(^{64-68}\)                      |
|------------|--------------|--------------------------------------------|
| MAD1445    | yA2 pabaA1 pacC14900 | Penas et al. 2007; Hervas-Aguilar et al. 2007 |
| MAD0305    | pabaA1 pacC14 | Caddick et al., 1986; Tilburn et al. 1995; Mingot et al. 1999 |
| MAD0306    | pabaA1 pacC202 | Tilburn et al. 1995; Mingot et al. 1999 |

Briefly, the gene replacement procedure to obtain alleles *pacC14* and *pacC202* can be described as follows. The parental strains with genotype *pabaA1* gatA2 palF15 fwA1 (*p*-aminobenzoate-requiring, lacking \(\omega\)-amino acid transaminase, mimicking growth at acidic pH, and having fawn conidial color) were mutagenized with ultraviolet treatment followed by growth selection on media supplemented with 5 mM \(\gamma\)-amino-\(n\)-butyrate in presence of 10 mM ammonium tartrate as nitrogen source\(^{69-71}\). The *pacC14* mutant strain was selected as it mimics the growth at alkaline pH values, downregulates the \(\gamma\)-amino-\(n\)-butyrate permease, decreases the \(\gamma\)-amino-\(n\)-butyrate level in cells, and thus relieves the \(\gamma\)-amino-\(n\)-butyrate-induced cytotoxicity. The *pacC202* strain was a spontaneous mutant strain derived from the
parental strains with genotype \textit{pabaA1 sasA60} (\textit{p}-aminobenzoate-requiring and semialdehyde sensitive), selected as it relieves the toxicity of 10 mM \( \gamma \)-amino-\( n \)-butyrate. The \textit{pacC} genomic sequences in \textit{pacC14} and \textit{pacC202} strains were then obtained by Polymerase Chain Reaction (PCR) amplification.

The procedure to obtain MYC\textsubscript{3}-tagged wild-type \textit{pacC} allele, designated as \textit{pacC900}, as well as the engineered allele \textit{pacC14900} can be described as follows. A null allele of \textit{pacC} was first constructed by transforming the orthologous \textit{pyr4} (orotidine 5' phosphate decarboxylase) gene of \textit{Neurospora crassa} flanked by 750 bp of upstream and 700 bp of downstream \textit{pacC} genomic sequences into a \textit{pyrG89} (resulting in pyrimidine auxotrophy) \textit{pacC14} strain. Transformation was carried out using linearized fragments containing the \textit{pacC} moiety of the plasmid. The long flanking sequence ensures high efficiency of homologous recombination-mediated gene replacement. The transformants displaying pyrimidine prototrophy were then selected and tested by Southern blot analyses to confirm that \textit{pacC14} was replaced by \textit{pyr4}. The \textit{pacC} null strain was then used as recipient strain and transformed with linearized MYC\textsubscript{3}-tagged \textit{pacC} carrying C2437A mutation flanked with genomic sequence 825 bp upstream of \textit{pacC} start codon and 925 bp downstream of stop codon\textsuperscript{72}. Transformants displaying \textit{pacC} colony morphology were tested for pyrimidine auxotrophy. The gene replacement was again confirmed using Southern blot analyses. The genotypes in the resulting \textit{pacC14900} strain were further confirmed by PCR sequencing. The protein expression was confirmed by Western blot analyses using anti-MYC antibody.

3.2 Identification of the Yeast Strain

Potato dextrose agar (PDA) that is nutritionally rich in carbohydrates and can stimulate vegetative growth of most fungi was chosen as growth medium. The yeast strain was obtained by culturing and purifying Active Dry Yeast (Hodgson Mill, Effingham, IL, USA) on PDA (Difco, BD Diagnostic Systems, Sparks, MD, USA). The purified fungal culture was grown on PDA for 7 days. Genomic DNA was extracted from the yeast cells with UltraClean Soil DNA Isolation Kit (MoBio Laboratories, Carlsbad, CA, USA). The nuclear ribosomal internal transcribed spacer (ITS) region, the universal fungal barcode marker, was amplified using the primers ITS1 and ITS4\textsuperscript{73}. PCR was performed with Taq 2X Master Mix (New England BioLabs, Maine, MA, USA). PCR cycling conditions for the ITS consisted of an initial denaturation step at 95 \( ^\circ \)C for 3 min, 35 cycles of 95 \( ^\circ \)C for 45 s, 52 \( ^\circ \)C for 45 s, 72 \( ^\circ \)C for 1 min and a final extension at 72 \( ^\circ \)C for 5 min. PCR products were purified with ExoSAP-IT (Affymetrix, Santa Clara, CA, USA) and sequenced with the PCR primers by Genscript, Piscataway, NJ, USA. The fungal strains were identified based on the search results of ITS sequences with BLASTn in GenBank as well as the morphological data.

3.3 Preparation of Mortar Specimens and Cement Paste Specimens

Series of mortar specimens were prepared for the survival test of the fungi in the environment of concrete by using Ordinary Portland Cement (CEM I 52.5N), standardized sand (DIN EN 196-1 Norm Sand) and tap water. The water-to-cement weight ratio was 0.5 and the sand-to-cement weight ratio was 3. The specimens were made according to the standard procedure NBN EN 196-1\textsuperscript{74}. They were then poured into 60 mm Petri dishes (9 ml per dish) and cured at 100\% relative humidity and 22 \( ^\circ \)C for 28 days.

Cement paste specimens were also prepared to investigate the pore size distribution of aging specimens. Ordinary Portland Cement (CEM I 52.5N) was mixed with tap water in a water-to-cement weight ratio of
0.5. Liquid paste was poured in molds with dimensions of 4 cm × 4 cm × 4 cm and cured at 100% relative humidity and 22 °C for 28 days. In addition, air-entrained cement paste specimens were also prepared to investigate the effect of air-entraining on the pore size distribution of the specimens. Eucon AEA-92 (Euclid Chemical, Cleveland, OH, USA) was dosed at a rate of 150mL per 100 kg of the total cementitious material.

3.4 Survival Test of Fungi in the Environment of Concrete

To check the effect of the highly alkaline environment of concrete on the fungal growth behavior, growth medium was prepared using PDA (Difco, BD Diagnostic Systems, Sparks, MD, USA) with or without the addition of the inert pH buffer 3-(N-morpholino)propanesulfonic acid (MOPS, 20mM, pH 7.0) (Fisher Scientific, Pittsburgh, PA, USA). 10 ml growth medium was overlaid onto each cured concrete plate. A mycelial disc with a diameter of 5 mm of each fungal strain was removed from 7-day-old cultures using a cork borer, and was aseptically deposited at the center of each 60 mm Petri dish containing growth medium with or without concrete. Sterile PDA plugs were used as the negative inoculum control. After inoculation, the Petri dishes were incubated in natural daylight conditions at 22 °C and 30 °C, respectively, for three weeks.

In summary, for each type of fungal strain, totally eight different types of plates were tested in this study, which were abbreviated as follows: PDA incubated at 30 °C (PDA30), PDA incubated at 22 °C (PDA22), PDA with MOPS incubated at 30 °C (MPDA30), PDA with MOPS incubated at 22 °C (MPDA22), PDA with concrete incubated at 30 °C (CPDA30), PDA with concrete incubated at 22 °C (CPDA22), PDA with both concrete and MOPS incubated at 30 °C (CMPDA30), and PDA with both concrete and MOPS incubated at 22 °C (CMPDA22). All the tests were done independently in triplicates. Radial growth measurements were recorded along two perpendicular diameters. The fungal samples were prepared by the tape touch method and observed with an optical microscope (Carl Zeiss model III, Zeiss, Jena, Germany). pH values were recorded after the plates were incubated for three weeks. The pH of each plate was measured by taking five independent measurements using an Orion double junction pH electrode (Thermo Fisher Scientific, Waltham, MA, USA).

3.5 Microscopic Characterization of Fungal Precipitates

3.5.1 X-Ray Diffraction (XRD)

The solid precipitates associated with fungal hyphae were identified by XRD analysis. A Siemens-Bruker D5000 powder diffractometer with Cu-Kα radiation in the θ/θ configuration was used. The diffractometer was operated at 40 kV and 30 mA. Measurements were made from 10° to 80° 2θ at a rate of 1°/min with a step size of 0.02° 2θ. Isolation of the fungal precipitates was performed by dissolving the fungi in NaOCl and repeated washings in methanol.

3.5.2 Scanning Electron Microscope (SEM)

The solid precipitates associated with fungal hyphae and the NaOCl-isolated crystals were analyzed using a Zeiss Supra 55 VP Field Emission SEM with an EDAX Genesis energy-dispersive X-ray spectrometer (EDS) at accelerating voltages of 5 kV to 20 kV. The fungal samples were completely dried in the oven at 50 °C for 2 days, and then were mounted on aluminum stubs and sputter-coated with carbon to ensure
electrical conductivity for the examination of crystal morphology and distribution. The elemental composition of the precipitates was investigated by EDS analysis.

3.5.3 Transmission Electron Microscope (TEM)

TEM images were obtained using a JEOL JEM-2100F TEM operating at 200 kV. The precipitated particles were collected by centrifugation (6000×g, 10 min) and washed four times with methanol to remove the growth medium. The particles were dried at room temperature then ground to fine powders by a pestle. The fine powders were then collected on copper grids overlain with a carbon-coated collodion film prior to the examination. Bright-field imaging (BFI), selected area electron diffraction (SAED), and high-resolution transmission electron microscopy (HRTEM) were used to characterize the calcite phases.

3.6 Pore Size Distribution of Cement Paste Specimens

High-resolution X-ray computed microtomography (µCT), a radiographic imaging technique to nondestructively characterize material microstructure in three-dimensional space, was used to characterize the pore size distribution in aged cement paste specimens. Images were acquired using UltraTom X-ray µ-CT system equipped with a LaB6 X-ray source and a CCD camera with resolution of 4000 × 2624 pixels. The X-ray beam geometry is conical, and as a consequence, larger objects will have a lower resolution and smaller samples a higher resolution. By moving the sample between the source and the detector, an appropriate magnification was chosen. Small cubic samples of 0.4 cm × 0.4 cm × 0.4 cm were used, for which the voxel size of the obtained images will be about 1.2 μm. Samples were firmly fixed on a thin cylindrical holder mounted on a rotating table using a non-intrusive adhesive paste. For all the measurements, acquisition parameters were set as follows: source 230 kV, voltage 90 kV, intensity 200-300 μA, filament LaB6, frame rate 0.4, and 8 averaging images. To impose small angular increments in order to cover 360° along the rotation axis, a total of 1664 phase contrast images were collected. The acquisition duration was approximately 30 min per sample. Based on the raw data measured by µCT, the reconstruction of inner microstructure will be carried out by using the Octopus Imaging software. Image processing is performed using public domain ImageJ software.

4. Results and Discussion

4.1 Mutant Sequence Changes and Resulting Proteins

The mutant sequence changes in pacC and the resulting proteins are summarized in Table 2. Note that c denotes constitutive phenotype, which bypasses the requirement for the pH signal and mimics alkaline growth conditions; and an asterisk denotes a stop codon. It can be seen that the pacC14 genotype carries a C2437A allele, causing an early stop codon that results in a C-terminal truncation of PacC protein at 492 amino acid. The pacC202 genotype has a deletion from 2353 bp to 2555 bp, resulting in an open reading frame shift after amino acid 464 and an early stop codon. The resulting pacC14900 strain has a MYC3-tagged pacC carrying the pacC14 mutation at the pacC locus.

Table 2. Mutant sequence changes in pacC and resulting proteins.

| Genotype | Phenotype | Mutation  | Mutant protein | Reference |
|-----------|-----------|-----------|----------------|-----------|
| pacC14    | c         | C2437A    | 1→492*         | Tilburn et al. 1995 |
4.2 Identification of the Yeast Strain

The yeast strain has 99% ITS sequence similarity to the *S. cerevisiae* ex-type culture CBS 1171 (NR_111007) and has the same morphological features of *S. cerevisiae*. Therefore, it is confirmed as the baker’s yeast, i.e., *S. cerevisiae*.

4.3 Fungal Growth in the Harsh Environment of Concrete

The fungal growth in each type of plate has been shown in Fig. 1. Optical microscopic analysis of each case has been shown in Fig. 2. The growth rates of all the tested species are showed in Table 3. The pH measurement results, as listed in Table 4, have shown that, due to the leaching of Ca(OH)$_2$ from concrete, the pH of the growth medium increased from 6.5 to 13.0. On the concrete plates, only one type of pH regulatory mutants of *A. nidulans*, i.e., MAD1445, has been found to be able to grow well. At 30 °C, its growth rates reached 3.2 mm/day in the case of CMPDA30. Abundant conidia were observed from the plates with concrete and had similar morphology compared to those produced on the plates without concrete, as shown in Fig. 3. However, no growth of *A. nidulans* (MAD1445) was found on any plates with concrete in the cases of CPDA22 or CPDA30. The other eight species had no growth on any plates with concrete, although they grew well on all the plates without concrete. Agar plug controls without any inoculum showed no fungal growth. It is important to note that of all these three strains, which carry pacC constitutive mutations and otherwise possess very similar phenotypes, only MAD1445 grows well on the concrete plates in the presence of MOPS. This could possibly be explained by the fact that other unknown genetic factors could lead to increased survival against high pH values. The three strains were obtained through either mutagenesis or homologous recombination. Although the pacC locus was sequence-verified, it is possible that mutations happened at other undesired loci of the MAD1445 strain resulted in increased survival in the alkaline environment.

Table 3. Average growth rates (mm/day) of the nine fungal species on PDA, MPDA, CPDA, and CMPDA at 22 °C and 30 °C, respectively, on day 21 after inoculation (n = 6).

|                | PDA30 | PDA22 | MPDA30 | MPDA22 | CPDA30 | CPDA22 | CMPDA30 | CMPDA22 |
|----------------|-------|-------|--------|--------|--------|--------|---------|---------|
| Aspergillus nidulans (ATCC38163) | 4.2 a  | 2.2 c  | 1.7 d  | 1.7 d  | 0      | 0      | 0       | 0       |
| Aspergillus nidulans (MAD1445)    | 3.9 a  | 1.9 c  | 1.2 d  | 1.2 d  | 0      | 0      | 3.2 b   | 0.9 e   |
| Aspergillus nidulans (MAD0305)    | 3.2 a  | 2.1 b  | 1.6 c  | 1.5 c  | 0      | 0      | 0       | 0       |
| Aspergillus nidulans (MAD0306)    | 2.1 a  | 1.4 b  | 0.3 c  | 0.3 c  | 0      | 0      | 0       | 0       |
| Aspergillus oryzae (ATCC1011)     | 4.3 a  | 2.8 c  | 3.3 b  | 2.3 d  | 0      | 0      | 0       | 0       |
### Table 4. pH measurement results of the nine fungal species on PDA, MPDA, CPDA, and CMPDA on day 21 after inoculation.

| Fungal Species                | PDA | MPDA | CPDA | CMPDA |
|------------------------------|-----|------|------|-------|
| *Aspergillus terreus*        | 4.6 | 2.8  | 1.8  | 1.7   |
| (ATCC1012)                   |     |      |      |       |
| *Rhizopus oryzae*            | 7.8 | 7.8  | 7.8  | 7.8   |
| (ATCC22961)                  |     |      |      |       |
| *Phanerochaete chrysosporium*| 7.8 | 7.8  | 7.8  | 4.2   |
| (ATCC24725)                  |     |      |      |       |
| *Saccharomyces cerevisiae*   | 1.9 | 1.9  | 1.9  | 1.5   |
| (Yeast)                      |     |      |      |       |
| *Aspergillus nidulans*       | 6.5 | 7.2  | 13.0 | 11.6  |
| (ATCC38163)                  |     |      |      |       |
| *Aspergillus nidulans*       | 6.4 | 7.1  | 13.2 | 11.2  |
| (MAD1445)                    |     |      |      |       |
| *Aspergillus nidulans*       | 6.3 | 7.1  | 13.0 | 12.1  |
| (MAD0305)                    |     |      |      |       |
| *Aspergillus nidulans*       | 6.2 | 7.1  | 13.1 | 11.3  |
| (MAD0306)                    |     |      |      |       |
| *Aspergillus oryzae*         | 6.3 | 7.1  | 13.6 | 12.2  |
| (ATCC1011)                   |     |      |      |       |
| *Aspergillus terreus*        | 6.3 | 7.0  | 13.1 | 13.0  |
| (ATCC1012)                   |     |      |      |       |
| *Rhizopus oryzae*            | 6.7 | 7.0  | 13.2 | 12.8  |
| (ATCC22961)                  |     |      |      |       |
| *Phanerochaete chrysosporium*| 6.8 | 7.0  | 13.3 | 12.1  |
| (ATCC24725)                  |     |      |      |       |
| *Saccharomyces cerevisiae*   | 6.3 | 7.0  | 13.0 | 12.8  |
| (Yeast)                      |     |      |      |       |
Figure 1. Only one type of pH regulatory mutants of A. nidulans, i.e., MAD1445, has been found to be able to grow well on the plates with concrete. In comparison, the other eight species did not grow on concrete.
Figure 2. Microphotographs of optical microscopy (1000X, Carl Zeiss model III) confirmed that only one type of pH regulatory mutants of A. nidulans, i.e., MAD1445, was able to grow well on the plates with concrete.
Figure 3. For the case of *A. nidulans* (MAD1445), abundant conidia were observed from the plates with concrete, which had similar morphology compared to those produced on the plates without concrete. The diameter of *A. nidulans* spores (round to oval in shape) appeared to be within the range of 3 μm to 4.5 μm.

### 4.4 Identification and Morphology of the Fungal Precipitates

The results from XRD analysis are shown in Fig. 4. The XRD data strongly suggested that the hyphae of *A. nidulans* (MAD1445) can promote calcium carbonate precipitation. For the precipitates associated with the fungal hyphae, the sharp peak at around 30° 2θ suggests the presence of highly crystalline phases of the calcium carbonate mineral calcite. The mortar specimens obtained from the parallel experiment performed with the agar control without fungi were mainly composed of highly crystalline phases of quartz and calcite. The carbonation results from the dissolution of carbon dioxide in the concrete pore fluid and its reaction with carbon hydroxide.
Figure 4. XRD results for crystalline precipitates collected from mortar specimens cured with and without the hyphae of A. nidulans (MAD1445). Reference diffractograms of quartz (SiO$_2$) and calcite (CaCO$_3$) mineral standards from the International Centre for Diffraction Data (ICDD) are included.

Fig. 5 illustrates SEM images of the calcium carbonate precipitation. As shown in Fig. 5(a), a large amount of mineral crystals grew in the A. nidulans (MAD1445)-inoculated medium. The particles have regular shapes with well-defined crystalline faces, indicating high crystallinity of the precipitates. As shown in Fig. 5(b), EDS analysis demonstrated that the crystals are composed of Ca, C, and O with an atomic percentage closely matching that of CaCO$_3$, implying that the crystals are composed mainly of calcium carbonate. In sharp contrast to the fungi-inoculated medium, the amount of formed crystals in the fungi-free control medium was much less, as evidenced by the absence of faceted particles, as shown in Fig. 5(c).
Figure 5. SEM and EDS spectra of the calcium carbonate precipitation: (a) A. nidulans (MAD1445)-inoculated medium; (b) EDS spectra; and (c) fungi-free medium.

TEM analyses, as shown in Fig. 6, further confirm the crystalline nature of the calcium carbonate. Fig. 6(a) shows a bright-field TEM image of the fragments. Fig. 6(b) is a SAED pattern from the collection of the particles shown in Fig. 6(a), which demonstrates the crystalline nature of the precipitated particles, as evidenced by the presence of well resolved diffraction rings of (104), (110), (11-6), (122), and (1010) planes, matching well with the calcite phase. Fig. 6(c) is a higher magnification TEM image of the precipitate particle, which shows the presence of crystalline lattice (moire fringes are also visible due to the overlapping of particles). Fig. 6(d) is a HRTEM image obtained from the area as marked with the black dashed square in Fig. 6(c). As shown in Fig 6(e), the interplanar spacing of the crystalline planes in Fig. 6(d) is measured to be 0.308 nm, which matches with the interplanar spacing of (104) of the hexagonal crystal of calcite with the lattice parameters of a=b=4.989 Å, c=17.062 Å, α=β= 90°, and γ=120°.
Figure 6. TEM analysis of the fragments of precipitated particles: (a) Bright-field TEM image of the fragments of crushed precipitated calcite; (b) SAED obtained from the crushed precipitate shown in (a), the indexing of the diffraction rings matches well with the crystal structure of the calcite phase; (c) Higher-magnification TEM image of the precipitated particles, showing the presence of crystalline lattice fringes; (d) HRTEM image from the area marked with the black dashed square in (c); (e) Measurement of the interplanar spacing by averaging ten lattice fringes marked in (d).

4.5 Discussion on Embedment of Healing Agents in Concrete Matrix

In this section, how to embed the healing agents, i.e., fungi spores and nutrients, into concrete will be briefly discussed. If the typical fungi spore is larger than the pore sizes in concrete, when the healing agents are directly put into cement paste specimens, the majority of spores will be squeezed and crushed due to the pore shrinkage during the hydration process, leading to loss of viability and decreased mineral-forming capacity.
Our results on μCT characterization of concrete microstructure have been shown in Fig. 7. The 3D renderings, as shown in Fig. 7(a) to Fig. 7(c), are particularly useful for qualitative assessment of phase distribution, which is complementary to higher resolution 2D microscopy imaging. From a typical 2D slice as shown in Fig. 7(d), it can be seen that the matrix pore sizes in 28 days cured specimens are much smaller than the typical diameters of fungal spores, which are usually larger than 3 μm, as shown in Fig. 3. The matrix pore sizes in 28 days cured air-entrained specimens are shown in Fig. 7(e). It can be seen that air-entraining agents could be utilized to create a large number of extra air voids in concrete matrix to provide the housing for the fungal agents.

Figure 7. A 3D rendering of a grayscale volume is shown in (a), the corresponding high-density particles and pores are shown in (b) and (c), respectively. (d) Pore size of cement paste specimens prepared with a water-to-cement weight ratio of 0.5 cured for 28 days measured by μCT. (e) Effect of air-entraining agent on pore size of cement paste specimens prepared with a water-to-cement weight ratio of 0.5 cured for 28 days measured by μCT.

5. Concluding Remarks and Future Work

In the current study, a new self-healing concept has been explored, in which fungi were used to promote calcium mineral precipitation to heal cracks in concrete infrastructure. The experimental results showed that, despite the drastic pH increase due to the leaching of calcium hydroxide from concrete, the spores of one type of pH regulatory mutants of *A. nidulans* (MAD1445) germinated into hyphal mycelium on concrete plates with growth only slightly weaker. In comparison, *A. nidulans* (ATCC38163), *A. nidulans* (MAD0305), *A. nidulans* (MAD0306), *R. oryzae* (ATCC22961), *P. chrysosporium* (ATCC24725), *A. terreus* (ATCC1012), and *A. oryzae* (ATCC1011) could not grow on concrete. To investigate whether and how fungal hyphae could promote CaCO₃ precipitation in concrete, we employed material characterization techniques including XRD, SEM, and TEM, all of which confirmed that the crystals precipitated on the fungal hyphae were mainly composed of calcite. As future work, the effects of the various factors influencing fungal calcium precipitation will be investigated, including pH, temperature,
ultraviolet light, growth medium composition, and fungal spore concentration. For example, varying concentrations of calcium and inorganic phosphate will be supplemented to the growth medium as they have been shown to play an important role in carbonate precipitation\textsuperscript{78,79}. In particular, phosphate can influence calcium mineralization in two ways, i.e., calcium oxalate mineral formation is promoted at high phosphate levels, and phosphate acts as a pH buffer to prevent drastic local pH variations, which has been proven to be critical for optimal microbial performance\textsuperscript{78,79}.

The research findings from this study will also shed light on many other applications, such as removal of radionuclides, bio-consolidation of soil and sand, and removal of calcium from industrial wastewater. (1) The discharge of radionuclide wastewater has resulted in serious contamination in the environment\textsuperscript{80}. Conventional methods, such as chemical reactions, ion exchange, as well as membrane technologies are either ineffective or costly. In comparison, microbial CaCO\textsubscript{3}-based coprecipitation offers a cost-effective solution\textsuperscript{81}. The current study will be helpful in providing insight on how fungi could effectively promote formation of radionuclide carbonate minerals. (2) Bio-consolidation is often involved in stabilization of erosion and increasing slope stability\textsuperscript{82}. Microbe-mediated CaCO\textsubscript{3} precipitation offers a reliable and low-cost method to bind sand grains together at the particle-particle level, leading to a tremendous change in the important mechanical properties of the sand, such as permeability, stiffness, compressibility, and shear strength\textsuperscript{83}. The current study will advance our understanding of how fungi could be used in such applications. (3) High concentrations of calcium ions in industrial wastewater pose serious challenges thanks to the clogging of pipelines, boilers, and heat exchangers\textsuperscript{84}. Microbially induced calcium carbonate precipitation offers a cost-effective and eco-friendly strategy for removal of inorganic contaminants from the environment\textsuperscript{81}. The fundamental knowledge advanced by the current study will be important to understand the role of fungi in removal of calcium from industrial wastewater.

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Author Contributions Statement

CJ and NZ conceived and initiated the project. RRM, JL, and XC designed and performed the experimental work. GZ, NZ, and CJ supervised the research tasks. RRM, JL, XC, ZL, GZ, NZ, and CJ co-wrote the manuscript. All authors reviewed the manuscript before submission.

Competing Interests
The authors declare that they have no competing interests.

**Data Availability Statement**

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

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