Screening of Fungi for Self-Healing of Concrete Cracks

Jing Luo1, Xiaobo Chen2, Jada Crump3, David G. Davies4, Guangwen Zhou2,3, Ning Zhang1,5*, Congrui Jin3*

1 Department of Plant Biology, Rutgers University, New Brunswick, NJ 08901, USA
2 Materials Science and Engineering Program, Binghamton University, NY 13902, USA
3 Department of Mechanical Engineering, Binghamton University, NY 13902, USA
4 Department of Biological Sciences, Binghamton University, NY 13902, USA
5 Department of Biochemistry and Microbiology, Rutgers University, New Brunswick, NJ 08901, USA

*Corresponding authors: ningz@rutgers.edu; cjin@binghamton.edu

Abstract

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 mineral precipitation to fill the cracks in concrete. Although many species of fungi have been reported to be able to promote calcium mineralization, they have not yet been investigated in the application of self-healing concrete, thus an initial screening of different species of fungi has been conducted. The experimental results showed that, due to the leaching of Ca(OH)$_2$ from concrete, the pH of the growth medium increased from its original value of 6.5 to 13.0. Despite the drastic pH increase, Trichoderma reesei (ATCC13631) spores germinated into hyphal mycelium and grew equally well with or without concrete. We employed material characterization techniques including X-ray diffraction (XRD) and scanning electron microscope (SEM), both of which confirmed that the crystals precipitated on the fungal hyphae were composed of calcite.

1. Introduction

We use infrastructure to support and facilitate our daily lives – the roads we drive on, the bridges that carry traffic across, the office buildings we work in, the dams that provide the water we drink, and the tunnels that transport people and freight. Unfortunately, our nation is facing the downfall of progressively aging infrastructure that needs rehabilitation. The latest America’s Infrastructure Report Card issued by the American Society of Civil Engineers in 2017 has rated the overall condition of our nation’s infrastructure as D+. From 2016 to 2025, each household will lose $3,400 each year in disposable income due to infrastructure deficiencies. If this investment gap is not addressed by 2025, the economy is expected to lose almost $4 trillion in gross domestic product, resulting in a loss of 2.5 million jobs in 2025.

In particular, concrete infrastructure suffers from serious deterioration. Cracking in concrete is very common due to the effect of various chemical and physical phenomena, such as drying shrinkage, alkali-silica reaction, freeze-thaw cycles, sulphate attack, reinforcement corrosion, and fatigue, etc. Without immediate and proper treatment, cracks tend to expand further and eventually require costly repair. Durability of concrete is significantly impaired by these cracks, since they provide an easy path for the transport of liquids and gasses that potentially contain harmful substances. If micro-cracks grow and
reach the reinforcement, not only the concrete itself may be attacked, but also the reinforcement will be corroded when it is exposed to water and oxygen, and possibly carbon dioxide and chlorides, leading to structural failure.

Therefore, inspection and maintenance techniques for concrete infrastructure have become the focus of increasing attention. However, the implementation of continuous maintenance is difficult owing to the considerable amount of labor and funds required. Moreover, repair work may become painful or impossible to be executed because of the existing conditions such as the location of the damage in the affected structure. Some infrastructure, such as highways and tunnels, is in continuous service, and in such cases repair work becomes very challenging to accomplish in a timely or cost-effective manner. Under such circumstances, self-healing of harmful cracks without onerous labor and high costs could be of great attraction. This idea was originally inspired by the amazing ability of the human body to heal itself of cuts, bruises, and broken bones.

For the damaged skins and tissues, the host can assimilate nutrients to produce new substitutes healing the damaged parts. Accordingly, for the self-healing of cementitious materials, the essence is to provide necessary products which can then fill in the cracks when damage happens. With respect to how to endow composite materials with self-healing property and improve the self-healing efficiency, many experimental studies have been conducted and generated many innovative strategies during the past decades. To date self-healing mechanism in concrete has been established mainly through three strategies: autogenous healing, encapsulation of polymeric material, and microbial production of calcium carbonate.

Autogenous healing is the natural process of repairing concrete cracks in the presence of moisture or water. Cracks are filled through hydration of unhydrated cement particles or carbonation of dissolved calcium hydroxide. Hydration of calcium oxide produces calcium hydroxide, which can react with carbon dioxide present in the atmosphere, resulting in production of calcium carbonate. However, this autogenous healing is limited to small cracks (less than 0.2 mm) and requires the presence of water.

Encapsulation of polymeric material can fill cracks in concrete by conversion of healing agent to foam in the presence of moisture. Although releasing chemicals from incorporated hollow fibers inside concrete can fill the cracks, these materials do not behave the same as concrete compositions in many conditions, e.g., filler and crack have different thermal expansion rates, and in some cases, they cause to extend the existing cracks. More importantly, the embedded capsules have to protect the healing agent for a long period of time and must not influence the concrete workability and mechanical properties. These requirements make encapsulation method a difficult practice for commercial applications.

Due to the above-mentioned drawbacks of autogenous healing and encapsulation of polymeric material, the use of the biological repair technique based on the application of mineral-producing microorganisms becomes highly desirable, as it provides a safe, natural, pollution-free, and sustainable solution to the serious challenge. It has been known for a long time that microorganisms, such as bacteria, fungi, and yeasts, are able to produce a wide range of minerals such as carbonates, sulphides, silicates and phosphates. In the presence of a calcium source, calcium carbonate, as one of the most suitable fillers for concrete due to high compatibility with cementitious compositions, can be precipitated through biologically induced mineralization process. This microbial approach prevails the other self-healing...
techniques due to superior microcrack-filling capacity, strong bonding between filler and crack, high compatibility with concrete compositions, favorable thermal expansion, and sustainability\textsuperscript{27}. Successful implementation of this innovative method will result in sustainable and resilient infrastructure systems that continually repair themselves without the need for human interference.

Although many microorganisms can contribute to the production of calcium carbonate through biomineralization, to date, only bacteria have been effectively studied for the application of self-healing concrete. Although bacteria, and particularly acid-producing bacteria, have been traditionally considered as harmful organisms for concrete\textsuperscript{18}, recent research has shown that some ureolytic bacteria, such as \textit{Bacillus sphaericus} and \textit{B. pasteurii}, have the ability to precipitate calcium carbonate through urea hydrolysis and thus can be used as a powerful tool to heal the cracks\textsuperscript{8-12}. Although this approach has proven to be successful, there are some drawbacks. In this approach, for each carbonate ion two ammonium ions are simultaneously produced which may result in excessive environmental nitrogen loading. It is estimated that remediation of 1 m\textsuperscript{2} of concrete needs 10 g/L of urea which produces 4.7 g of nitrogen, about one third of the nitrogen that is produced by each person every day\textsuperscript{15}. Furthermore, the presence of excessive ammonium in the concrete matrix increases the risk of salt damage by conversion to nitric acid.

To avoid these drawbacks, metabolic conversion of organic compound to calcium carbonate has been proposed\textsuperscript{18-20}. In this approach, aerobic oxidation of organic acids leads to production of carbon dioxide which results in carbonate production in an alkaline environment. The existence of a calcium source then leads to the production of calcium carbonate. Compared to ureolysis pathway, this metabolic conversion is more sustainable due to the absence of ammonium. However, high concentrations of calcium source are required for calcite production\textsuperscript{29}, and this may result in 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.

Another pathway to produce minerals is known as dissimilatory nitrate reduction\textsuperscript{23}. Denitrification defines as a respiratory process that results in reduction of nitrate to nitrite, nitric oxide, nitrous oxide, and nitrogen gas. Minerals are precipitated through oxidation of organic compounds by the reduction of nitrate via denitrifying bacteria. The most significant attribute of this approach is its application in anaerobic zones. However, it has been shown that the efficacy of denitrification approach is much lower than ureolysis in respect to the production of calcium carbonate\textsuperscript{30}. As a very young but promising technology, the use of the biological repair technique based on the application of mineral-producing microbes has been extensively studied but still not fully understood. While microbe is a term that defines a wide range of organisms, studies on self-healing concrete are still limited to prokaryotes\textsuperscript{8-27}. Of course, using bacteria has many advantages. For example, bacteria are easy to culture and handle in a laboratory setting and are typically harmless to humans\textsuperscript{31}. Moreover, collection and isolation of bacteria are not very complex, as during the years numerous selective media have been introduced for direct isolation of bacteria\textsuperscript{32}. On the other hand, however, bacteria do not generally possess sufficient resistance to survive the deleterious environment such as high pH, high temperature, and dry condition of concrete. So far there has been little success with respect to the long-term healing efficacy and in-depth consolidation, mainly due to the limited survivability and calcinogenic ability of the bacteria. Furthermore, from the economical point of view, the production of bacteria-based self-healing concrete
currently results in considerable costs due to the need of aseptic conditions to produce the microbial spores and the use of expensive growth media, making this approach unlikely to be applied in practical applications\textsuperscript{33}. In summary, there are still huge challenges to bring an efficient self-healing product to the concrete market with the guaranty that this product can both attain legislative requirements and be cost-effective.

2. Fungi-Mediated Self-Healing Concrete

Due to the above-mentioned problems, further investigation on other types of microorganisms having the ability to catalyze calcium mineral precipitation becomes of great potential importance. The overarching goal of the current study is to explore a revolutionary self-healing concept in which bacteria are replaced by fungi to promote calcium mineral precipitation on cracks in concrete infrastructure. Fungi are the most species rich group of eukaryotic organisms after insects with the magnitude of diversity estimated at 1.5M to 3.0M species\textsuperscript{34}. Fungi have long been investigated regarding their importance in organic matter degradation, and their link to inorganic constituents was mainly restricted to mineral nutrition through mycorrhizal symbiosis, mineral weathering of lichens as well as production of mycogenic oxalates. Although recent studies in the field of geomycology have demonstrated that fungi play an important role in calcium carbonate biomineralization, so far, to our knowledge, none of them have been studied for the application of self-healing concrete. The current study is driven by the following three hypotheses.

(1) It is hypothesized that some species of fungi are able to better adapt to the harsh conditions of concrete including high alkalinity, low humidity, and severe oxygen and nutrient limitation. Fungi are known for their remarkable ability to survive extreme environments such as those characterized by high salinity, high radiation, intense ultraviolet light, limited nutrients, extreme temperatures and pressures, and variable acidity\textsuperscript{35-48}. For example, some can be found in the Arctic and Antarctic cold deserts\textsuperscript{36}, the Sahara Desert in Africa\textsuperscript{37}, the hypersaline Dead Sea\textsuperscript{38}, as well as the deep-sea sediments of the Indian Ocean at depths of about 5000 m\textsuperscript{39}. Some can even survive the intense ultraviolet light and cosmic radiation encountered during space travel\textsuperscript{40}. Most relevantly, rocks are often considered as an extreme environment for fungi due to limited nutrient availability, exposure to ultraviolet light, and moisture deficit, but fungi have been reported in a wide range of substrates including sandstone, granite, limestone, marble and gypsum, where they constitute an important component of both epilithic and endolithic microbial munities\textsuperscript{41-48}. The ability of most fungi to form hard spores that can survive almost any natural environment is another important fungal adaptation\textsuperscript{49}. When exposed to environmental stresses such as nutrient deprivation, they produce dormant and highly resistant cells termed spores that can survive in this dormant state for long periods of time, waiting for more favorable conditions. Faced with the challenge of surviving prolonged periods of dormancy, spores have evolved various mechanisms to survive environmental assaults that would normally kill the fungi, and as a result, these spores can endure many years of hardship. For example, in 2004, spores from a fungus that lived roughly 400,000 years ago were germinated in a laboratory in India\textsuperscript{50}.

(2) It is hypothesized that some species of fungi are able to promote calcium mineralization in the harsh environment of concrete. According to Verrecchia\textsuperscript{51}, many near-surface limestones, calcic and petrocalcic horizons in soils are often secondarily cemented and indurated with calcite and whewellite. Although this phenomenon has partly been attributed to physico-chemical processes, the presence of calcified fungal filaments in limestone and calcareous soils from a wide range of localities implies that fungi may play a
prominent role in secondary calcite precipitation\textsuperscript{62-57}. It is widely known that oxalate salts, particularly whewellite and weddelite commonly occur in association with fungal hyphae in soils and leaf litter, as well as in lichen thalli\textsuperscript{58,59}. Verrecchia et al. proposed that oxalate can be degraded to carbonate, such as in semi-arid environments, where such a process may act in the cementation of preexisting limestones\textsuperscript{60}. The formation of biogenic fabrics in limestone by two fungi, \textit{Serpula himantoides} and a polymorphic fungal isolate from limestone identified as a \textit{Cephalotrichum} sp., was investigated by Burford et al.\textsuperscript{61}. X-ray diffraction of crystalline precipitates on the hyphae of \textit{S. himantoides} showed they were composed of a mixture of calcite and calcium oxalate monohydrate, and the crystals precipitated on the hyphae of the limestone isolate were composed solely of calcite or of calcite with some calcium oxalate dehydrate. These results provided direct experimental evidence for the precipitation of calcite and also secondary mycogenic minerals on fungal hyphae in low nutrient calcareous environments.

(3) It is hypothesized that using fungi in biogenic crack repair is more effective than bacteria due to their extraordinary ability to both directly and indirectly promote calcium mineralization. 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\textsuperscript{62-65}. Although the peculiar mechanisms leading to calcium mineralization by fungi remains incompletely understood, but it is widely believed that there are several different processes involved in the calcification of fungal filaments including the nucleation of calcite onto fungal cell walls and the formation of calcite through fungal excretion of hydrogen ions and/or organic acids which act in dissolution of the host rock\textsuperscript{66-68}. Cation binding by fungi can occur through metabolism-independent binding of ions onto cell walls and other external surfaces and is an important passive property of fungal biomass, leading to nucleation and deposition of mineral phases\textsuperscript{66,67}. Bound calcium cations could interact with soluble carbonate resulting in calcium carbonate deposition on the hyphae. Calcite formed in the aqueous phase could also nucleate onto the hyphae. Since the biosorption of metal ions onto fungal cell walls is a metabolism-independent process, dead and metabolically inactive fungal hyphae can also act as nucleation sites of further calcium carbonate precipitation\textsuperscript{68}. 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\textsuperscript{69}. According to Manoli et al., chitin is a substrate that significantly lowers the required activation energy barrier for nucleus formation so that calcite can readily nucleate and subsequently grow on it\textsuperscript{70}. In other words, the interfacial energy between the fungi and the mineral crystal is much smaller than the interfacial energy between the mineral crystal and the solution. On the other hand, the excretion of organic acids, particularly oxalic acid, by fungal hyphae plays an important role in the re-precipitation of secondary calcium-bearing minerals in the environments abundant in calcium carbonate\textsuperscript{51}. Oxalic acid produced by \textit{Aspergillus niger} grown in agar medium has been shown experimentally to react with calcium ions and calcium carbonate to form calcium oxalate by Sayer et al.\textsuperscript{71}. \textit{A. niger} and \textit{S. himantoides} were shown to precipitate calcium oxalate when cultured on agar medium amended with gypsum by Gharieb et al.\textsuperscript{72}. The fungal excretion of oxalic acid and the precipitation of calcium oxalate may result in the dissolution of the internal pore walls of the limestone matrix so that the solution becomes enriched in free carbonate. During passage of the solution through the pore walls, calcium carbonate recrystallizes as a result of a decrease in carbon dioxide, contributing to hardening of the material\textsuperscript{60}. Biodegradation of oxalate as a result of microbial activity can also lead to transformation into carbonate, resulting in precipitation of calcite in the pore interior, leading to closure of the pore system and hardening of the chalky parent material\textsuperscript{61}. During decomposition of fungal hyphae, calcite
crystals are released to act as sites of further secondary calcite precipitation\textsuperscript{51,60}. The carbon dioxide production results from both oxalate oxidation and fungal respiration can cause carbonate concentration in the local environment and thus favor more calcium carbonate precipitation. According to Verrecchia et al., a large amount of the secondary calcium carbonate found in soils and surficial sediments originates by such a process\textsuperscript{73}.

To test the hypotheses, this work presents a pilot study to investigate the feasibility of using fungi to promote calcium mineral precipitation to heal cracks in concrete infrastructure. Although many species of fungi have been reported to be able to promote calcium mineralization\textsuperscript{71-77}, they have never been investigated in the application of self-healing concrete, thus a wide screening of different species of fungi will be conducted.

3. Materials and Methods

The following criteria will be used to select the candidates of fungi for self-healing concrete. (1) They should be as eco-friendly and nonpathogenic as possible, i.e., pose no risk to human health and are appropriate to be used in concrete infrastructure. (2) The matrix of young concrete is typically characterized by pH values between 11 and 13 due to the formation of Ca(OH)$_2$, which is after calcium-silica-hydrate (C-S-H) quantitatively the most important hydration product. Fungi added to the concrete mixture thus do not only have to resist mechanical stresses due to mixing but also should be able to withstand a high alkalinity for prolonged periods. Most promising fungal agents therefore should likely be alkaliphilic spore-forming fungi. The fungal spores, together with nutrients, will be added into concrete during the mixing process. When cracks appear and water finds its way in, the dormant fungal spores will germinate, grow, and precipitate CaCO$_3$ to heal the cracks \textit{in situ}. When the cracks are completely filled 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 up again. (3) It is preferred if the genomes of the fungi have been sequenced and are publicly available so that the can be genetically manipulated to enhance their performance in crack repair.

Besides genetically engineered fungi, alkaliphilic fungi could also be found in nature. Through their evolution over millions of years, fungi have developed different primary strategies to survive and prosper in different environments. Many species of fungi can grow in alkaline environments where the pH value can often be consistently at about $10^{78}$. For example, \textit{Paecilomyces lilacinus} is described as being alkaliphilic and able to grow well between pH 7.5-10.0\textsuperscript{79}. Alkaliphilic \textit{Chrysosporium} spp. isolated from bird nests have a pH maximum for growth of $11^{78}$. In the current study, the best sources from which alkaliphilic fungi can be isolated will be examined and the field collection will be conducted.

To investigate whether and how fungal hyphae could promote CaCO$_3$ precipitation in concrete, we here will employ material characterization techniques including X-ray diffraction (XRD) and scanning electron microscope (SEM). XRD is an established technique to study mineral crystal structures, which has been extensively used to identify biominerals at fungi-mineral interfaces\textsuperscript{79,80}. SEM has been used for the surface visualization of fungal precipitates\textsuperscript{81,82}, and in this study SEM will be used to characterize the morphology and composition of the solid precipitates.

3.1 Isolation of Fungal Strains
The following six species of fungi have been selected and used for this pilot study: *Trichoderma reesei* (ATCC13631), *A. nidulans* (ATCC38163), *Cadophora interclivum* (BAG4), *Umbeliopsis dimorpha* (PP16-P60), *Acidomelanla panicicola* (8D), and *Pseudophialophora magnispora* (CM14-RG38). *T. reesei* and *A. nidulans* were purchased from the American Type Culture Collection (ATCC). The advantages of using *T. reesei* and *A. nidulans* are their well-understood genetics and a large range of mutants which are affected in a variety of metabolic pathways.

The other four fungal strains were isolated from the roots of plants that grew in nutrient poor soils. Native roots of pitch pine (*Pinus rigida*), rosette grass (*Dichanthelium acuminatum*), and switchgrass (*Panicum virgatum*) were collected from the New Jersey Pine Barrens in 2011, 2014, and 2016, respectively, and the Sprengel’s sedge samples (*Carex sprengelii*) were collected from a subalphine forest in Canadian Rocky Mountains in the province of Alberta, Canada in 2015. The New Jersey Pine Barrens represents one of a series of barrens ecosystems along the eastern seaboard of the United States and similar ecosystems around the world. The podzolic soil in this region is sandy, dry, and nutrient poor. However, while lacking nutrients, this habitat supports numerous species that have adapted to the harsh environment. Scarcely attention has been received on the studies of fungi in the pine barrens, and much remains unknown about fungal functions in this ecosystem. The soils in the subalphine forest are generally poorly developed, stony, shallow, and have low moisture-holding capacities. The pH of these soils is generally around 8.1 and moisture availability to plants is low. In this study, new fungal species uncovered from both the pine barrens and the subalphine forest will be tested in the application of self-healing concrete.

The collected plant root samples were kept on ice and transported to the laboratory for fungal isolation within 24 h. The roots were rinsed in tap water to remove soil particles and cut into 10-to-20-mm long segments. The segments were surface sterilized with 95% ethanol for 30 s, followed by 2 min in 0.6% sodium hypochlorite, 2 min in 70% ethanol, and two final rinses in sterile distilled water. Samples were further cut into 3-mm long small segments, air dried, and placed on 2% malt extract agar. Samples were incubated at room temperature and observed daily in the first two weeks, and then twice a week afterwards for 6 months. Fungal cultures were isolated and purified by subculturing from emergent hyphal tips. Spore morphology, if present, and colony characteristics were examined and recorded as morphological data for identification. Fungal cultures have been preserved at -80 °C in glycerol and 4 °C on agar.

### 3.2 Identification of the Isolated Strains

Potato dextrose agar (PDA) that is nutritionally rich in carbohydrates and can stimulate vegetative growth of most fungi was chosen as growth medium. Fungal cultures were grown on PDA (Difco, BD Diagnostic Systems, Sparks, MD, USA) for 7 days. Genomic DNA was extracted from fungal mycelium with the UltraClean Soil DNA Isolation Kit (MoBio Laboratories, Carlsbad, CA, USA) according to the manufacturer’s instructions. The nuclear ribosomal internal transcribed spacer (ITS) gene, the universal fungal barcode marker, was amplified using the primers ITS1 and ITS4. PCR was performed with Taq 2X Master Mix (New England BioLabs, Maine, MA, USA) according to the manufacturer’s instructions. PCR cycling conditions for the ITS consisted of an initial denaturation step at 95 °C for 3 min, 35 cycles of 95 °C for 45 s, 52 °C for 45 s, 72 °C for 1 min and a final extension at 72 °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 stains 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 ratio by mass was 0.5 and the sand to cement ratio by mass was 3. The specimens were made according to the procedure in the standard NBN EN 196-1. They were then poured into 60 mm Petri dishes (9 ml per dish) and cured at 100% relative humidity and 22 °C for 7 days.

Cement paste specimens were 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 1, 3, 5, 7, 14, or 28 days.

Air-entrained cement paste specimens were prepared to investigate the effect of air-entraining on the pore size distribution of the specimens. Ordinary Portland Cement (CEM I 52.5N) was mixed with tap water containing the air-entraining agent in a water-to-cement weight ratio of 0.5. Eucon AEA-92 (Euclid Chemical, Cleveland, OH, USA) was dosed at a rate of 100 mL to 260 mL per 100 kg of the total cementitious material. 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.

### 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. Radial growth measurements were recorded along two perpendicular diameters. The fungal growth was also evaluated via optical microscopy. The fungal samples were prepared by the tape touch method and observed with an optical microscope (Carl Zeiss model III, Zeiss, Jena, Germany).

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.

### 3.5 pH Measurements
The pH of the growth medium in each plate was determined by taking five measurements using an Orion double junction pH electrode (Thermo Fisher Scientific, Waltham, MA, USA). pH measurements were recorded after the plates were incubated for three weeks.

### 3.6 Microscopic Characterization of Biominerals Produced by Fungi

#### 3.6.1 X-Ray Diffraction (XRD)

The solid precipitates associated with fungal hyphae (from the *T. reesei* cases of CPDA30 and CMPDA30) were identified by XRD analysis. A Siemens-Bruker D5000 powder diffractometer with Cu-Kα radiation in the theta/theta configuration was used for measurements. 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 precipitates was done by dissolving the fungi in NaOCl and repeated washings in water and methanol according to the published protocol.

#### 3.6.2 Scanning Electron Microscope (SEM)

The solid precipitates associated with fungal hyphae (from the *T. reesei* cases of CPDA30 and CMPDA30) and the NaOCl-isolated crystals used in XRD analysis were examined 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 determined by SEM-EDS analysis.

#### 3.7 Pore Size Distribution of Cement Paste Specimens

To determine the pore size distribution in aged cement paste specimens, the mercury intrusion porosimetry (MIP) method was used to measure the matrix pore sizes in 1, 3, 5, 7, 14, and 28 days cured cement paste specimens using a Model AMP-30K-A-1 (Porous Materials, Ithaca, NY, USA). Aged cement paste specimens were cut to smaller pieces with a chisel with fragment sizes about 0.5 cm × 0.5 cm × 0.5 cm, which were frozen in liquid nitrogen and subjected to cryo-vacuum evaporation for at least two weeks for pore water removal prior to MIP tests. MIP tests were conducted according to the published protocol.

### 4. Results and Discussion

#### 4.1 Identification of BAG4, PP16-P60, 8D, and CM14-RG38

Strain PP16_P60 was isolated from the pitch pine in the Pygmy Pine Plains of the New Jersey Pine Barrens. It has 100% ITS sequence similarity to *Umbeliopsis dimorpha* ex-type culture CBS110039 (NR_111664), and thus it is identified as *Umbeliopsis dimorpha*. BAG4 was recovered from the Sprengel’s sedge samples (*Carex sprengelii*) collected from a subalphine forest in Canadian Rocky Mountains in the province of Alberta, Canada. The blast result indicates its phylogenetic position in the genus *Cadophora*, and it is further identified as *Cadophora interclivum* based on multigene and morphological analyses. 8D is associated with switchgrass roots in the New Jersey Pine Barrens. It has 92%
or less ITS sequence (KF874619) similarities to any known or described species with accessible ITS sequences in GenBank, such as *Mollisia fusca* CBS486.48 (AY259137) and *Loramyces macrosporus* AFTOL-ID 913 (DQ471005), and further identified as *Acidomelania panicicola*. CM14_RG38 was found from the rosette grass in Collier Mills in the New Jersey Pine Barrens. Its ITS sequence (KP769835) has 96% or less similarities to other *Pseudophialophora* species, such as *Pseudophilophora eragrostis* CM12m9 (KF689648) and *Pseudophilophora whartonensis* WSF14RG66 (KP769834), and it is identified as *Pseudophialophora magnispora*.

4.2 Fungal Growth in the 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 1. The pH measurement results, as listed in Table 2, have shown that, due to the leaching of Ca(OH)$_2$ from concrete, the pH of the growth medium in the cases of CPDA30 and CPDA22 increased from 6.5 to 13.0. Only *T. reesei* (ATCC13631) has been found to be able to grow well on the concrete plates. At 30 °C, its growth rates reached 2.6 mm/day in the cases of both CPDA30 and CMPDA30. Abundant conidia were observed from the concrete plates and had similar morphology compared to those produced on the plates without concrete, i.e., the cases of PDA30, PDA22, MPDA30, and MPDA22. However, no growth of *T. reesei* was found on any concrete plates at 22 °C, i.e., the cases of CPDA22 and CMPDA22. The other five species had no growth on any concrete plates, although they grew on most of the non-concrete plates. The MOPS buffer significantly decreased the fungal growth, which is probably due to the relatively high concentration used in the experiments. Agar plug controls without any inoculum showed no fungal growth.

*Table 1. Average growth rates (mm/day) of the six fungi species on PDA, MPDA, CPDA, and CMPDA at 22 °C and 30 °C, respectively, at day 21 after inoculation (n = 6).*

|                       | PDA30 | PDA22 | MPDA30 | MPDA22 | CPDA30 | CPDA22 | CMPDA30 | CMPDA22 |
|-----------------------|-------|-------|--------|--------|--------|--------|---------|---------|
| *Trichoderma reesei* (ATCC13631) | 2.6 a  | 2.6 a  | 1.0 b  | 0.8 b  | 2.6 a  | 0      | 2.6 a   | 0       |
| *Aspergillus nidulans* (ATCC38163) | 2.6 a  | 2.6 a  | 0.5 b  | 0.7 b  | 0      | 0      | 0       | 0       |
| *Cadophora interclivum* (BAG4) | 0.6 b  | 2.1 a  | 0      | 0.6 b  | 0      | 0      | 0       | 0       |
| *Umbeliopsis dimorpha* (PP16-P60) | 2.6 a  | 2.6 a  | 1.0 b  | 1.0 b  | 0      | 0      | 0       | 0       |
| *Acidomelania panicicola* (8D) | 2.1 a  | 1.9 a  | 0.9 b  | 0.9 b  | 0      | 0      | 0       | 0       |
| *Pseudophilophora magnispora* (CM14-RG38) | 2.6 a  | 2.1 a  | 0      | 0      | 0      | 0      | 0       | 0       |
Table 2. pH measurement results of the six fungi species on PDA, MPDA, CPDA, and CMPDA at day 21 after inoculation.

| Species                        | PDA | MPDA | CPDA | CMPDA |
|-------------------------------|-----|------|------|-------|
| Control                       | 5.1 | 6.8  | 13.1 | 11.5  |
| *Trichoderma reesei*<br>(ATCC13631) | 6.5 | 7.2  | 13.0 | 11.9  |
| *Aspergillus nidulans*<br>(ATCC38163) | 6.8 | 7.1  | 12.2 | 10.9  |
| *Cadophora interclivum*<br>(BAG4) | 6.3 | 7.1  | 12.0 | 11.4  |
| *Umbeliopsis dimorpha*<br>(PP16-P60) | 6.1 | 7.1  | 12.1 | 11.3  |
| *Acidomelania panicicola*<br>(8D) | 6.8 | 7.1  | 12.6 | 11.9  |
| *Pseudophialophora magnispora*<br>(CM14-RG38) | 6.9 | 7.0  | 12.0 | 11.0  |
Figure 1. T. reesei spores germinated on concrete into hyphal mycelium and grew equally well with or without concrete. In comparison, the other five species did not grow on concrete.
Figure 2. Microphotographs of optical microscopy (1000X, Carl Zeiss model III) showing that T. reesei spores germinated on concrete into hyphal mycelium and grew equally well with or without concrete.

4.3 Identification and Morphology of the Fungal Precipitates
The results from XRD analysis are shown in Fig. 3. The data strongly suggested that *T. reesei* hyphae can promote calcium carbonate precipitation. For the precipitates associated with the fungal hyphae, the sharp peak at around 30° 20 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 is the result of the dissolution of CO₂ in the concrete pore fluid and its reaction with Ca(OH)₂.

![XRD results for crystalline precipitates collected from mortar specimens cured with and without *T. reesei* hyphae. For comparison, reference diffractograms of quartz (SiO₂) and calcite (CaCO₃) mineral standards from the International Centre for Diffraction Data (ICDD) are included.](image)

As shown in the SEM images in Fig. 4, a large amount of crystals grew in the *T. reesei*-inoculated medium. The crystals showed evidence of fungal involvement. Wire-shaped traces having an average
thickness of 2 µm to 3 µm were found on the surface of the crystals, which presumably occurred in the space occupied by the fungi. These traces on the crystals also suggested that fungal hyphae served as nucleation sites during the mineralization process. Element composition analysis via EDS revealed that the crystal is primarily composed of calcium, carbon, and oxygen with a weight ratio closely matching that of CaCO₃, indicating that the crystal is CaCO₃ (the EDS spectra of the pure calcium carbonate crystals show 20 at.% of calcium ions, 20 at.% of carbon ions, and 60 at.% of oxygen ions, i.e., Ca:C:O = 1:1:3). In sharp contrast to the fungi-inoculated medium, the amount of formed crystals in the fungi-free control medium was much less. Furthermore, in the control medium, no sign of fungi involvement was observed during the production of crystals.

Figure 4. SEM and EDS spectra of the calcium carbonate precipitation in the T. reesei-inoculated medium.

4.4 Discussion on Embedment of Healing Agent 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, resulting not only in loss of viability but also in decreased mineral-forming capacity. As we measured by using the mercury intrusion porosimetry (MIP) method, the matrix pore diameter sizes in 28 days cured specimens decreased to less than 0.1 μm, as shown in Fig. 5(a), which cannot accommodate fungal spores with typical diameters larger than 3 μm, as shown in Fig. 6. Therefore, encapsulation or immobilization of fungi spores in a protective carrier becomes essential. Moreover, air-entraining agents could be utilized to create extra air voids in concrete matrix to facilitate the housing of the fungal agents. The matrix pore diameter sizes in 28 days cured air-entrained specimens are shown in Fig. 5(b). It can be seen that the amount of entrained air voids increases with increasing amount of air-entrained agents.

![Figure 5](image1.png)

**Figure 5.** (a) Effect of curing time on pore size of cement paste specimens prepared with a water-to-cement weight ratio of 0.5 determined by MIP tests. (b) Effect of the amount 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. MIP tests were conducted using a Model AMP-30K-A-1(Porous Materials, Ithaca, NY, USA).

![Figure 6](image2.png)

**Figure 6.** The diameter of T. reesei spores (round to oval in shape) used in the experiments appeared to be typically in the range of 3.5 μm to 4.5 μm.
5. Concluding Remarks

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. Although many species of fungi have been reported to be able to promote calcium mineralization, they have never been investigated in the application of self-healing concrete, thus an initial screening of different species of fungi has been conducted. The experimental results showed that, due to the leaching of Ca(OH)$_2$ from concrete, the pH of the growth medium increased from its original value of 6.5 to 13.0. Despite the drastic pH increase, the microscopic analysis showed that T. reesei (ATCC13631) spores germinated into hyphal mycelium and grew equally well with or without concrete. In comparison, A. nidulans (ATCC38163), C. interclivum (BAG4), U. dimorpha (PP16-P60), A. panicicola (8D), and P. magnispora (CM14-RG38) did not grow on concrete. To investigate whether and how fungal hyphae could promote CaCO$_3$ precipitation in concrete, we employed material characterization techniques including XRD and SEM, both of which confirmed that the crystals precipitated on the fungal hyphae were composed of calcite.

This research will also benefit many other applications as well, such as metal remediation, carbon sequestration, and enhanced oil recovery. (1) The discharge of heavy metals from mining and metal-processing industries has resulted in serious contamination in soil and groundwater. Conventional methods, such as chemical reactions, ion exchange, and membrane technologies are either ineffective or costly. In comparison, microbial CaCO$_3$-based coprecipitation offers a cost-effective and eco-friendly solution. This study will be helpful in providing insight on how fungi could effectively bind metal ions. (2) In recent years, CO$_2$ sequestration has become an attractive choice to mitigate CO$_2$ emission. Compared with geological sequestration, which has a potential risk of upward leakage of CO$_2$ through fractures, disturbed rock, or cement lining near injection wells, microbial CO$_2$ fixation offers an inexpensive, low-risk, and economically sustainable storage strategy. The current research will advance our understanding of how fungi could be used in such applications. (3) Enhanced oil recovery has become necessary to improve the overall extraction percentage of crude oil. However, the thermal or chemical flooding processes associated with enhanced oil recovery are environmentally hazardous and extremely expensive. In comparison, microbial enhanced oil recovery, in which microbes are used to plug high-permeability zones for a redirection of waterflood, therefore improving the yield of reservoir oil, offers a cost-effective and eco-friendly strategy. The fundamental knowledge advanced by the current study is essential to understand the role of fungi in enhanced oil recovery.

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