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Role of Fungi in the Formation of Patinas on Feilaifeng Limestone, China

Tianxiao Li[1] · Yulan Hu[1] · Bingjian Zhang[1] · Xiaoru Yang[2]

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Abstract

Feilaifeng is a cultural heritage site that contains unique Buddhist statues which date back to the Five Dynasties period (907 AD–960 AD). The site was inscribed on world heritage list by UNESCO in 2011. Various patinas, which may be caused by fungi, have covered the surface of the limestone and have severely diminished the esthetic value of the statues and altered the limestone structure. Culture-dependent method was used to isolate and identify the fungi. After incubation on modified B4 medium, the calcifying fungi were identified by optical microscopy and scanning electron microscopy combined with X-ray energy-dispersive analysis. *Aspergillus*, *Penicillium*, and *Colletotrichum* were observed as the biomineralizing fungi. X-ray diffraction showed that the patina consisted of calcite ($CaCO_3$), but the crystals synthesized by the identified fungi were whewellite ($CaC_2O_4{\cdot}H_2O$) for *Aspergillus* and *Penicillium*, and vaterite ($CaCO_3$) for *Colletotrichum*. In addition, the metabolites of *Colletotrichum* suppressed the transformation of vaterite to calcite, but Mg^{2+} could inhibit the function of the metabolites. The different crystal form between the patina and the products of fungi may suggest two different pathways of patina formation and provide important reference data for studies of the mechanisms of biomineralization, cleaning of the patina, and protection of the Feilaifeng statues.

Keywords Biodeterioration · Fungi-induced mineral precipitation · Transformation between calcite and vaterite · Feilaifeng limestone

Introduction

Outdoor immovable stonework is prone to severe deterioration. Microorganisms including bacteria, fungi, archaea, algae, and lichens are major causes of damage of stone monuments and have received serious attention from conservators and conservation scientists. Fungi have a wide range of habitats including sandstone, granite, limestone, marble, and gypsum and play an important role in the biodeterioration of cultural heritage statues and buildings [1, 2]. The excretion of H^+ and organic acid by fungi can enhance the dissolution of stone or the chelating of metal ions [3]. The ions generated by the bacterial or fungal activity can precipitate as calcium oxalate (CaC_2O_4) or calcium carbonate ($CaCO_3$) on the surface of stone as secondary minerals, which is termed patina [4–7].

Although photosynthetic organisms and bacteria play a significant role in calcrete formation, fungi may also play a crucial and more dominant role in carbonate transformation [5, 8–10]. Oxalate salts such as calcium oxalate and whewellite ($CaC_2O_4{\bullet}H_2O$) commonly occur in association with fungal hyphae and oxalic acid secreted by fungi in soils [11, 12]. Calcium oxalate can be transformed into calcium carbonate by oxalotrophic bacteria, which indicates a potential role of fungi in the precipitation of calcium carbonate [13, 14]. In addition, $CaCO_3$ can be directly synthesized by fungi, and there are two crystal types, calcite and vaterite, in the precipitated calcium carbonate [8, 15, 16]. It is reported that vaterite is a metastable polymorph of $CaCO_3$, an intermediate product of crystallization, and finally transforms to a stable form, calcite. However, biological metabolites, such as amino acid, protein, and sugar, can act as additives to control the shape and crystal polymorphology of precipitated calcium carbonate [17], which may keep the vaterite stable. These deposited

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✉ Bingjian Zhang
zhangbiji@zju.edu.cn

[1] Department of Cultural Heritage and Museology, Zhejiang University, Hangzhou 310028, China

[2] Monitoring and Management Center of Hangzhou West Lake World Cultural Heritage, Hangzhou 310007, China

secondary minerals may protect the stone from environmental damage due to their relative insolubility. However, they can cause esthetic damage and deterioration of the stone surface [9, 10].

Feilaifeng is located near West Lake in Hangzhou, China, and is the site of Buddhist statues that began during the Five Dynasties period (907 AD–960 AD). It was added to the UNESCO World Heritage List in 2011 for its graceful landscape and historical importance. The subtropical monsoon climate combined with raininess and high humidity is ideal for the deposition of growth of microorganisms on the statues, which may contribute to the ongoing deterioration of the cultural heritage [18]. It showed that the most obvious esthetic and structural damage was due to the patina on the surface. Our previous studies showed that *Crossiella*, *Rubrobacter*, *Bryobacter*, and *Sphingomonas* were the dominant bacterial community in the Feilaifeng limestone [19], but all of them did not belong to sulfate-reducing bacteria, organic acid-degrading bacteria, or the urease-secreting bacteria which were reported as the calcifying bacteria [20]. In addition, fungi also contribute to the biomineralization of calcite carbonate in a few studies. So, we considered the formation of the patina on the Feilaifeng limestone which may relate to fungi.

The causes and components of patina are diverse and complex [4–7]. To devise strategies that will be helpful in the conservation of the Buddhist statues in Feilaifeng, the present study analyzed the mineral composition of patina, isolated and identified the fungal community from the crust of stone with culture-dependent methods, and assessed the role of fungi in the formation of the encrustations. The results will help clarify the mechanisms of the formation of patina in Feilaifeng and provide useful data for conservators to formulate a way to clean the patina or prevent its growth.

Methods

Sampling Site and Sampling Collection

Feilaifeng is located near West Lake in China. It has a subtropical monsoon climate with an average annual temperature and relative humidity of 17.8 °C and 70.7%, respectively, and rainfall averages 1454 mm annually. The stones rich in patina located near the statues of the fifty-ninth and sixty-seventh niches were selected as sampling site and marked FLF59 and FLF67, respectively (Fig. 1).

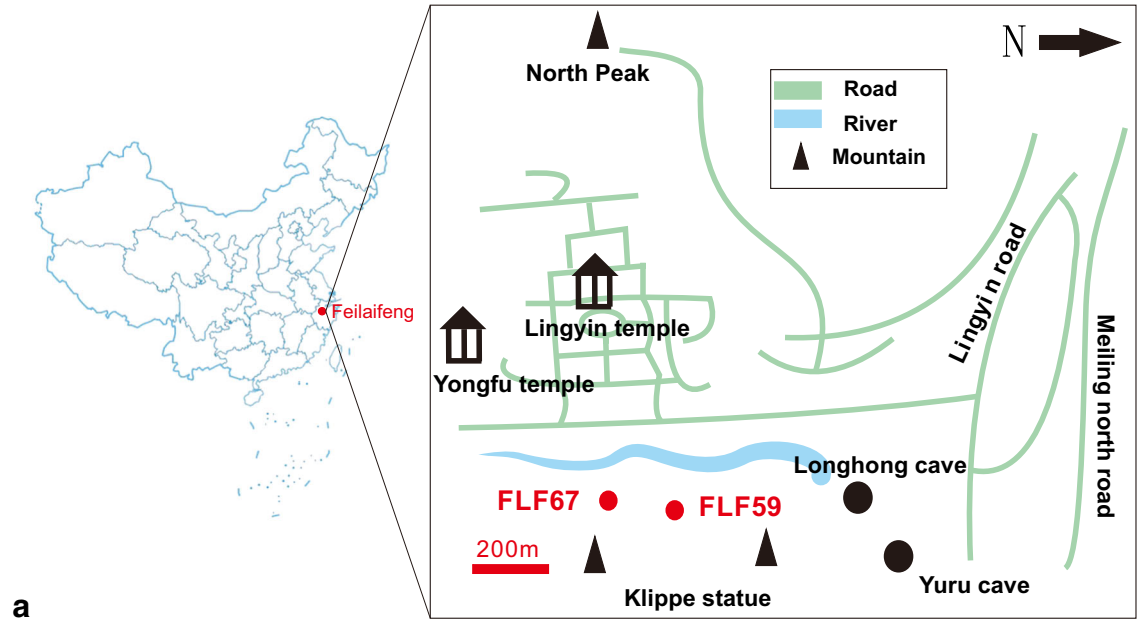
For each site, three independent patinas were randomly chosen and carefully collected with sterile scalpels (Fig. 1). Only a tiny amount of sample was collected each time due to the small area of patina, so the three samples collected from one site were mixed together. All the procedures of sampling followed strictly aseptic conditions and the samples were stored at 4 °C until further analysis. Each sample was divided into two parts: one was used for culturing and isolation of fungi and the other for chemical analysis by X-ray diffraction (XRD).

Isolation and Screening of Fungi

Fungi were isolated by two ways, one was the dilution plate method and the other was direct inoculation. For the dilution plates, a 100-mg sample was suspended in a sterilized tube with 1 ml deionized water. After blending with a pipette, the solution was subjected to serial dilution from 10^{-1} to 10^{-5}, and 100-μl aliquots of each dilution was inoculated on a PDA medium. For direct inoculation, particles of patina were sprinkled over the surface of the PDA medium. All samples were incubated on PDA in 90-diameter Petri dishes and grown at 28 °C in the dark for 4 days. Every experiment was conducted in triplicate.

Molecular Identification of Fungi

The isolated fungi were harvested by scraping and removing from the surface of the media. Identification involved PCR amplification and sequencing of the internal transcribed spacer (ITS) region for fungi. Total genomic DNA was extracted with the Power Soil® DNA Isolation Kit (MO BIO Laboratories, Inc., Carlsbad, CA, USA) according to the manufacturer protocols. The ITS region was amplified with the primers ITS1 (5′-TCCG TAGGTGAACCTGCGG-3′) and ITS4 (5′-TCCT CCGCTTATTGATATGC-3′). The procedures of PCR were: 5 min at 95 °C for initial denaturation, followed by 35 cycles of 1 min at 94 °C, 1 min at 55 °C and 1 min at 72 °C, and a final extension of 10 min at 72 °C. The PCR products were purified using a Gel Extraction Kit (Tiangen Co., Beijing, China) and the sequencing progress was performed by Genewiz (Jiangsu, China). Each sequence was compared with sequences from the GenBank database of the National Center for Biotechnology Information (NCBI) using the BLAST program. The most similar sequences that identify more than 98% were extracted from the GenBank database and a phylogenetic tree using the neighbor-joining method with the MEGA 7.0.14 software was constructed to clarify the species of the isolated fungi.



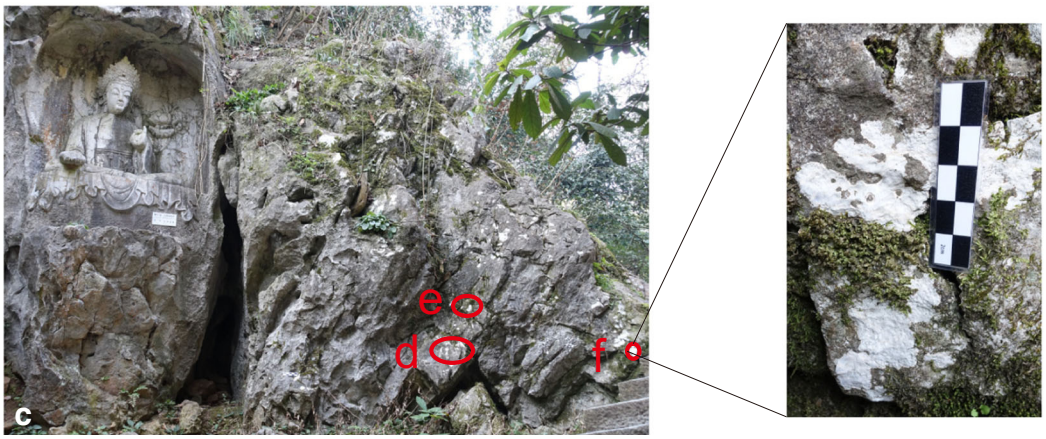


Fig. 1 Sampling site locations in Feilaifeng. **a** The geographical location of Feilaifeng and the sampling sites in China. **b**, **c** The sampling site of fifty-ninth and sixty-seventh niches in Feilaifeng, respectively. Samples a–c were collected from the fifty-ninth niche; they were mixed and named as FLF-59. Similarly, d–f were collected from the sixty-seventh niche and pooled into a sample as FLF-67

Identification and Cultivation of Calcium Salt Precipitation Fungi

Modified B4 medium consisting of 2.5 g calcium acetate, 4 g yeast extract, 10 g glucose, and 18 g agar per liter of deionized water was used to identify fungi capable of carbonate precipitation according to the widely used in previous studies about calcifying microorganisms and its components cannot affect the nucleation, growth, or transformation between the polymorphs of calcium carbonate [21]. After incubation at 28 °C in dark for 4 days, four fungi that could precipitate Ca^{2+} were chosen through the observation of crystals using optical microscopy (Fig. S1 in the Supplement). The selected fungi were incubated in the liquid-modified B4 medium on a rotary shaker at 150 r/min and 28 °C for 4 days. The pH of the solution was measured and biomass was harvested, washed with deionized water, and dried in a desiccator. Then, half of the biomass was grinded to a fine powder in a mortarfor XRD and the other was observed by scanning electron microscopy energy-dispersive X-ray spectroscopy (SEM-EDS).

The mineralogy of the samples of patina and minerals precipitated by selected fungi was determined using an X-ray polycrystal diffractometer (Rigaku D/Max 2550, Japan). The powder samples were firmly compacted on the reverse side of an aluminum specimen holder held against a glass side. Samples were analyzed over the range 10–80° 2θ at a scan rate of 1°/min in 0.1° increments.

The fungal biomasses cut into 2×2 mm pieces were mounted on a double-sided carbon adhesive tape on aluminum stubs. Samples were examined by SEM using a SU-8010 microscope (Hitachi, Tokyo, Japan) and a SIRION-100 device (FEI, Eindhoven, The Netherlands) equipped with an energy-dispersive spectroscopy (EDS) probe. Specimens were analyzed with an accelerating voltage of 25 kV for SEM and EDS.

Influence of Fungi on the Transformation Between Crystal Forms of Calcium Carbonate

FLF67-l was chosen for the evaluation of the role of fungi in the transformation between calcite and vaterite, since it is in the same genus as of FLF67-a and produces the larger amounts of biomass. The 4-day medium of FLF67-l was used to prepare aqueous $CaCl_2$ and Na_2CO_3 solutions, in which deionized water was the solvent of control. Calcium carbonate was precipitated by rapid pouring of 10 ml 0.1 mol/l Na_2CO_3 into a 50-ml tube containing equal volume of 0.1 mol/l $CaCl_2$. The reaction products were stirred for 5 min and analyzed by XRD. In addition, a liquid-modified B4 medium in which $Mg(NO_3)_2$ was added to yield a 1:1 concentration of Ca^{2+} and Mg^{2+} was used to incubate FLF67-l for 4 days at 28 °C. Mineral formed by the fungi was analyzed by XRD.

Results

Patina Profile

Patina samples collected from two different sites showed similar chemical composition. XRD showed that the patina was a precipitation of calcium carbonate without calcium oxalate or other salts (Fig. 2).

Identification of the Calcium Mineralization Fungi

Nine growing fungi were isolated from the FLF59 and 12 isolates were obtained from FLF67 samples. All the fungi were incubated in the modified B4 medium to analyze whether they could precipitate $CaCO_3$. Crystalline precipitates formed on fungal hyphae of FLF59-c, FLF59-g, FLF67-a, FLF67-b, and FLF67-l, but no crystals were observed in non-hyphal areas (Fig. S1).

Information on the fungal communities was obtained by PCR amplification and DNA sequencing. Ten fungal genera (*Aspergillus*, *Trichoderma*, *Pestalotiopsis*, *Penicillium*, *Colletotrichum*, *Cladosporium*, *Phoma*, *Phomopsis*, *Hannaella*, *Aschersonia*) were distinguished from the 21 isolates (Table S1). The five selected fungi belong to three genus *Aspergillus* (FLF59-c), *Penicillium* (FLF59-g and FLF67-b which were identified as one species), and *Colletotrichum* (FLF67-a, FLF67-l), and they were clustered into two branches of the phylogenetic tree (Fig. S2). The obvious gap between the branches of carbonate precipitation fungi and others could be due to one evolutionary event that promoted the formation of the function-precipitating carbonate.

Biomineralization by Fungi

FLF59-c, FLF59-g, FLF67-a, and FLF67-l were incubated on a liquid-modified B4 medium for further study. After 4 days culture, the pH of culture supernatant decreased from 6 to 4.9 and 3.4 for FLF59-c and FLF59-g because of the secretion of oxalic acid, and increased to 7.1 and 6.8 for FLF67-a and FLF67-l which may be due to the metabolic activity of fungi.

After incubation of the four strains in Ca^{2+}-containing medium for 4 days, the fungal biomass with precipitated mineral was extracted and analyzed by SEM-EDS. Fig. 3 displays the variety of precipitated crystals. The mineral formed on fungal hypha of FLF59-c was blocky and was rosette-like for FLF59-g. FLF67-a and FLF67-l

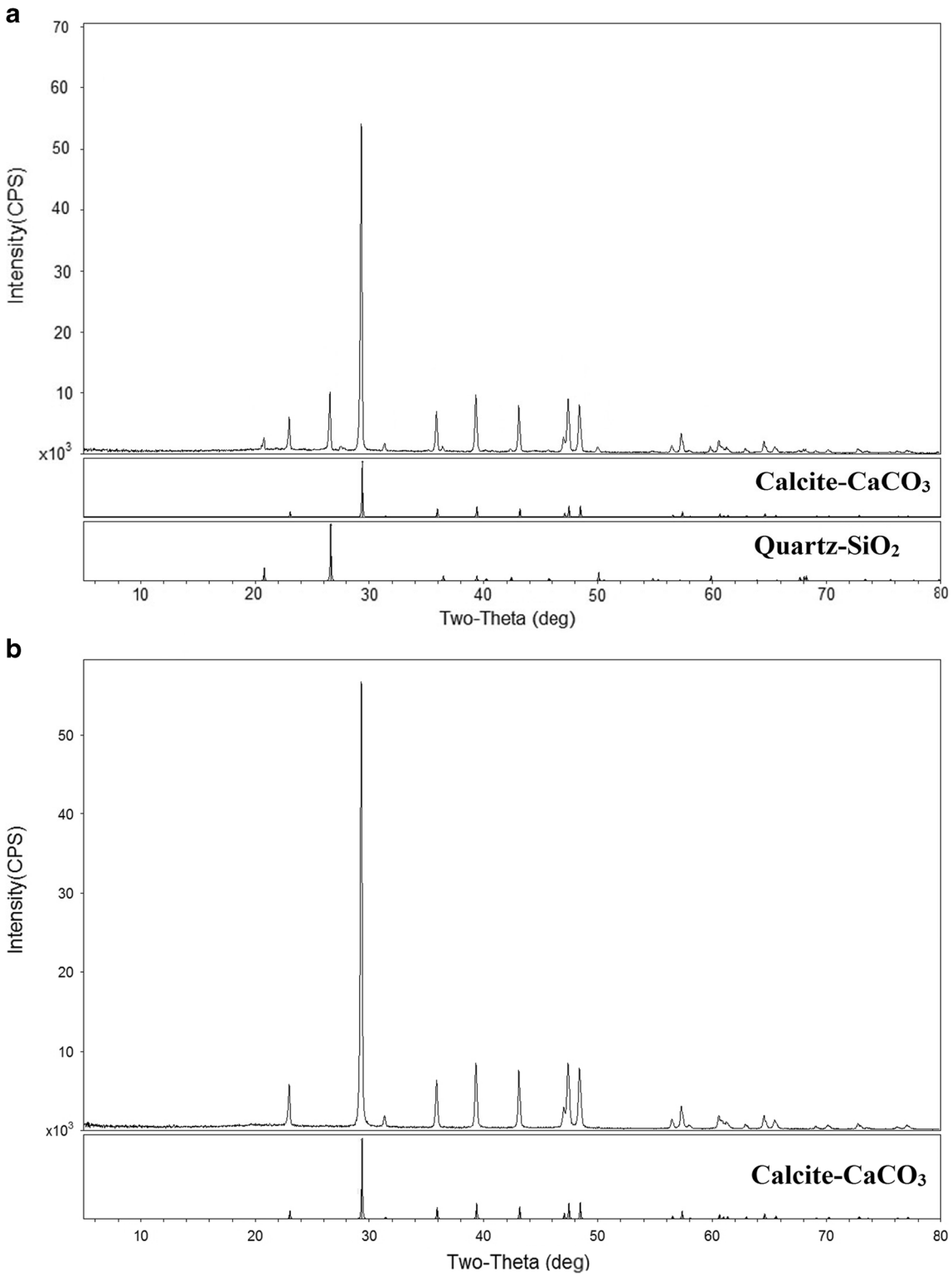


Fig. 2 XRD patterns of patina from Feilaifeng. **a** was sampled from FLF-59 and **b** from FLF-67

produced similar grape- and disk-like precipitation. All the crystals consisted same elements C, O, and Ca (Fig. S3).

There is some difference in the morphology of the formed mineral between Fig. 3 and Fig. S1 such as the shape or the size. The difference may be due to several reasons. Firstly, the crystal was formed under static condition in Fig. S1 but dynamic in Fig. 3, and stirring may promote the formation of a smaller-granularity crystal [22]. Secondly, the metabolisms of fungi should be the inhibitor of calcite carbonate and the concentration

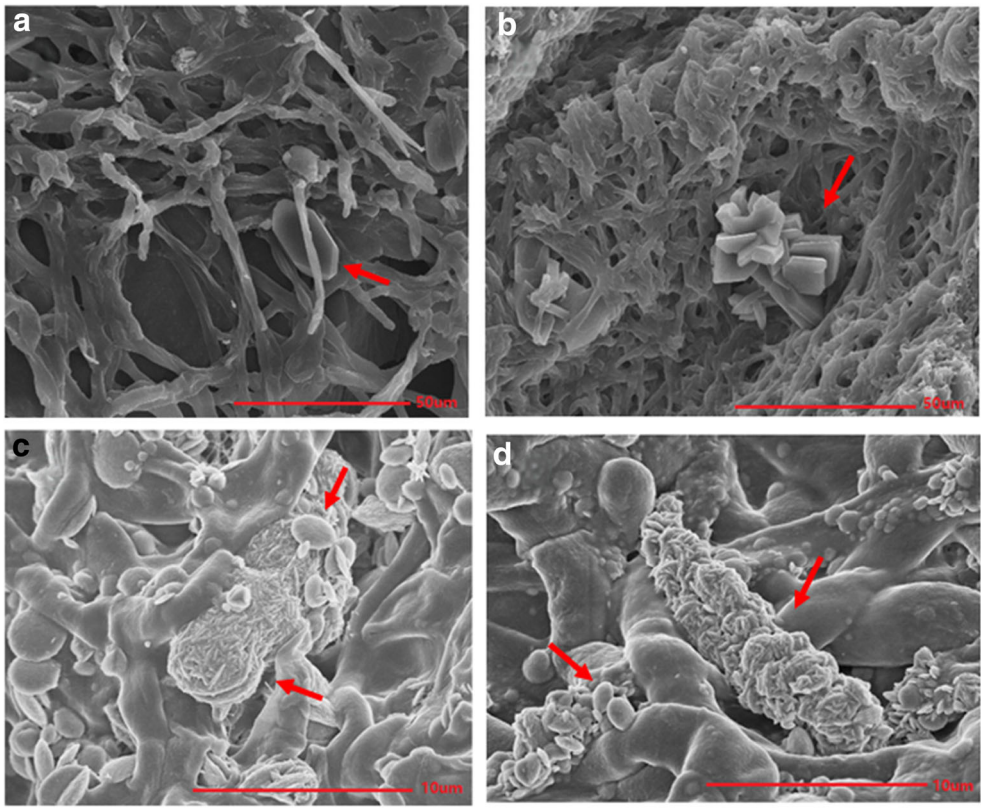


Fig. 3 SEM micrographs of CaCO3 crystals after 4 days of reaction of Ca^{2+} ions with fungi. The red arrow was the crystal formed on fungal hyphae. **a** *Aspergillus niger* (bar marker = 50 μm), **b** *Penicillium oxalicum* (bar marker = 50 μm), **c** *Colletotrichum acutatum* (bar marker = 10 μm), **d** *Colletotrichum gloeosporioides* (bar marker = 10 μm)

of these metabolisms, which the amounts in the liquid medium were larger than that in the solid medium, contributes to the morphology of crystal [22].

To further identify and characterize mineral formation, the mineral precipitates were analyzed by XRD. The crystals produced by FLF59-c and FLF59-g were both whewellite ($CaC_2O_4 \cdot H_2O$) (Fig. 4a, b), which may be related to the secreted oxalic acid. Although the mineral produced by FLF67-a and FLF67-l was calcium carbonate (Fig. 4c, d), the crystal type of vaterite was different from that of the patina collected from the Feilaifeng limestone. The difference of the crystals indicated a role of FLF67-a and FLF67-l in keeping the stability of vaterite.

Transformation Between Calcite and Vaterite

Calcite was the only crystal form in the chemical synthesis of calcium carbonate (Fig. 5a). However, after adding the metabolites of carbonate precipitation fungi, two kinds of calcium carbonate, calcite and vaterite, were detected, which indicated that the fungi could promote the stability of vaterite (Fig. 5b). However, the function of fungi may be inhibited and no any vaterite was observed when Mg^{2+} was added into the fungi medium (Fig. 6). Interestingly, a strong amorphous peak was observed at 2θ around 20° in Figs. 4b, c, d and 6. It may relate to the glass background or the organic matter of fungi. The XRD of amorphous calcium carbonate shows a similar peak at 2θ around 20°, but the amorphous peak should not be amorphous calcium carbonate because of the existence of oxalate acid in Fig. 4b, which may react with $CaCO_3$ to form CaC_2O_4.

Discussion

This study assessed the common damage patina on the surface of the Feilaifeng limestone. $CaCO_3$ was the major component of the patina, indicating a process of secondary crystallization of carbonatite, which may lead to a significant weathering of the cultural heritage. There were 10 genera of fungi isolated and identified from the surface on the patina. Here, we firstly find out that *Colletotrichum* could promote the formation of vaterite ($CaCO_3$). The different crystal form between the calcium carbonate produced by *Colletotrichum* and that of patina suggests that the metabolites of *Colletotrichum* play a role in the transformation of vaterite into calcite.

The patina from the Feilaifeng limestone was identified as $CaCO_3$. Previous studies have reported that calcium concentration, amount of dissolved inorganic carbon, availability of nucleation sites, and pH were the factors affecting the deposition of calcium carbonate [6]. There are two aspects in the formation of $CaCO_3$: chemical mineralization and biological mineralization. Chemical mineralization is a simple chemical reaction such as the form of stalactites. For biomineralization, fungi can directly affect the nucleation, growth, and morphology of the produced biominerals or release carbonate to combine with Ca^{2+} through

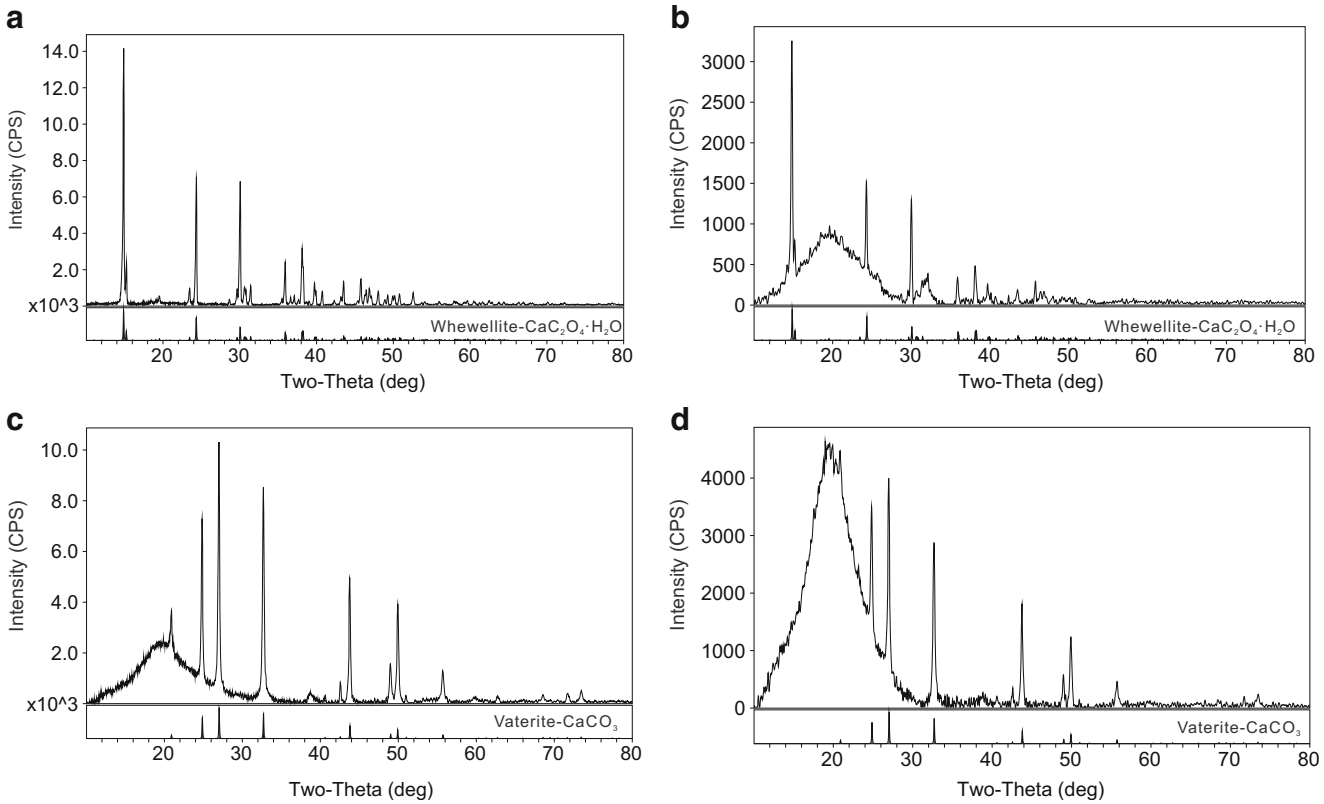


Fig. 4 XRD patterns of crystals on biomass synthesized by fungi. The crystal form of **a** *Aspergillus niger* and **b** *Penicillium oxalicum* was whewellite, but vaterite for **c** *Colletotrichum acutatum* and **d** *Colletotrichum gloeosporioides*

the cellular activities [15, 23]. The subtropical monsoon climate of the Feilaifeng limestone features copious rainfall and high humidity, which provides a suitable environment for the formation of $CaCO_3$. The patina was usually observed on the sloped limestone, which Ca^{2+} and HCO_3^- cannot be stored enough due to the gravitational action. Thus, it is hard to precipitate $CaCO_3$ only relying on chemical mineralization. This indicates a role of microbial community in the precipitation of $CaCO_3$.

Presently, the production of FLF59-c and FLF59-g, which were identified as *Aspergillus niger* and *Penicillium oxalicum*, reacted with Ca^{2+} to form $CaC_2O_4{\cdot}H_2O$. The result is consistent with previous studies of the formation of patina, which reported that *Aspergillus niger* and *Penicillium oxalicum* were the key in the weathering of limestone and calcium oxalate was the secondary biomineralization by the reaction of secreted oxalic acid with Ca^{2+} or $CaCO_3$ [12, 24–27]. However, the patina collected from Feilaifeng was identified as $CaCO_3$ rather than $CaC_2O_4{\cdot}H_2O$. It is reported that oxalotrophic bacteria are capable of using oxalic acid and its salts as sole carbon and energy source and degraded the calcium oxalate into calcium carbonate rapidly [28]. In addition, a model suggested that calcium oxalate was oxidized into calcium carbonate and carbon dioxide by the bacteria [13, 14]. Sahin reviewed that *Methylobacterium* is one of the oxalotrophic bacteria [29]. It was *Methylobacterium* that was widely distributed on the surface of the Feilaifeng limestone in our previous study [19], which indicated that there were some oxalotrophic bacteria on the Feilaifeng limestone. It provides a possibility that oxalic acid or its salts could be rapidly consumed by these bacteria and this may be the reason why we did not find calcium oxalate in the patina. The collective observations suggest that oxalate-producing fungi such as FLF59-c and FLF59-g with the participation of oxalotrophic bacteria provide enough dissolved inorganic carbon for the formation of $CaCO_3$ patina.

Colletotrichum, the genus of FLF67-a (*Colletotrichum acutatum*) and FLF67-l (*Colletotrichum gloeosporioides*), was first reported to promote the formation of $CaCO_3$ crystals in the present study. Vaterite was the mineral produced by *Colletotrichum* in the study. For crystallization and transformation of calcium carbonates, vaterite is the metastable polymorph which is formed by amorphous calcium carbonate and finally transforms to calcite [30]. Most previous studies reported that calcite was the production of fungal biomineralization [8, 15]. However, vaterite rather than calcite was the final crystal in the present study, which indicated that *Colletotrichum* is related to the crystallization and transformation of calcium carbonates. It is reported that biological metabolites, such as amino acid, protein, and sugar, can act as additives to control the shape and

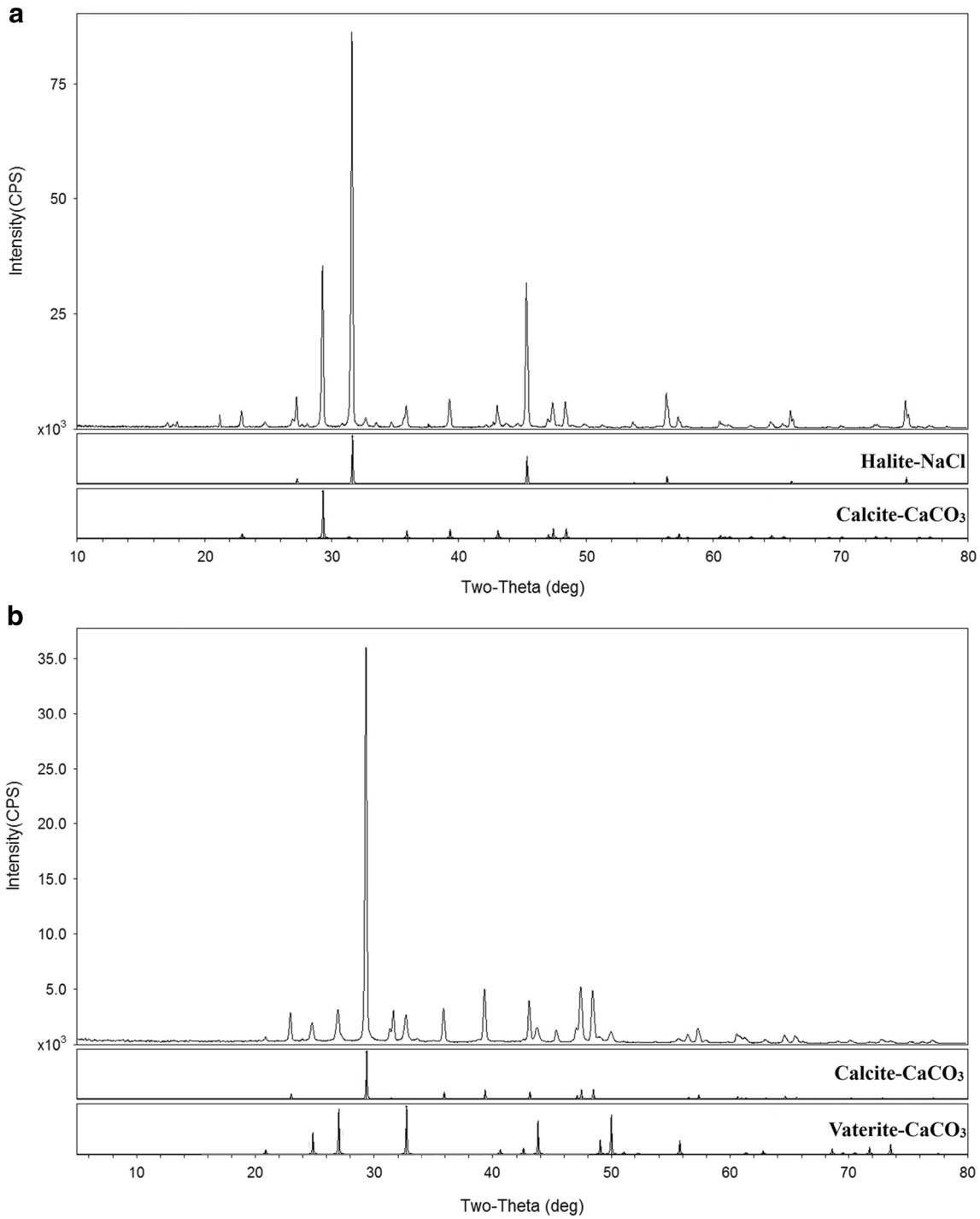


Fig. 5 XRD patterns of crystals synthesized by the reaction between $CaCl_2$ and Na_2CO_3. The solvent in **a** and **b** were water and culture medium of *Colletotrichum gloeosporioides*. The crystal form was calcite for **a** and calcite and vaterite for **b**

crystal polymorphology of calcium carbonate [17]. Rautaray et al. found that the protein produced by microorganisms defined the morphology of the $CaCO_3$ crystals formed, and when these proteins were removed, the crystal was just beginning to transform into a stable form [8]. Similarly, part of the synthesized $CaCO_3$ by $CaCl_2$ and Na_2CO_3 with the supernatant of *Colletotrichum* medium kept the metastable type in the present study (Fig. 5b). Microbially induced calcium carbonate precipitation is a process where an organism creates a local microenvironment. In the environment, the metabolisms rather than the composition of the

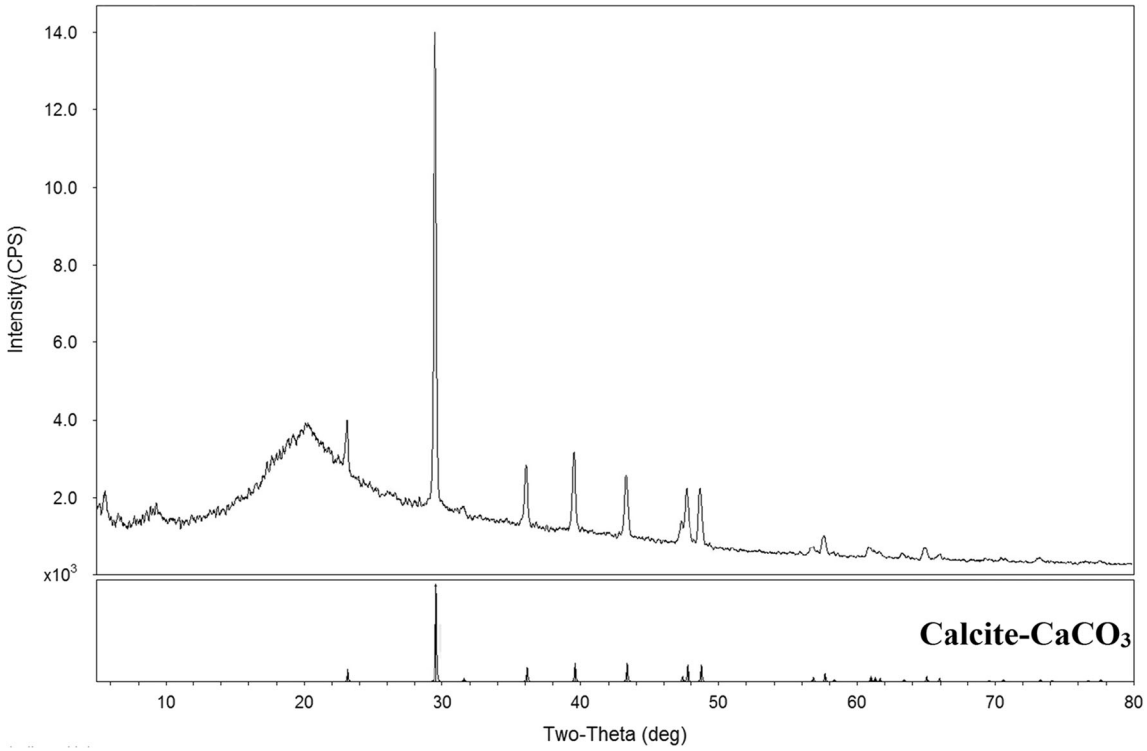


Fig. 6 XRD patterns of crystals on biomass synthesized by *Colletotrichum gloeosporioides* with the influence of Mg^{2+}. It shows that calcite was the product with the influence of Mg^{2+}

medium occupy a higher concentration, so we believe that the stability of vaterite relates to the fungi. It suggested that the metabolites of *Colletotrichum* may act as additives to inhibit the transformation of vaterite into calcite. Mg^{2+} is believed to influence the crystallization of calcium carbonate. It is reported that Mg^{2+} could promote the transformation of calcium carbonate from vaterite to calcite [31, 32]. Our results showed that calcite was the only product when Mg^{2+} was added into the medium of *Colletotrichum*. This suggested that the effect of Mg^{2+} for the crystallization and transformation of calcium carbonate is greater than the metabolites of *Colletotrichum*. MgO is one of the components of the Feilaifeng limestone [33], which may provide Mg^{2+} to inhibit the stability of vaterite and calcite becomes the final product after biodeterioration. The finding of *Colletotrichum* suggested that fungi may play a direct role in the formation of patina on the Feilaifeng limestone.

In conclusion, we clarified the patina distributed on the surface of the Feilaifeng limestone as calcium carbonate. *Colletotrichum* was first reported in the precipitation of calcium carbonate, which would expand the type of carbonate precipitation fungi. There were two pathways for fungi in the formation of patina on the Feilaifeng limestone. One was the oxalate-calcite cycle with the participation of oxalate-secreting fungi and oxalotrophic bacteria. The other was a direct synthesis of calcium carbonate by *Colletotrichum*. In addition, the results implicate the patina as a new marker to indicate the damage of stone by fungi for the protection of Feilaifeng cultural heritage statues. In order to remove the patina and prevent the fungi invasion, the mechanism of the precipitation of $CaCO_3$ as well as germicide and materials that will prevent the subsequent growth of microorganisms needs to be elucidated.

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