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Revision 1

Crystallization of Calcium Oxalate Hydrates by Interaction of Calcite Marble with Fungus Aspergillus Niger

Elena Rosseeva[1,2,†*], Olga Frank- Kamenetskaya[2*], Dmitry Vlasov[2],
Marina Zelenskaya[2], Katerina Sazanova[2], Alexey Rusakov[2] and Rüdiger Kniep[1]

[1]Max Planck Institute for Chemical Physics of Solids, Nöthnitzer Straße 40, 01187, Dresden, Germany.

[2] Saint Petersburg State University, Universitetskaya Nab 7/9, 199034 Saint Petersburg, Russia.

Present Address

†University of Konstanz, Department of Chemistry/Zukunftskolleg, Physical Chemistry, POB 714, 78457 Konstanz, Germany.

ABSTRACT. The crystallization of calcium oxalates (weddellite and whewellite) by interaction of calcite marble with fungus *Aspergillus niger*, one of the most active stone destructors, was studied under *in vitro* conditions. The temporal development of acid production of fungus as well as the sequence of formation and morphogenesis of the growing oxalate hydrates crystals were investigated in detail. Furthermore, the relationships between morphology and growth conditions of crystals within the biofilms on the surface of carbonate rocks are discussed.

KEYWORDS.

CRYSTAL GROWTH: crystallization, whewellite, weddellite

MINERALOGY: whewellite, weddellite, morphogenesis of calcium oxalate hydrates

GEOMICROBIOLOGY: microscopic fungi, *Aspergillus niger*, acid production, oxalate patina, bioweathering

INTRODUCTION

The significance of the problem of fungal activities during bioweathering and biotransformation scenarios of rocks and minerals is explained by the role of fungi in geomicrobiological processes, their deteriorative activity on natural rock and building materials (Ehrlich 1996; Burford et al. 2003; Burford et al. 2003a; Adeyemi and Gadd 2005; Vlasov and Frank-Kametskaya 2006; Gadd 2007; Frank-Kamenetskaya et al. 2009). Fungi are also of significance in the global carbon cycle. In addition, they permit potential biotechnological applications, e.g. in crystal engineering (Rautaray et al. 2003, 2004) as well as in the bioremediation processes of xenobiotic-, metal- and radionuclide-contaminated soils and wastes (Gadd 1999; Burford et al. 2006). Not at least, the study of the biochemical activity of fungi is of importance in development of effective procedures for the protection of stone monuments staying under the influence of urban environments (Frank-Kamenetskaya et al. 2012).

It is widely known that oxalate salts, particularly whewellite $CaC_2O_4 \cdot H_2O$ and weddellite $CaC_2O_4 \cdot 2H_2O$, are commonly present in biofilms on carbonate rock (marble, limestone) surfaces in association with lichen thalli, as well as fungal hyphae (Pinna 1993; Burford et al. 2003a; Gadd 1999, 2007; Magnuson and Lasure 2004; Rousakov et al. 2010). In addition, calcium oxalate hydrates can crystallize in the in biofilms on the surfaces of other calcium-containing rocks and minerals (Burford et al. 2003, 2003a; Gadd, 2007) and even on timber (Hastrup et al.

2012). This observation indicates a substantial contribution of microscopic fungus during oxalate patina formation, as already confirmed by a number of simulation experiments (Burford et al. 2006; Monte 2003, 2003a). The role of oxalate patina on the stone degradation process is controversial. It was demonstrated, that the removing of natural oxalate patina from the monument surfaces (especially in case of marble and limestone) induces the strong stone destruction (Bonaventura et al. 1999). However, the artificially formed oxalate patina might be also considered as a protection surface layer for the stone monuments (Doherty et al. 2007).

The present work continues research on the peculiarities of crystallization of calcium oxalates occured by interaction of carbonate rocks with organic acids excreted by fungus (Bonaventura et al. 1999; Frank-Kamenetskaya et al. 2003, 2012) by using fungus *Aspergillus niger* as an active stone destructor (Monte 2004; Barinova et al. 2010). Our interests were focused on: (a) temporal changes of acid production of *Aspergillus niger;* (b) sequence of formation and morphogenesis of calcium oxalate hydrates crystals. In addition, our in vitro results are compared with characteristic features of natural calcium oxalates associated with biofilms on the surface of damaged marble.

MATERIALS AND METHODS

Experiment conditions

For a detailed study of the growth of calcium oxalate hydrates under the influence of the microfungus *Aspergillus niger*, an *A. niger* Ch 4/07 strain was taken which was previously isolated from the surface of a damaged Proconesos marble of a monument column of the «Basilica in Basilica» was taken (Tauric Chersonesos, Crimea).

The Proconesos marble (Mezozoic age) was quarried near Constantinopole (Warren, 1999). Marble of columns of the «Basilica in Basilica» is a heterogranular, rarely slightly

dolomitized carbonate rock with some admixture of quartz (Figure 1). The calcite grains are generally xenomorphous, with the average size of about 0.2-0.3 mm. Macroscopically the rock has a parallel texture, however, under the microscope it appears massive. A parallel striation is observed on some grains. For our experiments, fungus was isolated from part of monument with signs of marble surface disintegration and development of lichen-fungal biofilm associated with oxalate crystals. The characterization of the strain was performed in the Research Center "Genomic technologies and cellular biology" of the All-Russian Research Institute of Agricultural Microbiology. The species identification of the strain was based on the sequence of the ITS region of rDNA (GenBank accession no - KF768341).

Two series of experiments were performed:

First series. For detailed investigation of organic acids produced by *A. niger* Ch 4/07 the fungus was cultivated on a liquid Czapek-Dox medium of composition (g/l): $NaNO_3$ – 3.0; KH_2PO_4 – 1.0; $MgSO_4 \cdot 7\ H_2O$ – 0,5; KCl – 0.5; $FeSO_4 \cdot 7\ H_2O$ – 0.015; glucose – 30.0; additionally $CaCO_3$ – 2.0 g/l. Non-shaken cultures were grown in 100-ml flasks containing 20 ml of the medium. The initial pH of the medium was 6.8. Inoculation was carried out by fungal conidia, using inoculum taken from the Czapek-Dox agar after 10 days of fungus cultivation. The organic acids and the pH of the cultural liquid were analyzed after 7, 15, 25 and 60 days of cultivation.

Second series. During the growth of *A. niger* Ch 4/07 fungus on the liquid Czapek-Dox medium (15 ml) filled in glass weighing bowls, a piece of homogenous Carrara calcite marble was put on the bottom of each bowl. Inoculation was carried out by fungal conidia, using inoculum taken from Czapek-Dox agar after 10 days of fungus cultivation. Investigation of the processes of calcite marble dissolution, the crystallization of calcium oxalate hydrates in the

fungal-marble system and the pH of the cultural liquid were investigated after 2, 4, 7, 9, 13 and 16, 21, 25 days of fungal cultivation (after inoculation).

Methods

The pH value of the cultural liquid was measured by using the pH-meter "pH-410". The analysis of the organic acids was carried out using a chromatography-mass-spectrometry (GC-MS) equipment. The result of the crystallization experiments were visualized by optical and scanning electron microscopy (SEM). In addition, elemental analysis (EDXS) and X-ray powder diffraction (XRD) were used. X-ray diffraction was carried out only when the amount of crystalline materials was high enough and prevailed over the amount of biomass (after 7 days of experiment). In general, the identification of calcium oxalate hydrate crystals was based on their morphological features (SEM) as reported in recent investigations (Zuzuk 2003; Thomas 2009; Thomas et al. 2012).

Chromatography mass spectrometry (GC-MS analysis). In order to separate the organic acids from sugars and other metabolites the culture broth was acidified by addition of 7% HCl to pH = 1.0 (displacement of oxalic acid from calcium oxalates). After that the sample was passed through a cation exchange resin (KU - 2-8) and then passed through an anion exchange column (AN-2FN) with a flow-rate of 1 ml / min. Anions of organic acids were displaced by NaOH and then again passed through a cation exchange resin.

GC-MS analysis was carried out by use of an Agilent mass spectrometer (mass selective detector MSD5975) and a HP-5MS, 30 m X 0.25 mm column. The organic acids were analyzed as TMS-derivatives. Helium was used as the carrier gas with a constant flow rate of 1.3 ml/ min. Evaporation was performed at 320° C. Linear temperature programming from 70 to 320° C (4 C/min) was used for analyses. Data were collected using the software Agilent ChemStation. Processing and interpretation of the mass spectrometric data were carried out by use of the

program AMDIS (http://www.admis.net/index.html) and the standard library NIST2005. Quantitative analysis of the chromatographic data was carried out by internal standardization of hydrocarbon C_{18} using UniChrom http://www.unichrom.com/unichrome.shtml.

Scanning electron microscopy (SEM) and Energy-dispersive X-ray spectroscopy (EDXS). Morphological investigations and elemental analysis (using EDXS) of samples (calcite marble blocks and fungal mycelia extracted from solution) were performed (in MPI CPfS, Dresden) by means of an ESEM FEI Quanta 200 FEGi system operated in a low vacuum (60 Pa) mode and at an acceleration voltage of 15 kV (FEI company, Eindhoven, NL). Samples were mounted on carbon tapes adhered to aluminum or brass holders. To increase the quality of the SEM images (to avoid charging effect) the samples were coated by a thin gold layer for 1 min (Gressington 108 Auto Sputter Coater). For EDXS measurements the microscope was additionally equipped with the system "Genesis 2000" (EDAX, AMETEK, Wiesbaden, Germany). The EDX spectra were analyzed by means the EDAX Genesis software package (semiquantitative analysis was performed by standard-less method which is in general reliable for elements with $Z > 10$).

X-ray powder diffraction (XRD). The determination of the phase composition of the crystallization products was carried out in the X-ray diffraction Centrum of Saint Petersburg state university by using a Rigaku powder diffractometer (CuKα radiation). X-ray diffraction patterns were collected at room temperature in the range of $2\theta = 10\text{-}50^{o}$ with a step of 0.04° 2θ and a counting time of one second per data point.

RESULTS

Acid production of *Aspergillus niger*

Acidification of the cultural liquid was started immediately after spore germination and was continued during the intensive growth of the mycelium (up to 25^{th} days of experiment). During

the first stage of cultivation the most excreted acid is gluconic acid (Figure 2). At the 7th day, also oxalic acid (3.3±0.7 μg/ml) and citric acid (3.6±0.6 μg/ml) were present.

At the 17th day the amount of gluconic acid was significantly decreased, while the amounts of oxalic and citric acids are increased (8.9±1.2 and 23.8±3.3 μg/ml respectively). The value of the pH of the cultural liquid during the period of 17 days decreased from 6.8 to 3.4. At the 25th day the lag-phase of mycelium growth comes to an end. The amount of gluconic acid is continuously decreased; the same is true for the amount of citric acid. The pH value continues to decrease, but at a slower rate (up to 2.8). Then, the mycelium grows at a slower speed (stationary phase of growth), the pH of the culture liquid stated slightly increase. After 25 days of cultivation the amount of gluconic acid continuously decreased, the amount of citric acid remains virtually unchanged. The amount of oxalic acid continuously is increased and reached 3.6 μg/ml at the 40th day. At this stage, malic (0.7 ± 0.2 μg / ml), fumaric (1.4 ± 0.2 μg / ml) and succinic (0.3 ± 0.1 μg / ml) acids are also formed. After 40 days the processes of autolysis of the mycelium started. The pH value continues to increase at a higher rate. After 60 days of cultivation the amount of oxalic acid remains constant, while the amounts of citric acid (1.9±0.6 μg/ml) and gluconic acid (4.8±0.8 μg/ml) decrease. Malic, fumaric and succinic acids are not detected at this stage.

Crystallization of calcium oxalate hydrates

Significant dissolution of marble under the influence of *A. niger* Ch 4/07 fungus is already observed after 4 days treatment. Fungal hyphae are fixed on the surface of the marble block and small pieces of marble are attached to the fungal mycelium (Figure 3). The surface of the marble particles within the fungal mycelium is strongly etched (Figure 3b). After 7 days the pH value of the cultural liquid decreased from 6.8 to 6. The surface of marble is became etched even stronger. In addition, elongated plate-like crystals are formed. XRD and EDXS analysis (Ca – 23

(1) wt.-%, P - 18 (1) wt.-%; Ca/P – 1.0(1)) reveal these crystals represent brushite, $Ca(HPO_4)\cdot 2H_2O$. No significant changes besides increasing marble dissolution and brushite growth are observed after 9 days. The pH value of the cultural liquid decreased to 5.5 during this period.

First crystals of tetragonal calcium oxalate dihydrate (weddellite) were found on the marble surface after 13 days of reaction (Figure 4a) at a pH value of 5. The crystals are almost isometrical dipyramidal and dipyramidal-prismatic (with dominant {101} pyramidal faces). Their size varies from 2 μm to 23 μm. The prismatic faces {100} are comparatively small (edge/length ratio [1 0 0]/ [0 0 1] ≈ 10). Multiple-headed weddelite crystals are also observed besides heavily destructed marble fragments incorporated in the mycelia (Figure 4b).

After 16 days of reaction the pH value had not changed (remaining at pH - 5). The formation of calcium oxalate hydrates is accompanied by intense dissolution of marble (Figures 5, 6). Tetragonal weddellite crystals with various sizes of their prism faces (edge length ratio [1 0 0]/[0 0 1] ≈ 2-10 μm) were grown on the marble surface (Figure 5). Some of the individuals reveal more pronounced prismatic faces {100} (Figure 5c). Besides the multiple-headed weddellite crystals, intergrowth structures and skeletal individuals are observed (Figure 5 b-d). At the same time, spherulite-like whewellite aggregates built up on intergrown lamellar individuals appeared on the fungal hyphae in the mycelium in solution (Figure 6 a-c). According to the results of XRD studies the amounts of weddellite and whewellite crystals are nearly equal (Figure 7).

The same process continues further in time (until the 25th day) and a massive amount of same morphological types appear, while the pH value remains equal to 5.

DISSCUSSION

It is well-known that the presence of organic acids plays a major role in the biochemical degradation of carbonate rock (Ehrlich 1996; Gadd 1999, 2007). Even weak acidic solutions (e.g. organic acids) formed by any organism via its metabolism are capable for dissolving inorganic carbonates. In addition, the metabolic generation of CO_2 (e.g. during respiration) may have the same effect.

Our experiments show that *A. niger* strain, in fact, is an active acid producer. The composition and quantity of the acids produced by fungus varies significantly over time (Figure 2). The highest total amount of acid produced by fungus is observed during the stage of active growth of the mycelium. With the exception of oxalic acid, the amounts of all other acids decreased with the aging of culture. The sequential changes in the pH of the medium (first decreasing, then remaining stable, and then increasing) correspond to acidification, oxalate crystallization, and autolysis of mycelium, respectively. Differences in the temporal changes of the pH value are related to the content of calcium carbonate in the medium, as the presence of $CaCO_3$ exerts stimulatory effects on fungi excretion of oxalic acid (Barinova et al. 2010).

During the first stage of the reaction most excreted acid by *A. niger* strain is gluconic acid (Figure 2). Gluconic acid is known to be well assimilated by fungi (Müller 1986; Elshafei 1989) and in case of a carbohydrate deficit previously excreted organic acids (except for oxalic acid) can be reused as a source for carbon (Gadd 1999). This may explain the observed decrease of citric and gluconic acids in the medium via assimilation by fungi because of increasing glucose deficit. In this way, the concentration of oxalic acid in the broth culture increases with time and leads to calcium oxalate hydrates crystallization between day 9 and day 13 (Figure 4).

Before that (between day 4 and day 7 of the reaction), the dissolution of calcium carbonate together with the presence of significant amounts of potassium dihydrogenphosphate KH_2PO_4 in the Czapek-Dox medium leads to the crystallization of brushite, $Ca(HPO_4)\cdot 2H_2O$.

Calcium oxalate crystallization starts at pH values between 5.5 and 5 with the formation of almost ideal dipyramidal and dipyramidal-prismatic (with dominant {101} pyramidal faces) tetragonal weddellite crystals (Figure 4). According to previous investigations (Thomas 2009; Thomas et al. 2012; Izatulina et al. 2014) the stabilization of metastable calcium oxalate dihydrate is connected with organic molecules present the solution , and witch are most strongly adsorbed by calcium biominerals at pH = 5 (Fleming et al. 2001). In the present case it is possible, that apart from organic acids, several other biomolecules produced by *A.niger* in small quantities, such as cellulase (Narasimha et al. 2006; Lee et al. 2011), amylase, lignocellulase (Villena and Gutiérrez-Correa 2007), xylanase (Soliman et al. 2012), pectinase (Joshi et al. 2006; Adeyemi et al. 2011), protease (O'Donnell et al. 2008; Devi et al. 2001), and even exopolysaccharides (glucanes) (Senthilkumar and Murugesan 2010), may attribute to the growth scenario.

The remaining steps of calcium oxalate crystal formation took place at the constant pH, equal to 5. After several days of fungal treatment the prismatic faces of dipyramidal weddellite crystals become more pronounced (Figure 5). Based on recent results on the formation and morphogenesis of calcium oxalate dihydrate in the presence of different amounts of polyacrylic acid (Thomas 2009; Thomas et al. 2012) it can be assumed, that the interaction of organic molecules with the prismatic faces of weddellite leads to an increased inhibition of the growth rate of these faces along [100], thereby promoting the elongation of the crystals along [001].

Calcium oxalate monohydrate, whewellite, appears in the medium as splitted (branched) spherulite-like aggregates between 13 and 16 days after the experiment was started (Figure 6a-c). The presence of such kind of aggregates as well as numerous multiheaded and skeletal weddellite habits gives evidence that at this step of reaction the supersaturation of the solution was high. The fact that the number of weddellite and whewellite crystals is nearly equal suggests

that the processes of formation and growth of crystals do not slow down at this step and occur at high speed along with of weddellite transformation to whewellite.

IMPLICATIONS

The present work contributes to the essential questions on calcium oxalate formation within biofilms on carbonate rocks under the influence of microscopic fungi. Most of the works published on this subject up to now are restricted to the characterization of calcium oxalate hydrates aggregates found in the natural oxalic patina on the surface of marble and limestone monuments only, which, however, cannot definitely explain the mechanism of their formation. To solve this problem, the crystallization of calcium oxalate hydrates on marble surfaces under the influence of fungus *Aspergillus niger* (one of the most active stone destructors) was studied under *in vitro* conditions. It was shown that *A. niger* is an active producer of organic acids, among which gluconic-, citric- and oxalic- acid were detected as a function of time. The highest total amount of acid production by fungus was observed during the stage of active growth of the mycelium. In the aging cultures, apart from the oxalic acid, the amount of all other organic acids significantly decreased. The temporal changes of acid production of this fungus significantly affected the sequence of formation and the morphogenetic peculiarities of the calcium oxalate hydrates (both weddellite and whewellite) crystals and aggregates. The sequence and, in most cases, the morphogenetic peculiarities of oxalate hydrate crystals formed under the influence of *A. niger* are well explained by adsorption interactions of the organic components with the growing individual. Morphology and size of the tetragonal weddellite crystals at the first stage of the reactions are closely related to crystals observed on the surface of damaged Proconesos marble of monuments in Tauric Chersonesos (Crimea) (Figure 8). Besides, as in our experiment whewellite is often found in natural oxalate patina in form of intergrowths and aggregates

(Frank-Kamenetskaya et al. 2012). However, multiheaded and skeletal weddellite crystals have not been observed in natural biofilms on the surface of carbonate rocks. Additionally, microbial community (rather than one type of microorganisms) is nearly always present in biogenic oxalate patina. Also, the speed of formation of oxalate patina in environmental conditions is about an order of magnitude slower than in our experiments. This distinction is probably due to the fact that the ionic supersaturation of the solutions and the crystal growth speed under the natural conditions are not as high as in our *in vitro* experiments.

The close morphological relationships between grown crystals and aggregates and natural weddellite and whewellite specimens found in oxalate patina allows to get deep inside into the their formation mechanism. These investigations also demonstrate that the formation of oxalic patina is associated with acid production by fungi and results in destruction of carbonate rocks (marble and limestone). This creates doubt on the protective properties of the natural oxalate patina (of biogenic origin) on the surface of cultural heritage monuments.

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LIST OF FIGURE CAPTIONS

Figure 1. Light microscope image in polarized light of the Proconesian marble (thin section): a - 1N, b - N+. The diameter of the microscopic field of view is 1.2 millimeters. (Mosyagin et al. 2009)

Figure 2. Content of organic acids in the culture broth of *A. niger* Ch 4/07 and pH values of the solution during the cultivation process (up to 60 days).

Figure 3. SEM images of fungal mycelium on the surface of marble (a) and in the solution mycelium (b) after 4 days of reaction (fungal treatment).

Figure 4. SEM images of tetragonal calcium oxalate dihydrate (weddelite) crystals on the surface of marble (a) and in the solution mycelium (b) after13 days of reaction (fungal treatment).

Figure 5. SEM images of weddelite crystals on marble surface after 16 days of fungal treatment: (a) - the overview of the marble surface, (b, c) – enlarged Figure (4a) illustrating dipyramidal-prismatic crystals with different sizes of the prismatic faces as well as intergrown individuals, (d) - skeletal dipyramidal crystal.

Figure 6. SEM images of spherulite-like intergrowth aggregates of whewellite crystals on hyphae in solution mycelium on the 16-th day of reaction (fungal treatment).

Figure 7. XRD pattern (CuKα) of crystallization products after 16 days of reaction (fungal treatment). Key: *Wh*-whewellite, *Wed*- weddelite.

Figure 8. SEM images of natural tetragonal weddelite crystals observed on the surface of marble monuments in Tauric Chersonesos (Ukraine, Crimea)

ASSOCIATED CONTENT

Abbreviations

SEM, Scanning Electron Microscopy; GC-MS Gas Chromatography Mass Spectrometry; EDXS, Energy-dispersive X-ray spectroscopy.

428 **FIGURES**

429 **Figure 1.**

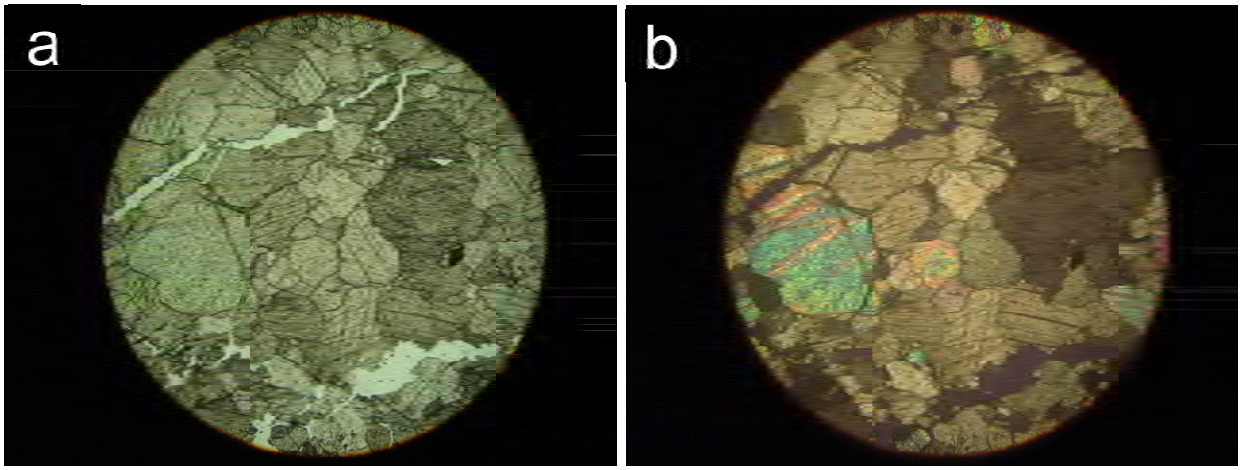

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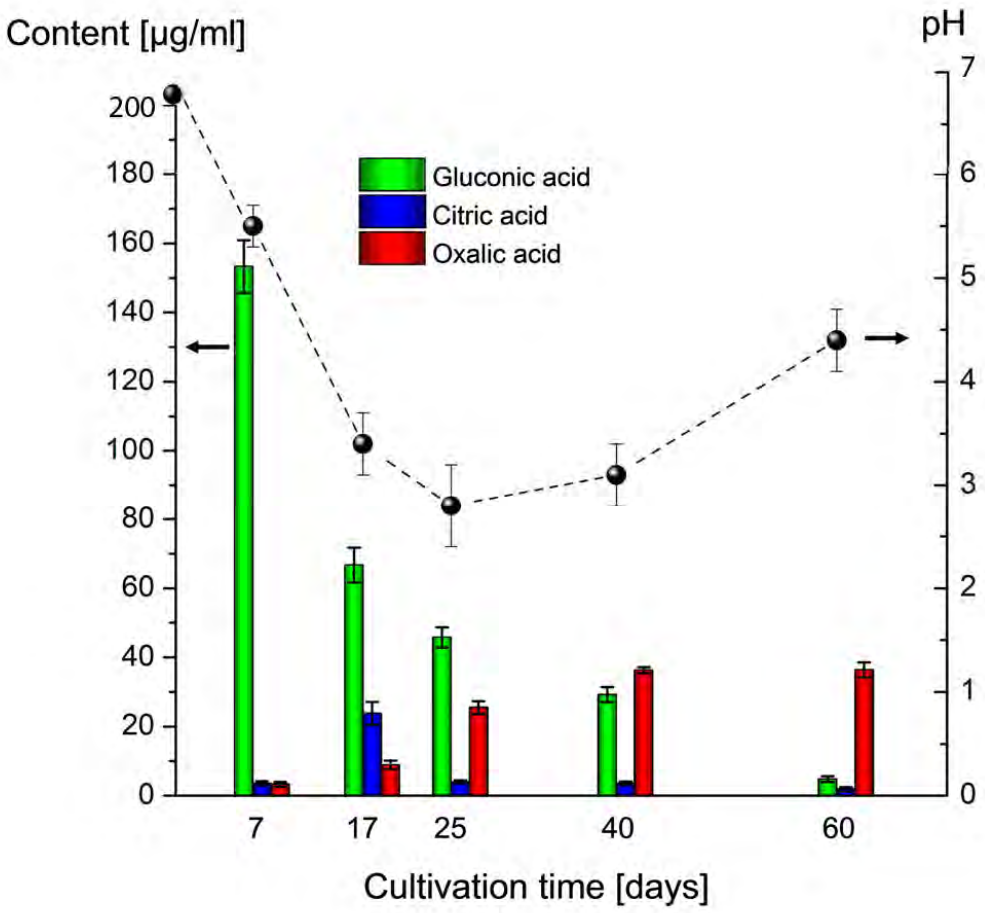
Figure 2.



435 **Figure 3.**

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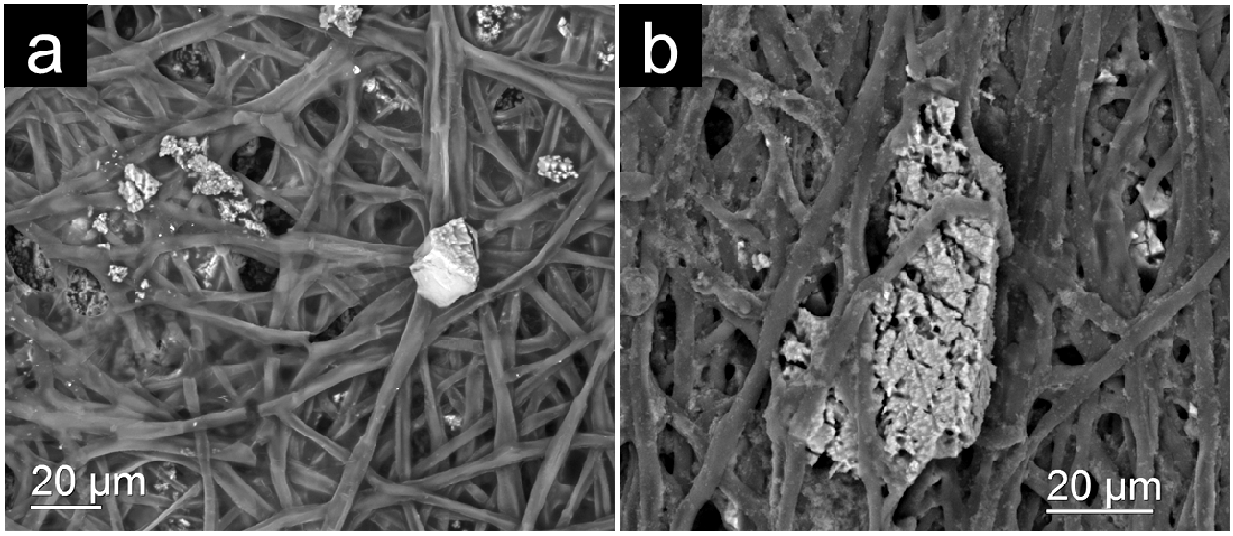

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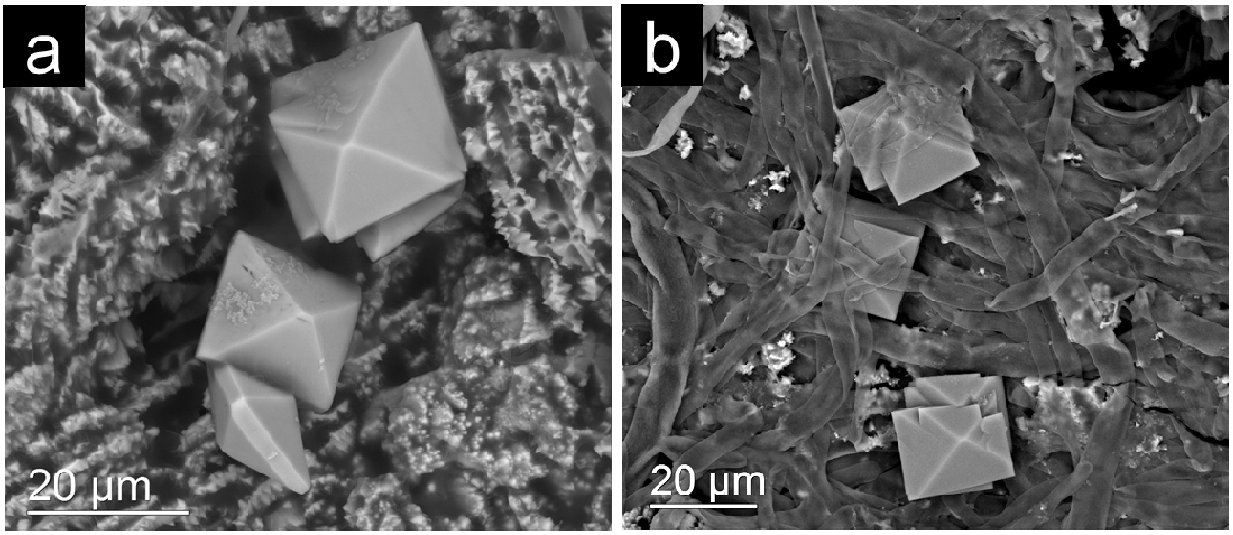
439 **Figure 4.**



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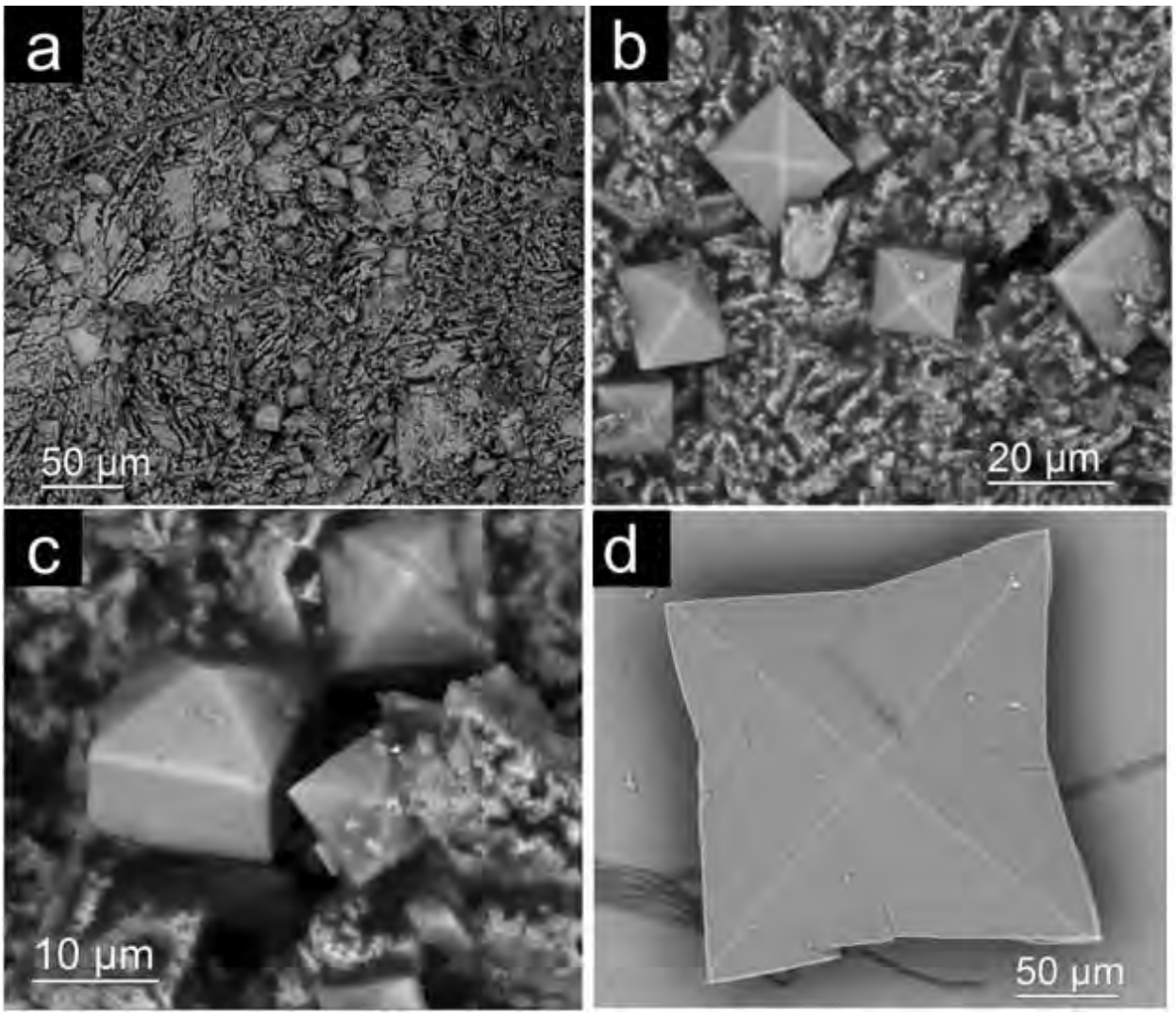

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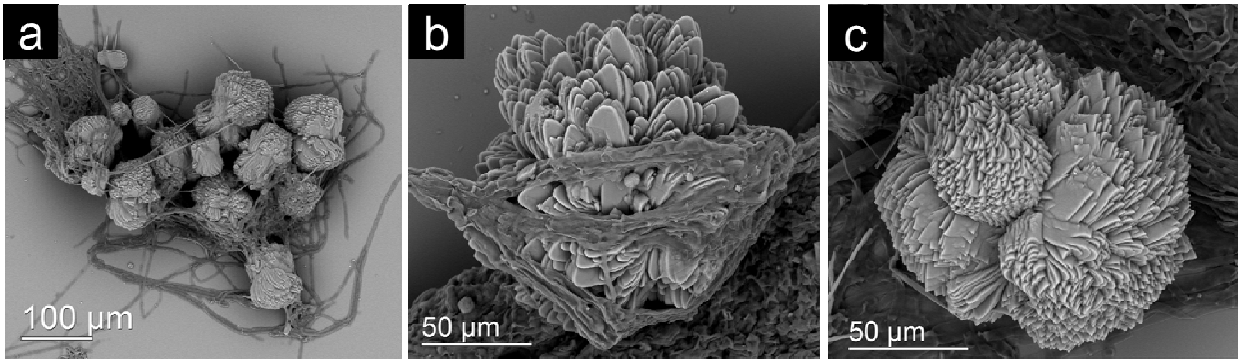
445 **Figure 6.**



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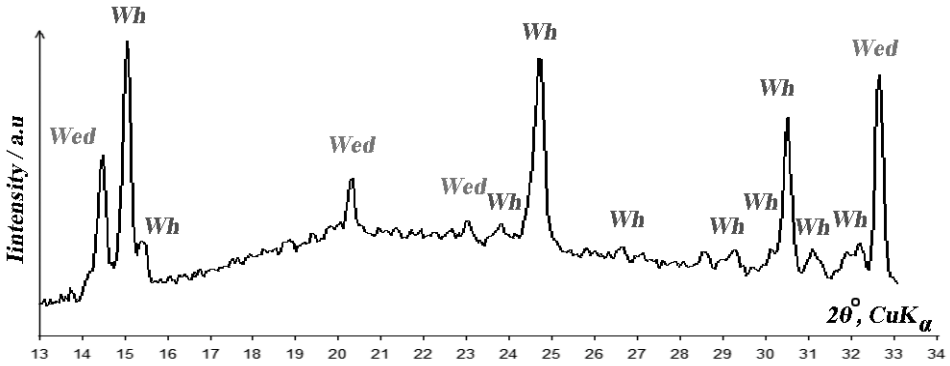
448 **Figure 7.**



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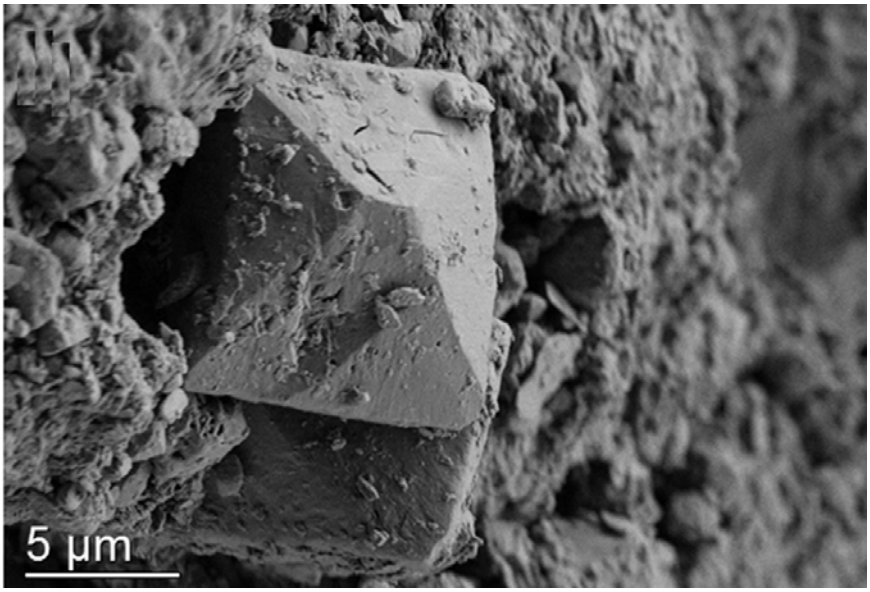
452 **Figure 8.**



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