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Biomineralization Potential of a Ureolytic Fungus Isolated From Mawsmai Cave in Meghalaya

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Abstract

Meghalaya is home to numerous caves of which many are still undiscovered. Geomicrobiological studies so far have focused on the bacterial composition and diversity of these caves. The present study was undertaken to isolate calcifying ureolytic fungi from the Mawsmai cave in Meghalaya, India for their calcium biomineralization efficacy. Out of 77 isolates, 25 were selected based on microscopic observations. In addition, 5 isolates that tested positive for urease were undertaken to further test for biomineralization potential. The isolate UF3 showed highest removal of Ca^{2+} when grown in liquid media supplemented with different calcium salts (calcium chloride, calcium nitrate and calcium oxalate). The medium supplemented with calcium chloride showed highest production of fungal biomass. A similar trend was observed for the fungal isolate UF3 showing better Ca^{2+} precipitation in agar and low soluble Ca^{2+} in liquid media. Upon phylogenetic analysis, the isolate UF3 showed high similarity with Aspergillus versicolor. The findings of this preliminary study on the ureolytic fungi isolated from Mawsmai cave suggest that the isolate UF3 could play an important role in biomineralization.

Keywords: Cave, Fungi, Urease, *Aspergillus versicolor,* Biomineralization.

Introduction

Humankind from the early times have depended on caves for shelter and even expression of art (Valladas *et al.*, 2001). Gillieson (1996) defined a cave as "any natural space below the earth's surface that extends beyond the twilight zone, and is accessible to humans". The behavior of seeking shelter in caves has been observed in other animal species as well (Chelini *et al*, 2011; Fišer *et al.*, 2019). The subterranean environment harbors a variety of organisms ranging from microscopic bacteria and fungi to organisms with regressive evolution of optical morphological features owing to the low light environment of caves (Tobler *et al.*, 2009; Pérez-Moreno *et al.*, 2018; Stern *et al.*, 2018). Caves are exemplary sites serving as natural and experimental systems for fundamental geomicrobiological studies because of the easy accessibility (Barton and Northup, 2007). Geomicrobiological interactions in the cave environment influence microbial diversity and their activities that contribute to calcium carbonate ($CaCO_3$) precipitation (Boquet *et al.*, 1973; Buczynski

and Chafetz, 1991).

Based on the process of formation, caves can be of many types. The most common type of caves, known as karst caves are formed by chemical dissolution of the host rock consisting of dissolvable minerals, namely calcite, dolomite, gypsum and rock salt (Lauritzen, 2018). Across the world, carbonate karst is the most common type followed by gypsum karst (formed by evaporation of calcium sulphate deposits on the land surface) (Ford and Williams, 2007).

Due to lack of light in the cave environment, carbohydrate synthesis that would usually occur by photosynthesis is restricted resulting in limitation of resources (Sarbu *et al.*, 1996). This would make the cave environment extremely unfavourable for any living organism to survive. However, cave ecosystems have adapted to deriving energy from organic matter brought in by allochthonous sources (percolating water, wind, bats or birds, etc.) (Simon *et al.*, 2003). Microorganisms are key players in mediating energy transfer between organic sources and the subterranean environment influencing biogeochemical cycles via metabolic pathways that convert minerals into bioavailable forms for use by other organisms (Simon *et al.*, 2007). Furthermore, microbes are known to regulate precipitation of carbonates in various natural environments, which induce formation of cave geological structures such as stalactites, stalagmites and cave wall deposits (Zhu and Dittrich, 2016).

Extensive study of minerals like $CaCO_3$ formed by the process of biomineralization is necessary due to the significant contribution of these minerals to the global biogeochemical cycles (Van Cappellen, 2003). Microbial biomineralization has been discussed widely with much focus on prokaryotic (particularly bacteria) metabolic processes (Rusznyák *et al.*, 2011). However, several studies report that fungi are responsible in shaping biogeochemical cycles and help in biomineralization (Oggerin *et al.*, 2014; Dhami *et al.*, 2017; Pasquale *et al.*, 2019). Fungi are also responsible for organic matter recycling, mineral weathering and production of organic acids such as oxalic acid leading to formation of various metal-oxalate complexes (Gadd, 1999; Hoffland *et al.*, 2004). Furthermore, fungi can influence Ca^{2+} concentrations, either directly or indirectly. Fungal hyphae require Ca^{2+} in high concentrations for their apical growth but concentration of free Ca^{2+} in the cytoplasm is much lesser. To maintain this gradient, fungi either sequester the Ca^{2+} in organelles (mitochondria or endoplasmic reticulum) or pump it out of the cell or bind it to calmodulins (Ca^{2+}-binding protein) (Pitt and Ugalde, 1984). Fungal cell wall components like chitin and glycoproteins as well as secreted exopolymeric substances (EPS) can adsorb Ca^{2+} thereby initiating $CaCO_3$ nucleation and subsequent organo-mineralization (Manoli *et al*., 1997). Li *et al*. (2014) confirmed that urease-positive fungi could induce $CaCO_3$ biomineralization similar to ureolytic bacteria. In another study, Li *et al*. (2015), isolated fungi from calcareous soils, suggesting that ureolytic fungi present in these environments may contribute to $CaCO_3$ formation/stability. This is an important aspect of biomineralization since transformation of urea into ammonium leads to pH increase,

which is one of the important factors for $CaCO_3$ precipitation (Korchef and Touaibi, 2019). Cave microbiology is an interdisciplinary field of microbiology, geology and chemistry that studies microorganisms in caves and their various influences on cave processes. The recognition of microorganisms (bacteria, in particular) in geological processes in caves has been noteworthy in our understanding of the cave ecosystems (Barton and Jurado, 2007). With various literature available to prove that fungi contribute to the formation of cave structures, its study may unveil different dimensions related to geomicrobiology. In this context, the present study was proposed to isolate and characterize fungi from a cave, Krem Mawsmai (Krem translates to "cave" in the local language) located in the Khasi Hills, Meghalaya. Furthermore, the fungal isolates were studied for their calcification potential under *in vitro* conditions to relate to the process of biomineralization.

Study area and Geology

Meghalaya is blessed with diverse natural wonders like caves. In 2016, a group of cave explorers in the East Khasi Hills district of Meghalaya discovered Krem Puri, the longest (8,269 m) sandstone caves in India (Kharpran-Daly, 2016). Other caves found in Meghalaya are Krem Mawsmai, Krem Phyllut, Krem Mawpun, Krem Mawmluh, Krem Liat Prah, Krem Umthloo, Tetengkil Balwakol and Siju–Dobhakhol.

Meghalaya Plateau is a northeastern extension of the Indian Peninsular Shield, with an elevation of 600–1800 m. The cave investigated in this study is located in the East Khasi Hills (N 25814.680 ; E 91843.480), bounded by Ri-Bhoi District on the North, Karbi Anglong district of Assam on the North-East, Jaintia Hills district on the East, Bangladesh on the South and West Khasi Hills district on the West (Ghosh *et al*., 2005). With huge deposits of limestone and abundant rainfall, Meghalaya has abundant Karst cave formations. The limestone band runs from the West Garo Hills in the west through the West Khasi Hills, East Khasi Hills and into the Jaintia Hills in the east. The limestone deposits in the East Khasi Hills are located in the Mawmluh-Mawsmai Hills south of lower Sohra extending to about 1.40 km area. The deposit is made up of limestone in the upper part and dolomite in the lower part (Harries *et al.*, 2008).

Krem Mawsmai is located at a distance of around 6 km from Sohra and is surrounded by thickly forested zone. The cave is 160 m long, 15 m high and 4–10 m wide and is completely aphotic with an abundance of stalactites and stalagmites. The moist environment and the dense canopy cover makes the cave a suitable shelter for certain species.

India has a large number of unexplored caves and few caves in Meghalaya are among the largest mapped caves across the world. These caves have so far not attracted scientific attention with reference to microbial diversity and fungi in particular. The present study was therefore aimed at isolation and characterization of fungi from Mawsmai cave located in East Khasi hills, Meghalaya (India) and to study various parameters that test their biomineralization (BIM) efficacy.

Methodology

Sampling

Samples were taken from minimally contaminated (undisturbed by human/anthropogenic activities) areas of the Mawsmai cave and were collected using sterile disposable gloves, forceps, spatula and autoclaved sampling bottles. Samples were collected from multiple sites within the cave which include lime deposits inside the cave, middle cave substratum, stony rock surface, water flowing inside the cave, cave exit, sandy substratum in the cave and forest soil. The samples were stored at 4˚C until analyzed.

Isolation of fungi

1 gram (soil sample) and 1 ml (water sample) was measured and added to 9 ml of 0.85% (w/v) sterile physiological saline and vortexed vigorously to make a uniform suspension followed by successive dilutions. Medium used for fungal isolation was Potato Dextrose Agar (PDA) (infusion from potatoes 200 gL-1, dextrose 20 gL-1, agar 15 gL-1 (HiMedia, India). The PDA plates (supplemented with chloramphenicol) were inoculated with aliquots of 100 µl from the suspension dilutions of 10^{-2}, 10^{-3} and 10^{-4}. All plates were incubated at 28˚C for 7 days and subcultures were made from resultant colonies.

Staining using Lactophenol Cotton Blue Stain

The fungal cultures were stained using Lactophenol cotton blue and observed under a bright-field microscope.

Urease Test for Selection of Ureolytic Fungi

Christensen Urea agar media (peptone 1 gL-1, dextrose 1 gL-1, sodium chloride 5 gL-1,disodium hydrogen phosphate 1.2 gL-1, potassium dihydrogen phosphate 0.8 gL-1, phenol red 0.012 gL-1, agar 15 gL-1) (HiMedia) is used for this test. Phenol red acts as a pH indicator where the hydrolysis of urea produces ammonia with an increase in pH resulting in colour change of media from yellow to bright pink. 2% urea (membrane sterilised) is then added to the sterilized media before pouring into the glass vials aseptically. The slants after solidifying were inoculated with pure isolated fungal cultures and incubated at 28˚C in an incubator for a day and observed for any colour change.

Biomass Estimation in different Calcium Salts

For biomass estimation, the fungal cultures were grown in Potato Dextrose Broth (PDB) media supplemented with different calcium salts (100 mM calcium chloride, 100 mM calcium oxalate, 100 mM calcium nitrate) separately. Membrane-sterilized 2% urea was then added to the sterilized PDB media. Fungal cultures were inoculated into the media in aseptic conditions (laminar air flow chamber) and incubated in a shaker incubator at 30 ˚C for 7 days. After 14 days, the fungal biomass was separated from the broth by filtering it through Whatman filter paper no. 1. The biomass was transferred onto pre-weighed petri plates and weight was taken which accounts for the wet weight of the biomass. The

biomass was dried and the weight was taken again which accounts for the dry weight. The difference between wet weight and dry weight gives the actual weight of the biomass.

Calcium Precipitation Efficacy

Malt Extract Agar (MEA) medium (malt extract 20 gL^{-1}, agar 15 gL^{-1}) (HiMedia, India), 2% urea supplemented with 100 mM calcium chloride. Membrane-sterilized 2% urea was then added to the sterilized MEA medium. The MEA plates were inoculated with pure isolated ureolytic fungal cultures, incubated at 28^0C and checked for calcium precipitation efficacy after 5 days.

Soluble Calcium Estimation

The method of determining calcium is by EDTA titration method described in Stocks-Fischer *et al.* (1999). AP1 medium was used to grow the fungal cultures. Membrane-sterilized 2% urea was then added to the sterilized AP1 medium prepared as per the protocol of Li *et al*. (2015). The fungal cultures were then inoculated in the medium and incubated in a shaker incubator at 30 ºC for 14 days.

Molecular Characterization and Phylogenetic Tree Analysis

Genomic DNA of the fungal isolates was isolated by using the HiPurA™ Fungal DNA Purification Kit followed by amplification of the Internal Transcribed Spacer (ITS) regions of fungal ribosomal DNA (rDNA) using the universal primers ITS1-F [5'-CTT GGT CAT TTA GAG GAA GTA A-3'] and ITS4-R [3'-CAG ACT T(G/A)T A(C/T)A TGG TCC AG-3'] to amplify the highly variable ITS1 and ITS2 sequences. The amplification was performed at a volume of 50 µL comprising 10 mM Tris-HCl, 50 mM KCl, 1.5 mM MgCl2, 0.25 mM each of deoxynucleoside triphosphates (dATP, dCTP, dGTP, and dTTP), 0.2 µM primers, 0.25 µL of 3 U/lLTaq DNA polymerase (Bangalore Genei, India), and 3 µL of the template DNA (approximately 100 ng). The reaction parameters were denaturation for 5 min at 94˚C, followed by 35 cycles consisting of denaturation for 1 min at 94˚C, annealing at 55˚C for 1 min, elongation at 72˚C for 2 min and cycling was completed by a final elongation step for 5 min at 72 ˚C. A control tube containing sterile water was used as a negative control. The pure amplified samples of the fungal ITS sequences were then sent to the Macrogen for sequencing (Seoul, South Korea). The sequencing results were compared using the Basic Local Alignment Search Tool (BLAST) program on NCBI and ITS gene sequence homology analysis was done using GenBank data. A phylogenetic tree was constructed using the neighbor-joining model of the MEGA 6.0 program.

Results and Findings

Isolation of fungi: The total number of pure fungal isolates obtained after plating on potato dextrose agar (PDA) media from each site is presented in Table 1.

Table 1: Isolates obtained from different sample sites from Mawsmai cave

Serial no.	Sample site	Number of pure isolates
1	Lime deposits inside cave	5
2	Middle cave substratum	22
3	Stony rock surface	9
4	Water flowing inside cave	7
5	Forest soil	12
6	Cave exit	17
7	Sandy substratum inside cave	5
Total isolates		77

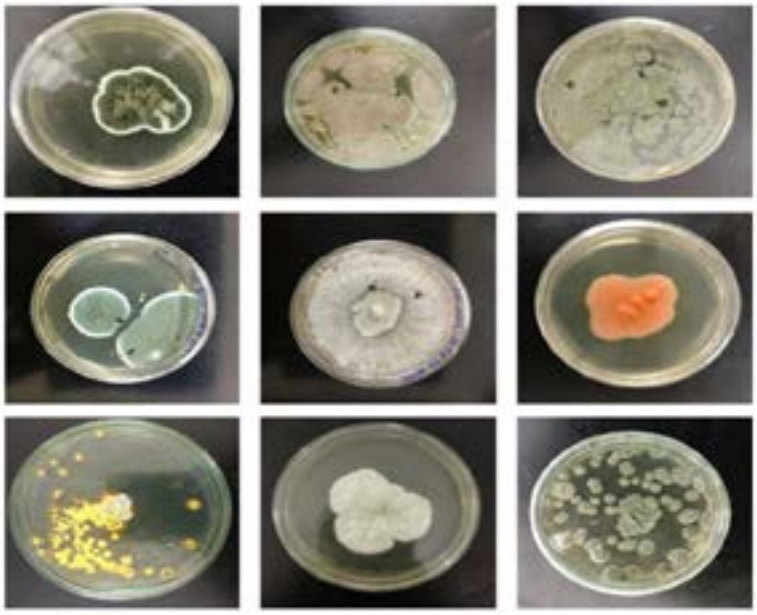

Fig. 1. Fungi isolated from Mawsmai cave

Fungal Staining using Lactophenol Cotton Blue Stain

The pure fungal isolates were stained using lactophenol cotton blue stain to study their characteristic morphology such as shape, size and arrangement of spores and hyphae. Upon staining and observation under the microscope the following genera of fungi were notably observed – *Aspergillus, Penicillium* and *Mucor*. Among the 77 fungi isolated, 25 isolates were subsequently selected based on differences in morphological characteristics observed under light microscope.

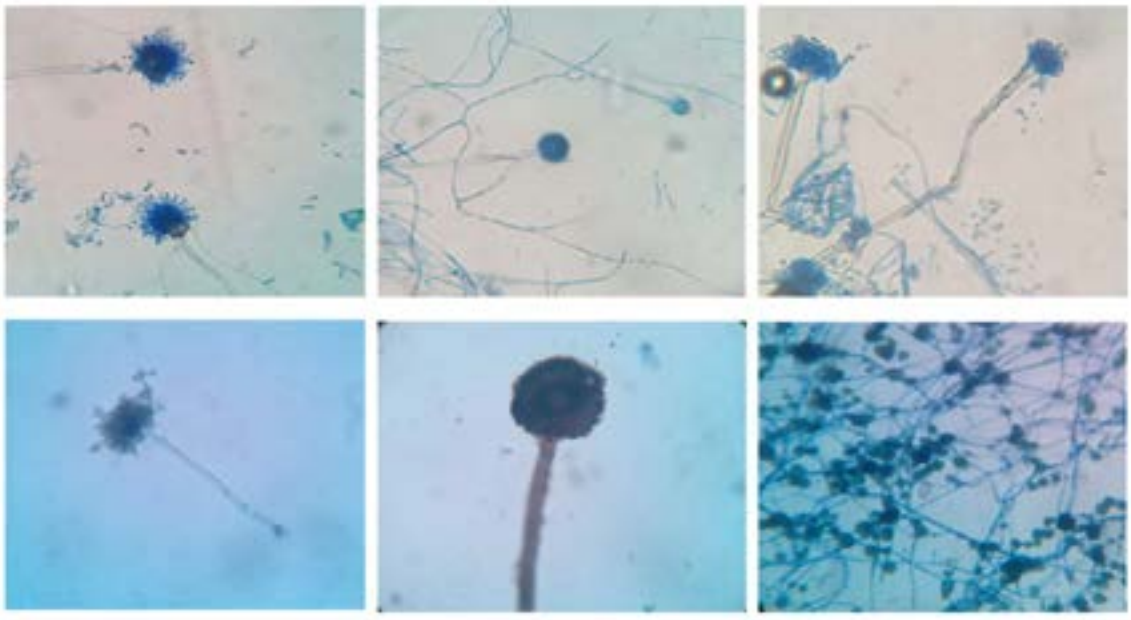

Fig. 2. Fungal isolates at 40X magnification

Urease Test for Selection of Ureolytic Fungi

After incubation in the isolation media, around 77 fungi were isolated amongst which 25 strains were subsequently shortlisted based on differences in morphological characteristics observed by light microscopy. Upon selection in urea media, 5 isolates (UF1, UF2, UF3, UF4 and UF5) displayed ureolytic activity based upon formation of pink colour. These cultures were then further investigated for calcification potential.

Fig. 3. Ureolytic fungi using Christensen Urea Agar media

Biomass Estimation Using Different Calcium Salts

As varying calcium sources have been reported to significantly affect microbial growth, the effect of different commonly used calcium sources (calcium chloride, calcium nitrate and calcium oxalate) on fungal biomass was investigated.

Table 2: Wet weight of fungal biomass in different calcium sources

Isolates	Weight of centrifuge tube (gm) (a)	Weight of centrifuge tube + wet fungal Biomass (gm) (a)	Wet Weight (gm) (b - a)
For Calcium Chloride			
UF1	16.66	18.51	1.85
UF2	16.32	16.11	2.79
UF3	15.50	17.40	1.9
UF4	15.51	16.97	1.46
UF5	15.95	17.13	1.18
For Calcium Nitrate			
UF1	14.28	16.47	2.19
UF2	15.56	16.72	1.16
UF3	14.40	15.72	1.32
UF4	14.45	19.10	4.65
UF5	No growth was observed		
For Calcium Oxalate			
UF1	16.67	18.92	2.25
UF2	16.18	17.51	1.33
UF3	15.18	16.16	0.98
UF4	15.84	17.13	1.29
UF5	15.96	16.64	0.68

Table 3: Dry weight of fungal biomass in different calcium sources

Isolates	Weight of centrifuge tube (gm) (a)	Weight of centrifuge tube + wet fungal Biomass (gm) (b)	Wet Weight (gm) (b - a)
For Calcium Chloride			
UF1	16.66	17.06	0.4
UF2	15.32	16.58	1.26
UF3	15.50	15.93	0.43
UF4	15.51	16.49	0.98
UF5	15.95	16.68	0.73
For Calcium Nitrate			
UF1	14.28	15.73	1.45
UF2	15.56	16.20	0.64
UF3	14.40	14.89	0.49
UF4	14.45	18.69	4.24
UF5	No growth was observed		
For Calcium Oxalate			
UF1	16.67	18.15	1.48
UF2	16.18	16.98	0.8
UF3	15.18	15.58	0.4
UF4	15.84	16.60	0.76
UF5	15.96	16.16	0.2

Table 4: Actual weight of fungal biomass in different calcium sources

Isolates	Wet weight (gm)	Dry weight (gm)	Actual weight (Wet weight - Dry weight) (gm)	% Biomass Ca^{2+} uptake (Actual weight/ wet weight)*100
For Calcium Chloride				
UF1	1.9	0.4	1.47	77.36
UF2	2.79	2.26	0.53	19
UF3	1.85	0.43	1.45	78.4
UF4	1.46	0.98	0.48	32.9
UF5	1.18	0.73	0.45	38.1
For Calcium Nitrate				
UF1	2.19	1.45	0.74	33.8
UF2	1.16	0.64	0.52	44.8
UF3	1.32	0.49	0.83	62.9
UF4	4.65	4.24	0.41	8.8
UF5	No growth was observed			
For Calcium Oxalate				
UF1	2.25	1.48	0.77	34.2
UF2	1.33	0.8	0.53	39.8
UF3	0.98	0.4	0.58	59.1
UF4	1.29	0.76	0.53	41.1
UF5	0.68	0.2	0.48	70.6

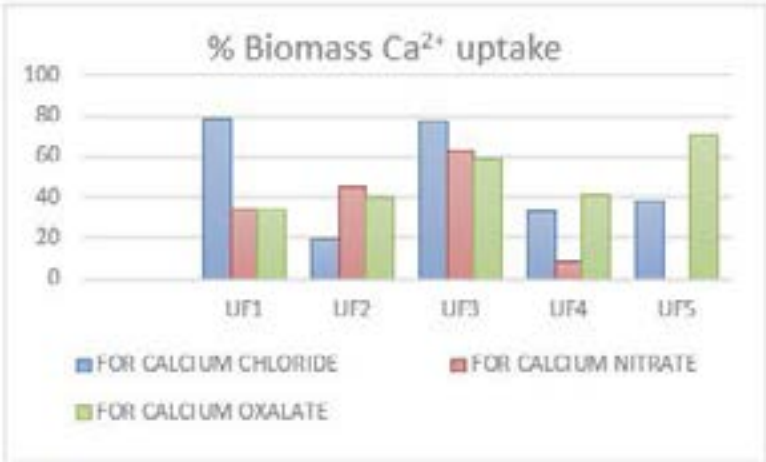

Fig. 4. Biomass Ca^{2+} uptake in different fungal isolates

After incubating the fungal isolates at 30°C for 14 days, the biomass production was calculated. It was observed that UF3 had the highest amount of biomass production among other isolates with Ca^{2+} removal of 78.4% in calcium chloride-supplemented media and 62.9% in calcium nitrate-supplemented media. However, it did not show the highest biomass production in calcium oxalate-supplemented media. Calcium oxalate-supplemented media was efficiently utilized by UF1. The isolate UF3 was followed by UF1 and UF4 in biomass production. Furthermore, calcium chloride is an overall preferred calcium source for growth among other calcium sources.

Calcium Precipitation Efficacy

It was observed that calcium chloride was better utilized by the fungal isolates. Hence, calcium carbonate precipitation efficiency was performed using calcium chloride as the calcium source. Upon incubation at 30°C for 5 days, precipitation zones were observed around the fungal cultures UF3 and UF1 on MEAU (MEA with urea) plates supplemented with calcium chloride. UF4 showed moderate precipitation zones. However, no calcium carbonate precipitation efficiency was observed in UF2 and UF5.

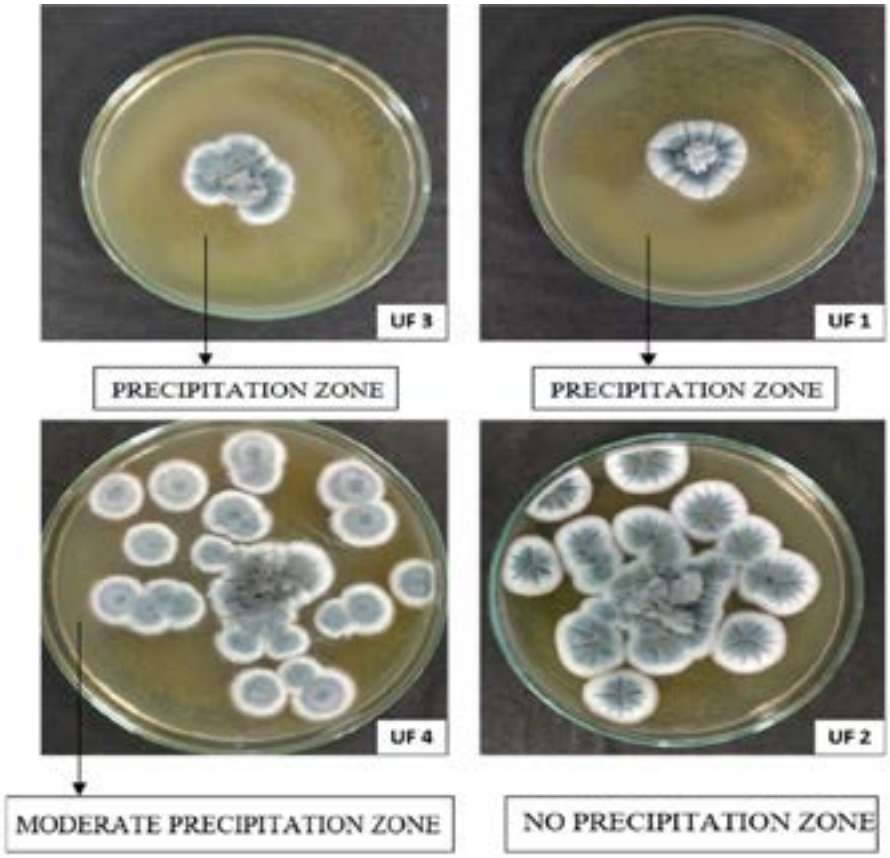

Fig. 5. Precipitation zones formed by different fungal isolates on MEAU plates

Soluble Calcium Estimation

Analysis of Ca2+ concentration was done by EDTA titration method.

Table 5: Estimation of soluble Ca^{2+} in the media for different fungal isolates

Isolates	Mass of Ca^{2+} in the solution (gm)	% Decrease in soluble Calcium content
UF1	0.008	68 %
UF2	0.014	44 %
UF3	0.008	68 %
UF4	0.013	48 %
UF5	0.011	56 %

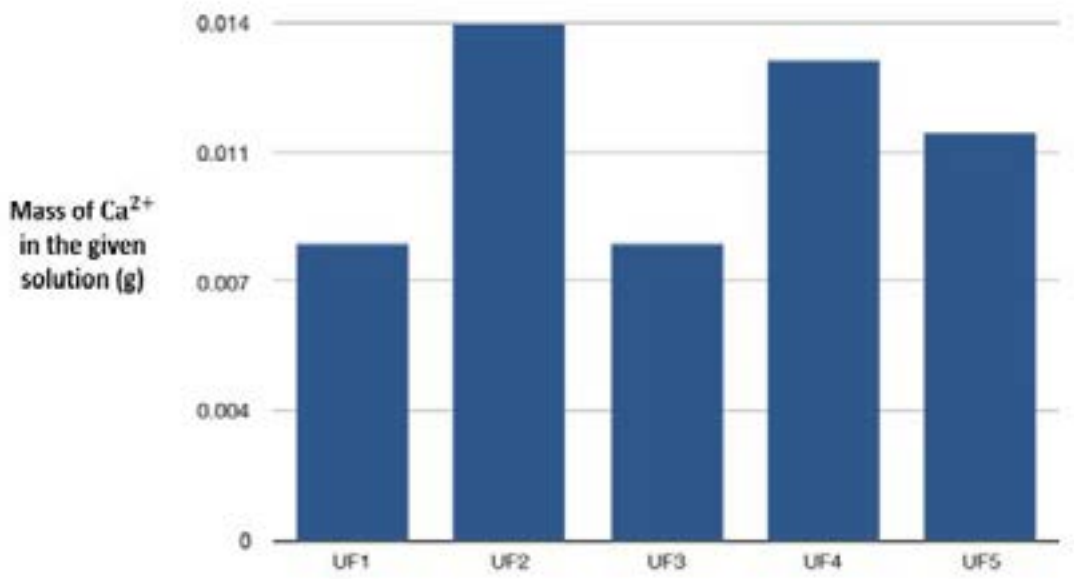

Fig. 6. Comparison of soluble Ca^{2+} in the media for different fungal isolates

The concentration of soluble Ca^{2+} was found to decrease in all fungal samples. When related with ureolytic profile, it was noticed that in this case lower soluble Ca^{2+} content was seen in case of more efficient ureolytic cultures. Lowest soluble calcium was noticed in case of UF3 and UF1 followed by UF5, UF4 and UF2. UF3 and UF1 showed 68% decrease in soluble calcium content. As the focus of the study was to select most efficient ureolytic cultures with high calcium precipitation efficiency, based upon the results above UF3 was chosen for molecular identification.

Molecular Characterization and Phylogenetic Analysis

ITS region amplified by polymerase chain reaction (PCR) from the genomic DNA of UF3 using universal primer pair ITS1 and ITS4 were sequenced and the sequencing results were compared using the Basic Local Alignment Search Tool (BLAST) program on NCBI and ITS region sequence homology analysis using GenBank data. Homology analysis of UF3 showed that the degree of sequence similarity of this strain to Aspergillus versicolor exceeded 99%. As shown in the phylogenetic tree constructed 7using MEGA 6, the strain UF3 is related to Aspergillus versicolor.

Fig. 7. Neighbour-joining phylogenetic tree based on ITS sequences of the isolate UF3

Discussion

Caves are important sources of novel fungi that can biomineralize metals. There are very few literature that have explored cave fungi of Meghalaya from the perspective of biomineralization. In this study, a variety of morphologically distinct fungal cultures were isolated from multiple sites within the Mawsmai cave. Following isolation and obtaining pure fungal cultures, lactophenol cotton blue staining was performed to study their characteristic morphology such as shape, size and arrangement of spores and hyphae under microscopic conditions. Fungal strains such as *Aspergillus, Penicillium* and *Mucor* were identified upon staining. In addition, 25 were selected based on differences in morphological characteristics. Preliminary studies were conducted in order to check for calcification potential of the isolated fungi under *in vitro* conditions to relate to the biomineralization potential. The isolated fungal cultures were evaluated for urease activity, calcium precipitation efficacy along with their potential to grow in various calcium sources and soluble calcium uptake capability. Based upon the performance of the fungal strains in the various tests, a single fungal isolate (UF3) was selected for molecular characterization. After the homology study and phylogenetic tree construction, the strain UF3 showed relation with *Aspergillus versicolor.*

Precipitation of carbonates has been observed to occur through microbial pathways such as ureolysis, denitrification, ammonification, photosynthesis, methane oxidation and sulfate reduction. Among these, ureolysis has been found to be the most energy efficient and having a high calcification potential, with urea being the nitrogen source of many organisms (Zhu and Dittrich, 2016). In this process, urea is hydrolysed by urease to produce carbonate, which is then hydrolysed to ammonia and carbonic acid. Bicarbonate is formed as a result when these products react with of water whereby releasing ammonium and hydroxide ions leading to pH increase. These reactions are pre-requisites in the process

of carbonate precipitation and are dependent on the availability of calcium and a high pH (Dhami *et al.*, 2017). Hence, screening and isolation of ureolytic fungi was done using Christensen Urea Agar Medium. Fungal cultures with high ureolytic activity were further study on calcification potential.

Biomass estimation was carried out for the selected ureolytic fungal cultures in different calcium sources supplemented with urea in order to determine the best calcium source for growth. It was observed that calcium chloride was the preferred calcium source over calcium nitrate and calcium oxalate. Since calcium chloride supported better fungal growth, further calcification studies were carried out with it. Calcium carbonate precipitation efficacy was determined using calcium chloride as calcium source. Carbonate precipitation was higher in UF3 followed by UF1 and UF4. UF2 and UF5 did not show any zones of precipitation. In addition, maintenance of alkalinity is a major driving force in calcium carbonate precipitation (Stocks-Fischer *et al.*, 1999). UF3 and UF1 showed the highest decrease in soluble Ca^{2+} (up to 68% decrease). Along with urease, carbonic anhydrase may also play an important role in carbonate mineralization (Li *et al*., 2004).

As per the BLAST analysis, UF3 showed 99% sequence identity with *Aspergillus versicolor*. Neighbour joining phylogenetic tree with an outgroup organism, *Rhizoctonia* sp., was constructed. *Aspergillus versicolor* is a slow-growing filamentous fungus commonly found in damp environments. Similar calcifying fungi can bear immense potential for several applications in environmental and civil engineering as removal of heavy metals, radionuclides, calcium carbonate cements, CO_2 sequestration through biomineralization.

Conclusion

From the preliminary investigation, it is evident that the isolated fungus is able to precipitate minerals. However, to confirm this finding, further tests such as SEM-EDX and other mineralogical analysis like energy dispersive X-ray spectrum (EDS) are required. In addition, quantitative estimation of urease and carbonic anhydrase will provide a better understanding of the fungus and its biomineralization potential. Identification of such fungi present an avenue of applications like bioremediation of heavy metals and in the microbially induced calcium carbonate precipitation (MICP) technology where robust calcitic minerals are designed for use in the construction industry.

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