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Research Article

Epilithic Microalgae Isolated from Biofilm on Borobudur Temple Stone

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ABSTRACT
Borobudur Temple is a historical heritage building located in an open area and made of porous building materials (stone materials). This condition makes the Borobudur Temple susceptible to various problems related to degradation and weathering. Biodeterioration of Borobudur Temple may be caused by activities of living organisms present in the biofilm of stone. Continuous monitoring and evaluation need to be carried out by observing and isolating the growth of micro-organisms, including epilithic microalgae. Therefore, this study aims to isolate and identify epilithic microalgae from the biofilm on Borobudur Temple stones. Epilithic microalgae were isolated to obtain a uni-algae and maintained under culture conditions. The morphological of microalgae were observed using light microscopy, while the 18S rRNA gene sequence determined the molecular identification of microalgae for eukaryotic and 16S rRNA sequence for prokaryotic. A total of nine epilithic microalgae were successfully isolated from the biofilm of Borobudur Temple stones. The isolated were identified as *Ankistrodesmus falcatus*, *Tetraselmis cordiformis*, *Pseudendoclonium arthropyreniae*, *Anabaena cylindrica*, *Nostoc gelatinosum*, *Oscillatoria limnetica*, *Messastrum gracile*, *Stigeoclonium aestivale*, and *Scenedesmus acuminatus*. This is the first study for the identification of microalgae from Borobudur temple stones. The isolates will be collected and will be used as a source for further study.

Keywords: 16S rRNA gene, 18S rRNA gene, epilithic algal, molecular identification, phylogeny, subaerial

INTRODUCTION
Borobudur Temple is located in Borobudur Village, Borobudur District, Magelang Regency, Central Java Province. This historical heritage temple compound was built in an open area on a modified hill, with a height of 265 meters above sea level, with a length of 121.66 meters, width 121.38 meters, and a height of 34.50 meters. The structure of the Borobudur Temple consists of nine terraces and a main stupa at the top. There are six rectangular terraces and three circular terraces, covering the Kamadhatu, Rupadhatu, and Arupadhatu levels. Borobudur Temple built using the andesite stone material from rivers around the Borobudur Temple with a total of ± 2,000,000 pieces of stones (Banindro, 2015; Salazar, 2018).

The restoration of Borobudur Temple has been carried out twice, the Dutch East Indies government carried out the first restoration under the leadership of Van Erp, and the second restoration was carried out by the Indonesian government chaired by Soekmono (Voûte & Voute, 1973; Gunarto, 2007; Banindro, 2015). Rehabilitation of Borobudur Temple has also been done after Mount Merapi's eruption in 2010 to clean up volcanic ash, which is chemically acidic and can damage this historic temple stone. Demolition of stone blocks has been carried out to improve the clogged water and drainage system, which is clogged with volcanic dust mixture mixed with rainwater, followed by reforestation and planting of trees in the surrounding environment to stabilize the temperature (Yulianto *et al.*, 2013; Khoirunnisa, Warsono, & Suryaningsih, 2014). After the Borobudur Temple has been restored and rehabilitated, it does not mean that the temple maintenance has been completed. There is no

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guarantee that the Borobudur Temple is free from damage, degradation, and weathering processes. Therefore, continuous monitoring and evaluation need to be carried out by observing and isolating the growth of micro-organisms, including epilithic microalgae covered on Borobudur Temple stones.

Epilithic microalgae are part of a group of peripheral microalgae that live attached to various substrates such as rocks/stones, corals, gravel, and other hard objects. The development and ability of epilithic microalgae are very dependent on the presence and condition of the substrate. Microalgae attached to temple stone are more permanent than microalgae attached to a living substrate because the living substrate will experience development and death. In contrast, in the substrate, inanimate objects do not experience changes such as damage or death. The presence of epilithic microalgae on surfaces over time can cause substantial damage, including physicochemical damage and aesthetic discoloration of stone objects such as facades of buildings and monuments (Garside, 2010; Bertuzzi *et al.*, 2017; Matteucci *et al.*, 2019).

Several studies have been reported that eukaryotic green micro-algae are the dominant organisms on the biofilm of monumental stones of temperate regions, where microalgae like the Cyanophyta group predominantly occur on similar substrates in the tropics (Song, Kim & Lee, 2012; Keshari & Adhikary, 2013; Nakajima, Hokoi & Ogura, 2015; Villa *et al.*, 2016). Research on the isolation and identification of microorganisms associated with moss on the Surface of the Borobudur Temple Stone has been carried out on the Actinomycetes group, as one of the monitoring and exploration of microorganism biodiversity in Borobudur Temple (Putri, Purbani, & Habibi, 2020). However, studies of epilithic microalgae isolated from biofilms in Borobudur Temple stones have never been reported. Studies on microbial populations living, including epilithic microalgae on stones of Borobudur Temple, need to be done as a starting point for successful conservation management and control. One of the most important steps in studying the epilithic microalgae ecology of Borobudur Temple stones is to identify the microalgae involved in biological damage/ biodeterioration. Therefore, this study aims to isolate and identify epilithic microalgae from the biofilm on Borobudur Temple stone, as a database that can complement studies on micro-organism in particular that can weather and reduce the aesthetic value of temples in Indonesia.

MATERIALS AND METHODS

Materials

The materials were used for this study: a sterile swab, scalpel, screw cap and bottles, Pasteur pipette, object and cover glass, rubber bulb, microscope (Olympus CKX41 and Olympus BX5), Olympus DP26 cameras, corning well cell, fluorescent lamps, shaker incubator, shaking bath, centrifuge, vortex mixer, microtube 2.0 ml, beat bitter, micropipette and tips, thermal cycler (PCR), electrophoresis apparatus, UV transilluminator, Gel Doc, BG11 medium, Genomic DNA mini kit (Plant) Geneaid, Go Taq Green kit, and universal primer 18S rRNA and 16S rRNA gene.

Methods

Samples were collected from the exposed biofilm stone surfaces of the different tiers of Borobudur Temple by gently scrapping the surfaces of stone with a sterile swab and scalpel. Each sample was placed into the BG11 medium (Keshari & Adhikary, 2013). Epilithic microalgae were isolated to obtain a uni-algae culture using the capillary micro-pipetting method under the light microscope (Olympus CKX41). The culture was maintained for 10-14 days at 25^{o}C below 30 µmol m^{-2} s^{-1} fluorescent light with 12h light cycle:12h dark in a shaker incubator (Anderson, 2005; Barsanti & Gualtieri, 2014). Morphological characteristics of epilithic microalgae were observed regularly under a microscope using Olympus BX5 light microscopy linked to the Olympus DP26 camera and a personal computer with the CellSens Standart application. The epilithic microalgae characteristics were then analyzed descriptively using a microalgae identification book (Naselli-Flores & Barone, 2009; Barsanti & Gualtieri, 2014; Kaštovský *et al.*, 2019).

Microalgae were identified based on the 18S rRNA gene sequence (for eukaryotic) and 16S rRNA gene sequence (for prokaryotic). Firstly, as much as 10 ml of microalgae culture was centrifuged for 4 minutes at 3000 rpm, a temperature below 10^{o}C. Genomic DNA was extracted using the Genomic DNA mini kit (Plant) Geneaid, following Genetica Science manufacturing protocol. Amplification was performed using 18S rRNA primers, 18SF, and 18SR for eukaryotic microalgae (Tale *et al.*, 2014). A partial gene sequence of 16S rRNA was amplified using universal primers, Forward 27F Algae, and Reverse 1510R for prokaryotic microalgae (Marsh & Nakatsu, 2014). The PCR composition was 12.5 µl GoTag® Green Master Mix, 10 µl Nuclease Free Water (NFW), 0.5 µl primer, 0.5 µl DMSO, and 1 µl DNA template. The PCR condition includes pre denaturation at 94°C for five minutes, 35 cycles of

denaturation at 94°C for one minute, annealing at 63°C (for eukaryotic) and 55°C (for prokaryotic) for one minute, and extension at 72°C for one minute, then final extension at 72°C for 10 minutes, and storage at 4°C (Ma *et al.*, 2008; Tale *et al.*, 2014).
The PCR products were visualized on 1% agarose gel under UV-transilluminator using Mupid Electrophoresis. Then, the gene fragment was sequenced by Macrogen. inc. The DNA sequence similarities were analyzed using the BLASTN program on the NCBI database server (http://www.ncbi.nlm.nih.gov/BLAST). A phylogenetic tree was constructed using Neighbor-Joining (NJ), 1000 bootstrap welding methods with the application of the Molecular Evolutionary Genetics Analysis (MEGA) program (Kumar *et al.*, 2018).

RESULTS AND DISCUSSION

We successfully isolated and purified nine isolates of epilithic microalgae from the biofilm on Borobudur Temple stone during this study. Based on isolates' morphological characteristics under a light microscope, the isolates identified to the division Chlorophyta and division Cyanophyta. Among the nine isolates, six isolates belong to Chlorophyta (or green algae) division, and three isolates belong to the Cyanophyta division. The isolates code belongs to the Chlorophyta division were M18-BR1, M18-BR2, M18-BR5, M18-BR13, M18-BR16, and M18-BR20, while the isolates code belongs to Cyanophyta were M18-BR7, M18-BR8, and M18-BR12 (Figure 1).

Molecular identification was carried out to support the identification of morphology and determine which types of isolates to the species level. Six isolates were identified based on the 18S rRNA gene sequence, and three isolates were identified based on the 16S rRNA gene sequence. The 18S rRNA gene sequence was determined, and BLAST analysis was performed, which confirmed that the isolate M18-BR1 has similarities with *Ankistrodesmus falcatus,* M18-BR2 has similarities with *Tetraselmis cordiformis*, M18-BR5 has similarities with *Pseudendoclonium arthropyreniae*, M18-BR13 has similarities with *Messastrum gracile,* M18-BR16 has similarities *Stigeoclonium aestivale*, and M18-BR20 has similarities with *Scenedesmus acuminatus*. The similarity values of these isolates were between 99.06-99.68 of the closest strain type (Table 1.).

Three isolates (M18-BR7, M18-BR8, and M18-BR12) were identified based on the 16S rRNA gene. These isolates have similarities with *Anabaena cylindrica* (M18-BR7), *Nostoc gelatinosum* (M18-BR8), and *Oscillatoria limnetica* (M18-BR12). The isolates have a homology percentage of 100, 99.31 %, and 99.04 % of the closest strain type (Table 2).

The Neighbor-Joining method's phylogeny tree construction was made with 1000 bootstrap replications in the Kimura 2 Parameter model, to

Figure 1. Microscopic photographs of epilithic microalgae in the biofilm of the Borobudur Temple stone.

Table 1. Identification result of epilithic microalgae isolates based on 18S rRNA gene.

Isolate code	Number of nucleotides	Sequence result	Percentage of homology
M18-BR1	609	*Ankistrodesmus falcatus* (MK159026.1)	99.06
M18-BR2	610	*Tetraselmis cordiformis* CCAC 0051 (MK460468.1)	99.93
M18-BR5	601	*Pseudendoclonium arthropyreniae* SAG467-2 (MF034609.1)	99.66
M18-BR13	609	*Messastrum gracile* CCMA UFSCar 622 (KT833593.1)	99.34
M18-BR16	624	*Stigeoclonium aestivale* EP SAG477-20 (KU948222.1)	99.64
M18-BR20	606	*Scenedesmus acuminatus* (AB037088.1)	99.68

Table 2. Identification result of epilithic microalgae isolates based on 16s rRNA gene.

Isolate code	Number of nucleotides	Sequence result	Percentage of homology
M18-BR7	1377	*Anabaena cylindrica* NIES19 (AF247592.1)	100
M18-BR8	1390	*Nostoc sp.* Leptogium gelatinosum cyanobiont (DQ185232.1)	99.31
M18-BR12	1381	*Oscillatoria limnetica* MR1 (AJ007908.1)	99.04

emphasize the identification process of the Basic Local Alignment Search Tool (BLAST). A phylogenetic tree showed that microalgae isolates were affiliation to nine species, the results obtained as shown in Figures 2 and 3.

Ankistrodesmus falcatus is a species of Chlorophyta in the family Selenastraceae. It is needle -like in shape, with gradually tapering ends. As seen in the morphology of the M18-BR1 isolate in Figure 1. Cells mostly of four arranged in cruciate flocky mucilaginous groups could be two to many cells in a colony. M18-BR2 isolate was identified as *Tetraselmis cordiformis* which belongs to the phylum Chlorophyta. These isolates are characterized by chloroplasts of intense green color, their marked cell bodies, the presence of pyrenoids in the chloroplasts, and skeletal walls produced by scales. Isolates M18-BR5 has a green appearance of cell morphology, unipolar or bipolar germination, the irregular shape of the filament, with branching cylinder attached to the surface, can be split into different directions and has a parietal chloroplast with a pyrenoid. Molecularly, this isolate was identified as *Pseudendoclonium arthropyreniae*.

M18-BR13 was identified as *Messastrum gracile* which belongs to phylum Chlorophyta in the family Selenastraceae. Morphologically, the cells are narrow, fusiform to semilunate in shape, the ends gradually tapered, curved. Colonies are solitary or multicellular with irregularly separated cells. There is one parietal chloroplast with a cell wall covered by a thin layer of diffuse mucus. M18-BR16 isolate has morphological characteristics of branched filamentous thalli, without rhizoid. The filamentous cells are cylindrical or spherical, containing chloroplasts of the parietal plate with pyrenoids. The branches are unilateral or alternating, not opposite, ending in sharp cells or hairs, with a thick gelatinous sheath. This isolate was identified as *Stigeoclonium aestivale*. InaCC M131 isolate is a green cell, with an elliptical and spindle shape, and has chloroplasts containing pyrenoid. This isolate was identified as *Scenedesmus acuminatus*.

In this study, we are showing for the first time the biodiversity of epilithic microalgae on the biofilm of Borobudur Temple Stone, identified by morphological and molecular traits. Chlorophyta was the dominant taxa from the biofilm on Borobudur Temple stone, with six genera (*Ankistrodesmus, Tetraselmis, Pseudendoclonium, Messastrum, Stigeoclonium,* and *Scenedesmus*). The genera are mostly soil algae. Biological colonization of the stone containing micro-organisms such as microalgae can originate from the surrounding soil, contaminating the stone after excavation. Also, stone inoculation can occur due to increased infiltration of groundwater and windblown dendrites. According to Soares *et al.* (2019), the Chlorophyta division is a cosmopolitan type that is easy to breed and adapt. They often occur in stone monuments and building stone walls, which are anthropogenic surfaces. Some of them are tolerant of harsh environmental conditions, including temperature fluctuations. This trait also causes Chlorophyta to be more diverse than other groups.

Two orders from the Cyanophyta group were also found from the biofilm in Borobudur temple stone, namely Nostocales and Oscillatoriales. *Anabaena* and *Nostoc* included in the Nostocales group were isolated from samples examined from the Borobudur temple stone's biofilm. Anabaena has

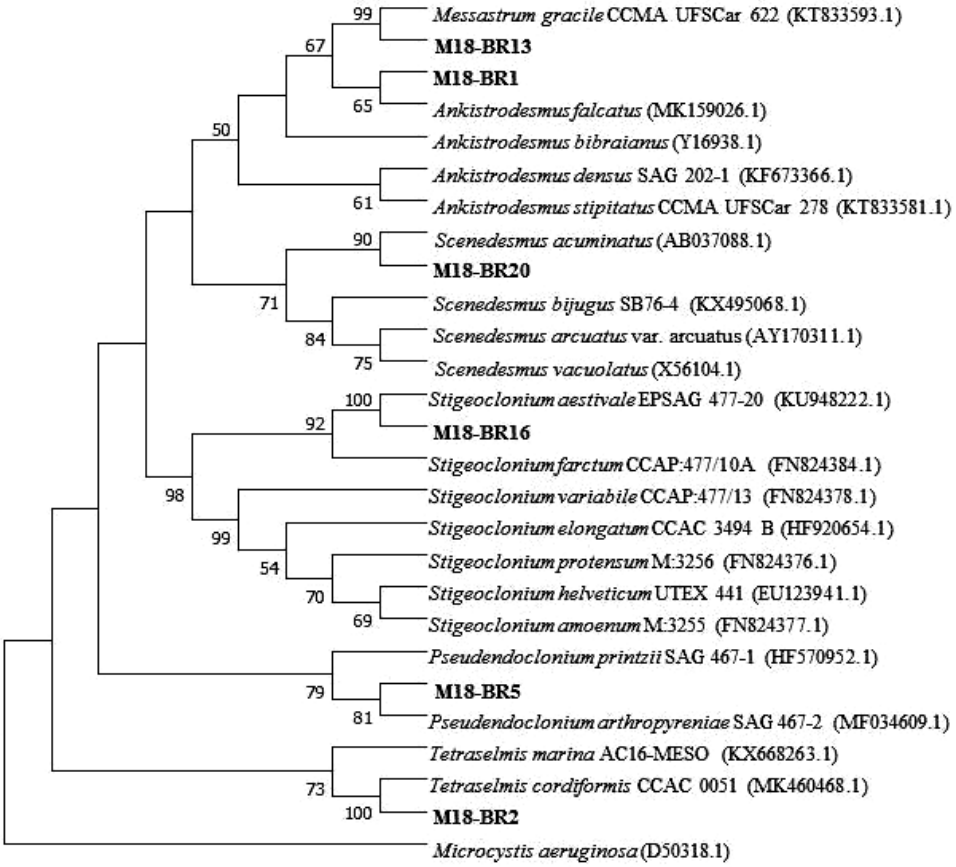

Figure 2. The phylogenetic tree of six selected epilithic microalgae isolates based on 18S rRNA gene sequences.

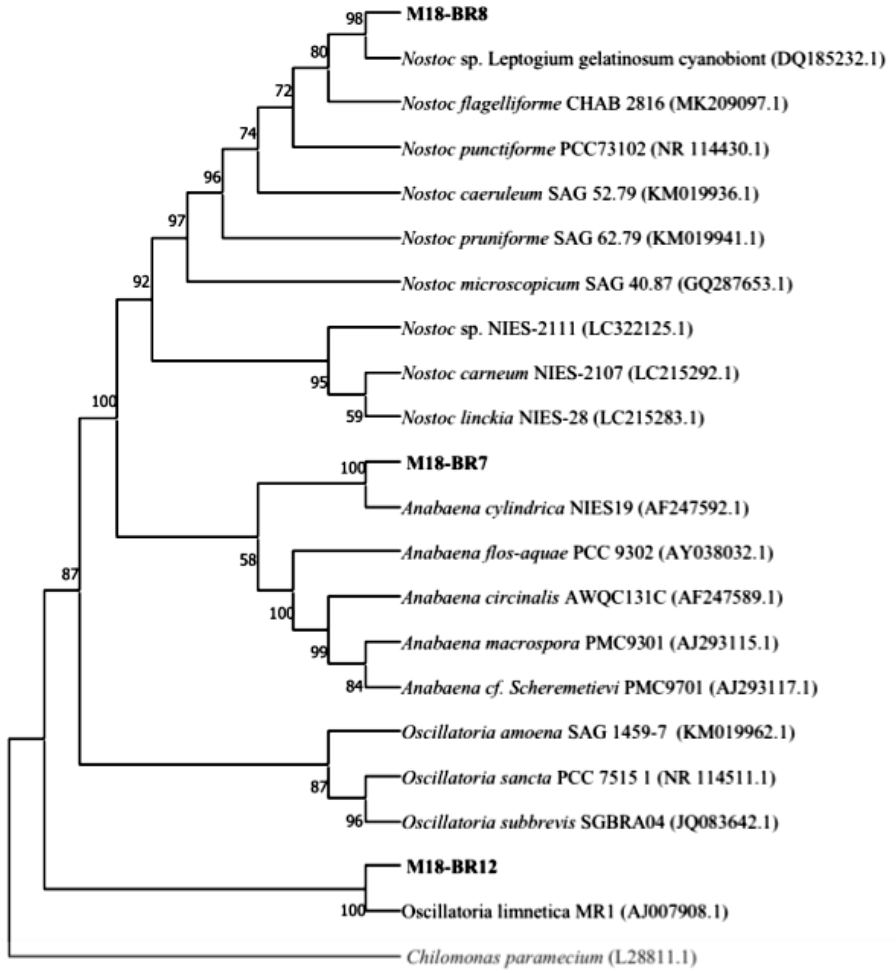

Figure 3. The phylogenetic tree of three selected epilithic microalgae isolates based on 16S rRNA gene sequences.

characteristic filaments with a long chain of vegetative cells ranging from square to round, or cylindrical. Heterocyst forms with a slightly higher length compared to vegetative cells. Nostoc also has cells arranged in beaded chains. Nostoc also has cells arranged in bead chains in the form of unbranched filaments. There are heterocysts with a filamentous structure that is twisted and folded by itself to form a spherical structure. Kaštovský *et al.* (2019) stated that epilithic Cyanophyta, such as Nostocales have thick outer envelopes and protective pigments to survive extreme environmental conditions such as cold, hot, and dry stone surfaces along with cryptoendollithic lichens, fungi, and bacteria. The *Oscillatoria limnetica* identified in this study completely lacks a sheath, and the cells may contain small vacuoles. The cells are typically considerably longer than broad, although the length to width ratio varies. Average filament length is quite steady across seasons. Occasionally the filaments are slightly curved, but never tightly coiled. The cells touch each other but are not closely joined as in most *Oscillatoria* species. Adhikary (2000) and Pandey (2011) reported that the Cyanophyta group belonging to the genera *Gloeocapsa*, *Lyngbya*, *Oscillatoria*, and *Tolypothrix* is the main component of the biofilm. These epilithic microalgae are enveloped by a colored sheath layer and occurred binding with finely textured soil particles on the temple stones. The microalgae communities that inhabit these stones are filamentous microalgae that can induce carbonate formation and cement deposition. In the present study, the Cyanophyta that colonized the temple stones were dominated by filamentous forms such as *Oscillatoria, Nostoc,* and *Anabaena*.

According to several studies, Chlorophyta and Cyanophyta can develop easily on stone surfaces because of their photoautotrophic nature. They are considered pioneering inhabitants of stone colonization, giving rise to colored patinas and incrustations (Tomaselli *et al.*, 2000; Vázquez-Nion *et al.*, 2016; Villa *et al.*, 2016; Popović *et al.*, 2018; Gallego-Cartagena *et al.*, 2020). The epilithic microalgae identified in this study are known to have a water reservoir in the form of a gelatinous sheath, bound by a strong molecular force, thus allowing the microalgae to colonize stone even in dry conditions. This sheath causes adhesion to the substrate. Sometimes the sheath can be pigmented and colored, which is an expression of various ecology and environmental adaptation stages, such as light intensity, temperature, nutrient availability, and cell age. Reduction in chlorophyll and phycocyanin, as well as increased carotenoids under low nitrogen conditions, can cause the epilithic microalgae to turn yellow-brown. Colored patinas and incrustations because of epilithic microalgae cause aesthetic damage to the temple building (Wynn-Williams *et al.*, 2002; Mayer, Dubinsky, & Iluz, 2016; Sonina *et al.*, 2018).

Many factors can cause the high number of taxa contained in the biofilm on the Borobudur Temple stone. One of them is the physicochemical properties of rocks that support the formation of photosynthetic communities such as epilithic microalgae that live in it, especially light, which affects the total community biomass. The availability of water also determines the successful colonization of green algae and Cyanophyta and allows subaerial biofilms to be formed by micro-organisms. According to Young *et al.* (2008) and Pinheiro *et al.* (2019), the population or community of immobilized micro-organisms on the surface of a stone, including green algae and Cyanophyta, can live in biofilms because biofilms can introduce large amounts of water into their structures so that the humidity and temperature balance is maintained. They play a role in binding cells to the substrate (adhesion) and cells to other particles together (cohesion).

Epilithic microalgae from biofilms in Candi rocks can be considered deteriogenic, because patinas that produce various colors can be aesthetically damaging (Javaherdashti *et al.*, 2009). Macedo *et al.* (2009) stated that the inhibition of epilithic microalgae colonization reduces the growth and heterotrophicity of fungi and bacteria and supports the accepted colonization sequence. These photosynthetic micro-organisms can provide nutrients for other communities' growth through the accumulation of the resulting biomass. They contribute to stone breaking directly and through synergistic interactions with heterotrophic micro-organisms such as fungi and bacteria. So the isolation and identification of epilithic microalgae are very important for further research on the control and prevention of biodeteriogenic processes.

CONCLUSION

Chlorophyta was the dominant taxa from the biofilm on Borobudur Temple stone, consisting of *Ankistrodesmus falcatus, Tetraselmis cordiformis*, *Pseudendoclonium arthropyreniae*, *Messastrum gracile, Stigeoclonium aestivale*, and *Scenedesmus acuminatus*.. Three species from the Cyanophyta group were also found, namely *Anabaena cylindrica*, *Nostoc gelatinosum*, and *Oscillatoria limnetica*. This study may help evolve a managerial plan for preventing the growth of epilithic microalgae in exposed temple stones and historic buildings to prevent damage. The isolated epilithic microalgae were then preserved and stored in the Indonesian Cultural Collection (InaCC) for further study on the ecology and physiology of

certain species of this micro-organism to understand their role in the process of stone colonization and biodeterioration.

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