MORPHOLOGICAL, MOLECULAR CHARACTERIZATION AND CONTROL OF CYANOBACTERIA FROM WALL PAINTINGS AT NIAH, SARAWAK, TEMBURONG AND PERAK CAVES IN MALAYSIA

M. A. Aisha[1] Fatma. M. A. El-Garj[2*] and W.O.Maznah[3]

[1,2]*Al-zintan University. Faculty of science, Zoology Department. Azintan – Libya,* [3]*School of Biological Sciences, University Sains Malaysia 11800 Penang. Malaysia,*

[*]*Al-zintan University. Faculty of science, Zoology Department. Azintan – Libya,*
E-mail:fatmagorj@yahoo.com

Corresponding Author: -
fatmagorj@yahoo.com

ABSTRACT

Malaysia is a country of numerous natural wonders, which include a collection of impressive cave formations. Found all over the country, these caves contain rock art and wall paintings. The samples were collected from wall surfaces painted caves of Niah, Sarawak, and Tempurung, Perak. Three cyanobacterial genera, have been characterized using a polyphasic approach by comparing phenotypic characteristics, using a light microscopy and scanning electron microscopy (SEM). Molecular characteristics, by confirmed their identification based on 16S rRNA gene sequences. The microflora from both sampling locations was represented by three cyanobacteria morphospecies, Synechococcus sp. (18.78%), Gloeocapsa sp. (12.20%), Chroococcus sp. (4.94%). The photomicrographs of all the dominant microalgae recorded. Synechococcus sp. a cylindrical oblong elliptical unicellular or sometimes 2 to 4 cells as a result of cell division. Under SEM Synechococcus sp. cells were cylindrical to oblong 2.6 µm in diameter and 3.8µm long. The cells were enclosed in hyaline mucilage and with cells usually aggregating in large numbers to form colonies. Gloeocapsa sp. cells were spherical, blue- green homogeneous contents, enclosed by wide colorless sheaths, of diameter 2-4µm, with sheaths 4-8µm. Chloroococcus sp. cells were spherical, sometimes hemispherical after division, united cell in colonies of two sheath, sheath is colorless and the individual cells were large; 20-28µm in diameter. According to DNA sequence analysis, cyanobacteria l was closely related to Synechococcus sp, (99% similarity), cyanobacteria ll was found to be similar to Gloeocapsa sp. (97% similarity) and cyanobacteria lll was Chroococcus sp. (95% similarity). This study, used chemicals agents to control cyanobacteria from both sampling locations, by (NaOCl), (Ca(OCl)2) and (H_2O_2). The result showed that, 5% H_2O_2, Ca(OCl)2 and NaOCl gave a significant difference ($p<0.05$) on the growth of cyanobacteria. However, the effect of 15% H_2O_2 was not significantly different ($p>0.05$) with Ca(OCl)2 and NaOCl at 10, 15, and 30 minutes exposure, only 5 minutes exposure was significant ($p<0.05$).

Keywords: *Malaysian caves, cyanobacteria, NaOCl, H_2O_2, Ca(OCl)2.*

INTRODUCTION

Malaysia has so many caves in terms of formation, size, age and limestone landscapes. Archaeological sites in the largest caves are been found throughout Malaysia. In the Niah Caves, situated in Sarawak, have been found wall paintings, wooden boat shaped coffins, and the site of a Palaeolithic human skull which is at least 40,000 years old [1,2]. But these archaeological resources, are exposed to growth of lampenflora detracts the natural beauty of rock paintings in cave walls, and threatens their archaeological value. So the paintings are damaged by the growth of microorganisms, such as through the loss of pigment, cracking, the disintegration of paint layers, and the formation of a biofilm on the painted surface. This is a real problem in Malaysian caves [3].

Cyanobacteria are Gram-negative prokaryotes as they lack inner organelles; the autotrophic cyanobacteria were once categorized as blue-green algae due to their apparent similarity to eukaryotic green algae. Later, with the introduction of electron microscopy and many other sophisticated instruments and techniques, it was realized that the blue-green algae are actually Gram-negative bacteria in terms of cell structure and chemistry [4,5]. Cyanobacteria have a rich and highly organized system of internal membranes which function in photosynthesis [6- 8].

Sources, where the relative humidity reaches 100%, cyanobacteria and ferns appear later, with cyanobacteria dominating. When microflora are covered with calcium carbonate, the amorphous mix of dead phototrophs and carbonate destroys heritage paintings and other objects of cultural value as it has been confirmed that eukaryotic microalgae can successfully and quickly colonize new stony surfaces around lamps. [9] reported that phototrophic organisms in Belgian caves were made up of 54% Cyanobacteria, 16% Chlorophyta and 30% Chrysophyta. In another study of caves in Italy, the make-up constituted 50% Cyanobacteria, 29% green algae and 21% diatoms. [10, 11] reported that in Slovenian caves, cyanobacteria contributed 69% of the identified taxa, while Chlorophyta (19%) and Chrysophyta (12%) represented the minor part of the community.

The chemical substances suitable for controlling the growth of microorganisms must fulfill the criteria of minimum side effects on the cave environment and its organisms, while providing high efficiency in suppressing the phototrophs. It is necessary to destroy microorganisms by chemical agents when a microorganism proliferates on large spaces, irregular surfaces, sediments or large surface areas. It is important to carry out a chemical control method in caves where it is difficult to destroy microorganisms with physical methods. There are many chemical agents for the elimination of microorganisms, such as Sodium hypochlorite NaOCl, Calcium hypochlorite $Ca(OCl)_2$ and Hydrogen peroxide H_2O_2. NaOCl is commonly known as bleach or liquid bleach. It is frequently used as a disinfectant or a bleaching agent [12]. $Ca(OCl)_2$ has a strong chlorine odour, which may not give sufficient warning of hazardous concentrations. Using H_2O_2 to control microorganisms will not damage the environment because hydrogen peroxide decomposes into water and oxygen. This research Objective to identify and eliminate the growth of the cyanobacteria inhabiting the cave walls of Tempurung and Niah caves.

MATERIALS AND METHODS

Study Area

The area selected for this study was the Niah Caves, situated in Miri, Sarawak to the north of Borneo, Malaysia (3.802 N-113.773 E), and Tempurung Cave, the Tempurung Cave is situated in Gopeng, Perak.

Isolation, Purification Cyanobacteria

Samples were collected from the walls of 19 sites in both caves with a scraper, toothbrushes and plastic sample bottles, and were kept in dark boxes. 5 g was inoculated into 250 ml sterile Erlenmeyer flasks containing 100 ml of Blue-Green Medium (BG-11) to grow the cyanobacteria that were possibly in the samples. The flasks were incubated for 30 days under dim conditions (about 0.27 μmol. photons m^{-2} s^{-1}) and room temperature, some of the flasks were incubated for 30 days under continuous illumination at an average intensity of 1500 Lux (24.75μmol photon.m^{-2}.s^{-1} or micro Einsteins, μE). Three replicates were prepared for isolation.

Morphological identification of cyanobacteria

Light microscopic identification of the cyanobacterial samples were all conducted by magnification of 10x, 20x, 40x or/and 100x, a small of the cyanobacterial solution drop was observations under light microscopy and Then recorded some pursuits such as color, size and cell shapes. SEM is used to examine surface topology and distribution of specimens as well as relationship among organisms at a high magnification. Conventional SEM preparation techniques include air drying; chemical fixation followed by critical point drying or freeze fixation followed by freeze-drying and critical point dried. The hexamethyldisilazane (HMDS method) method was used to prepare the cells of cyanobacteria grown in the liquid media [13,32-34].

Molecular identification

DNA extraction

Approximately 1 mg (fresh weight) of actively grown axenic cyanobacterial biomass was used to extract total DNA following the manufacturer's protocol, the cyanobacterial DNA was extracted using Wizard® Genomic DNA Purification Kit (Promega). DNA products were checked by agarose gel electrophoresis (0.8%). The resultant DNA was

stored at -20℃ until further use.

Polymerase chain reaction (PCR) amplification and DNA sequencing

The extracted cyanobacterial DNA was used for polymerase chain reaction (PCR) for the 16S rRNA gene using cyanobacteria-specific primers, we used forward and reverse recommended primers by [35]. The PCR reaction was performed avolume of 50 µl containing 1.5µl of 50mM MgCl2, 1.0µl of 10 mM dNTP mix, 0.5µl of DNAtemplate, 2µl of 5µ/µl Taq DNA polymerase, 2µl of 25 pmole of 16S primers [35], 5.0µl of 10X PCR buffer, and deionized water to a total volume of 50µl were added and mixed. Amplification cycles consisted of initial denaturing step at 94 °C /5 min, followed by 30 cycles of 94 °C denaturation /45 sec, 55 °C annealing / 30 sec, 72°C extension /1 min, and a final extension step at 72 °C /5 min. The PCR products obtained were analyzed using electrophoresis on 1 percent agarose gel. Then the purified products were sent for sequencing by First Base Laboratory Sdn. Bhd. The analysis of sequences was with the help of BLAST which is the Basic Local Alignment Search Tool (BLAST) provided by National Centre for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov/genbank). The DNA sequences were used to compare with other sequences in the GenBank using BLAST.

Chemical control of cyanobacteria growth

For this study, this chemical agents were selected to assess its anticyanobacteria effectiveness: sodium hypochlorite (NaOCl), calcium hypochlorite (Ca(OCl)2) and hydrogen peroxide (H_2O_2), were diluted to concentrations of 1, 5, 10, 15, 30 and 60 % to be tested against the cyanobacteria (Table 1).

Preparation of cyanobacteria inoculum for test chemical

1) Test media: BG-11 media was used for cyanobacteria.

2) Inoculum preparation: The cyanobacteria, which was grown in a 2L Erlenmeyer flask with 800 ml of specific media (BG-11), was incubated under suitable conditions (30 days under continuous illumination at an average intensity of 1500 Lux (24.75µmol photon.m^{-2}.s^{-1} or micro Einsteins, µE) provided by a white-cool fluorescent lamp at 25 ± 2°C) and after 4 to 5 days, the growth was visible. The growth phase was determined by plotting a growth curve (based on cell enumeration).

3) Chemical concentrations: from Table 1, 5 ml of [NaOCl, Ca(OCl)2 or H_2O_2] was added to 95 ml of BG-11 media with culture to obtain a concentration of 5%; the concentration of 15% was obtain from 15 ml of [NaOCl, Ca(OCl)2, or H_2O_2] with 85 ml of BG-11 media with culture; 30% was obtain from 30 ml of a chemical with 70% of BG-11 media with culture, and 60% was obtain from 40 ml of a chemical agent with 60% of BG-11 media with culture. The control sample was 100% of BG-11 media without chemicals, and with a young culture.

4) The cells were counted at intervals of 1, 2, 5, 10, 15, 30, 60 and 120 minutes for each chemical concentration (0, 5, 15, 30 or 60%) under light microscope (20x to 40x magnification). The data were recorded.

5) Statistical analysis**:** A scatter chart was inserted to compare pairs of values. The data were analyzed using a general linear model (univariate) (SPSS, 20) to test for possible significant differences in the independent variables (concentration, time and chemical agents). A p-value of less than 0.05 ($P< 0.05$) was considered significant [14- 17].

RESULTS

Morphological and molecular identification of the isolated cyanobacteria

Morphological identification of cyanobacteria

Across the both sampling locations, a three of cyanobacteria genera were identified using morphological characteristics. The most frequent genera by relative abundance of cell densities across all sites were *Synechococcus* sp (18.78%) this genus was abundant in the samples, *Gloeocapsa* sp. (12.20%), *Chroococcus* sp. (4.94%). The three cyanobacteria morphological characteristics were similar with the descriptions by [18,19]. Plates 1–3 show the photomicrographs of all the dominant cyanobacteria recorded. Plate 4 shows the SEM observation of *Synechococcus* sp. cells were cylindrical to oblong 2.6 µm in diameter and 3.8µm long. The cells were enclosed in hyaline mucilage and with cells usually aggregating in large numbers to form colonies.

Molecular identification of cyanobacteria

In this reaserch, isolated and morphologically identified three strains of cyanobacteria belonging to genera *Synechococcus* sp., *Gloeocapsa* sp. and *Chroococcus* sp., and confirmed their identification based on 16S rRNA gene sequences. The resulting sequences were analyzed using BLAST search from NCBI website. According to DNA sequence analysis, cyanobacteria l was closely related to *Synechococcus* sp, (99% similarity), cyanobacteria ll was found to be similar to *Gloeocapsa* sp. (97% similarity) and cyanobacteria lll was *Chroococcus* sp. (95% similarity). The summary of the information of the strains and BLAST search verses are presented in table 2.

Chemical Control of Cyanobacteria

Sodium hypochlorite (NaOCl)

NaOCl eliminated the cells of cyanobacteria, with 5% concentration (Table 3), 99.5 % of the cells were eliminated after 5 minutes of exposure. Treatment with the same concentration reduced cyanobacteria cells, from 3647.6 cell/ml to

zero after 10 minutes of exposure. With a 15% concentration, the cells were reduced from 3147 cell/ml to zero after 5 minutes of exposure (Table 4). With 30% NaOCl concentration, colonies of cyanobacteria was eliminated at all the exposure duration times (Figure 1). Post hoc analysis suggested that 5% of NaOCl was found to be statistically significantly different ($p<0.05$) from 5% H_2O_2 at all exposure duration, and there was no statistically significantly different ($p >0.05$) with 5% Ca(OCl)2. 15% of NaOCl was not significantly different ($p>0.05$) from Ca(OCl)2 at all exposure duration. Ca(OCl)2 at 15% concentration was not significantly different ($p>0.05$) with 15% H_2O_2 at 10, 15 and 30 minutes duration. Comparing the effect of the three chemical agents on the cyanobacteria at concentration of 5% and 15%, NaOCl was significantly different ($p<0.05$) fromonly H_2O2.

Calcium hypochlorite Ca(OCl)2

Five percent of Ca(OCl)2 concentration eliminated 99.5 % of cyanobacteria (Table 3) after just 1 minute of exposure. For all other concentrations, the colonies were removed entirely following treatment at all exposure duration (Figure 2). The growth of cyanobacteria treated with 5% Ca(OCl)2 was significantly different ($p<0.05$) with 5% H_2O_2 at all exposure duration, but the difference was not significant ($p>0.05$) when compared with 5% NaOCl. The effect of 15% Ca(OCl)2 on cyanobacteria growth was not significantly different ($p >0.05$) with 15% H_2O_2 and Ca(OCl)2 at 10, 15 and 30 minutes exposure. Comparing the effect of 5% and 15% Ca(OCl)2 with other two chemical agents on the cyanobacteria of the same concentration showed that the difference was significant ($p<0.05$) only from H_2O2.

Hydrogen peroxide (H_2O2)

Concentration of 15% H_2O_2 (Table 4) eliminated 98.1% of cyanobacteria after 2 hours and 92.2% were eliminated after treatment of 5% H_2O_2 for 2 hours (Table 3). (H_2O_2) was more effective in eliminating cyanobacteria (Figure 3). Post-hoc analysis showed that the treatment with 5% H_2O_2, Ca(OCl)2 and NaOCl gave a significant difference ($p<0.05$) on the growth of cyanobacteria However, the effect of 15% H_2O_2 was not significantly different ($p>0.05$) with Ca(OCl)2 and NaOCl at 10, 15, and 30 minutes exposure, only 5 minutes exposure was significant ($p<0.05$).

DISCUSSION

Morphological and molecular identification of cyanobacteria

Cyanobacteria is a widely distributed , often found in xeric habitats with high UV light levels [20-22] which agree with this study, also cyanobacteria was found to prevail especially in the low light zone, so their primary common stress factors are low light, humidity, nutrient deficiencies, rate of humidity and low temperature [10,26]. A cave is a place characterized by low temperature and humidity, which is more or less constant throughout the year [23]. Also these conditions made Tempurung and Niah Caves a suitable environment for the invasion of cyanobacteria. This study agreed with the study conducted by [21] who reported growth of *Synechococcus* sp. together with *Chroococcus* sp. and *Gloeocapsa* sp. In the dark, with light intensity of $0.01\mu E\ s^{-1}m^{-1}$ Chroococcacea species also increase under low levels of irradiation. Occurrence of *Chroococcus* sp. and *Gloeocapsa* sp. were reported in the dim and also on monuments exposed to daylight [24,25] reported the existence of dominant filamentous cyanobacteria in stable conditions of low light intensity in different caves. Cyanobacteria in the caves can survive even at photon flux densities lower than their photosynthetic compensation point [11]. The use of artificial lights and influx of visitors in Tempurung and Niah caves facilitates the growth of these microorganisms. It was reported that visitors brought a substantial amount of soil and organic compounds present in their breath and sweat increased the concentration of carbon dioxide in the caves [27]. These microorganisms are nuisance and responsible for the destruction of mural paintings on cave walls. Discoloration of the paintings was due to the pigments and metabolic products excreted by the microorganisms [28].

Cyanobacteria can be grouped morphologically into unicellular, spherical shapes that form singly, in pairs or colony, and filamentous that are unbranched, falsebranched or branched form [26]. According to the above mentioned morphological characterization, in this study isolated and morphologically identified three of cyanobacteria belonging to genera *Synechococcus* sp., *Gloeocapsa* sp. and *Chroococcus* sp. and confirmed their identification based on 16S rRNA gene sequences. the sequence analysis of genes encoding smallsubunit ribosomal RNA (16S rRNA) is the most promising approach to be used in identification, comparison, and phylogenetic classification of prokaryotes, and can be found relationships among all cyanobacterial species [36]. The previous studies on cyanobacteria identification based on 16S rRNA gene with percentage similarity ranging between ≤ 99%≥ 95% to define a genus [37], according that, The BLASTn result analysis of 16S rRNA for the three cyanobacteria isolates in this study, thus were determined to be within the genera *Synechococcus* sp. *Gloeocapsa* sp. and *Chroococcus* sp.

Chemical control of cyanobacteria

Sodium hypochlorite and Calcium hypochlorite are bleaches, strongly oxidative chemical compounds and used as a disinfectant [38]. There are several studies have been used different concentrations of Sodium hypochlorite and Calcium hypochlorite solution to eliminate cyanobacteria communities in caves [16,29,30]. The results of the present study demonstrated the treatment with 5% sodium hypochlorite concentration effectively eliminated cyanobacterial colonies/cells, compared to the colonies in the control, 10 min treatment, also 5% of Calcium hypochlorite concentration was sufficient to destroy the cyanobacterial colonies/cells, compared to the colonies in the control, 5 min treatment. These results concur with those reported by [29] and [30] an average cleaning efficiency of 80% was achieved by applying a 5% solution of sodium hypochlorite. Also, the results of the present study demonstrated the treatment with 15% sodium hypochlorite and 15% Calcium hypochlorite concentration reduce time of exposure. In many caves,

treatment with sodium hypochlorite does not cause any significant damage to the formations and has a satisfactory end result [16]. Contrary to that, [39] reported that with ten minutes of treatment and after 17 hours, 41 mg/m^2 of rock was dissolved. [29,31] reported that the use of sodium hypochlorite may represent a negative burden for the environment in the cave, as the products resulting from its oxidation are carbon dioxide, water and chloride ions, and it may also cause the release of chlorine gas. For these reasons, several authors have suggested hydrogen peroxide as an alternative agent. [31] suggested use of hydrogen peroxide for suppressing lampenflora growth, by a 15% solution of hydrogen peroxide, is sufficient to kill phototrophic organisms if it is applied many times. The results of the present study demonstrated the treatment with 5% and 15% of hydrogen peroxide enough to destroy cyanobacteria, 120 min treatment (Table 3 & 4 respectively). [17] suggested the following procedure to eradicate lampenflora by spraying with hydrogen peroxide and washing is only required once every six months to a year.

CONCLUSIONS AND RECOMMENDATIONS

Based on morphology and 16S rRNA gene sequence data analysis. We isolated and identified three cyanobacterial strains attained from selected caves of Niah, Sarawak, and Tempurung, Perak, Malaysia. The closest related taxa to the three strains were *Synechococcus* sp., *Gloeocapsa* sp. and *Chroococcus* sp. as determined by 16S rRNA gene sequence analysis using the BLAST search program.

These cyanobacteria are corroding the cave paintings. To control this harmful growth of cyanobacteria, disinfectants such as sodium hypochlorite, calcium hypochlorite and hydrogen peroxide were tested on the cyanobacteria to study their efficiency in eradicating the cyanobacteria. (NaOCl) and Ca(OCl)2 were the most appropriate for use as a chemical agents to eliminate the cyanobacteria. However, these chemical agents emit a very strong odour and thus, unsuitable for use inside the caves, which might not be well-aerated. An open space which is well-aerated would be a better place for the application of these two types of chemical treatment. (H_2O_2) was less toxic compared with (NaOCl) and Ca(OCl)2. Chemical methods used to control the growth of microorganisms should not pollute the environment of caves. For this reason, to control the growth of cyanobacteria, this study suggests investigating other methods such as physical and biological methods.

REFERENCES

[1] Price, L. (2006). Caves and caving in Malaysia. *Caves and limestone hills of malaysia*http://www.cavesofmalaysia.com/

[2] Barker, G., Piper, P. J., & Rabett, R. J. (2009). Zooarchaeology at the Niah Caves, Sarawak: Context and Research Issues. *International Journal of Osteoarchaeology*, *19*, 447–463.

[3] Rajendran, R., & Prasad, N. (2012). A study on the indoor and outdoor microflora associated with the biodeterioration of mural paintings at Sekharaipuram Vishnu temple, Adakkaputhur, Palakkad, Kerala, India *International Journal of Environmental Sciences and Research,1*(4), 104-108 .

[4] Sigee, D. C., Glenn, R., Andrews, M. J., E.G.Bellinger, R.D.Butler, H.A.S.Epton, et al. (1999). Biological control of cyanobacteria: principles and possibilities. *Hydrobiologia* 161–172.

[5] Muthukumar, C., Muralitharan, G., Vijayakumar, R., Panneerselvam, A., & Thajuddin, N. (2007). cyanobacterial biodiversity from different freshwater ponds of thanjavur, tamilnadu (india). *Acta botanica malacitana 17*, 17-25.

[6] Prescott, G. W. (1962). *Algae of the western great lakes area with an lllustrated key to the genera of desmids and freshwater diatoms* U. S. A.: WM. C. Brown Company Publishers pp 932-977.

[7] Fay, P., & Baalen, C. V. (1987). *The cyanobacteria* New York, Oxford: Elsevier 87-608.

[8] Sincy, J. (2005). ecological and biochemical studies on cyanobacteria of cochin estuary and their application as source of antioxidants. india. pp 159-218.

[9] Garbacki, N., Ector, L., Kostikov, I., & Hoffmann, L. (1999). Contribution a` l"e´tude de la flore des grottes de Belgique *Belgian Journal of Botany*, *132*(**1**), 43-76.

[10] Smith, T., & Olson, R. (2007). A Taxonomic Survey of Lamp Flora (Algae and Cyanobacteria) in Electrically Lit Passages within Mammoth Cave National Park, Kentucky. *International Journal of Speleology, 36*(**2**), 105-114.

[11] Mulec, J., Kosi, G., & Vrhovsek, D. (2008). characterization of cave aerophytic algal communities and effects of irradiance levels on production of pigments. *Journal of cave and karst studies, 70* (**1**), 3–12.

[12] Cahn, A. (Ed.). (1994). Proceedings of the 3rd World Conference on Detergents on Detetgents:Global perspectives. *World Conference on Detergents: Global Perspectives (3rd: 1993: Montreux, Switzerland,* 279.

[13] Al-Fawwaz, A. T. S. (2010). Biosorption of heavy metals from aqueous solutions by green algae isolated from rivers in Penang, Malaysia. PhD Thesis, Universiti Sains Malaysia, Penang, Malaysia. 183pp.

[14] Eliopoulos, G. M., & Eliopoulos, C. T. (1988). Antibiotic combinations: should they be tested. *Clinical microbiology 1*(**2**)139–156.

[15] Michael E. Klepser, Erika J. Ernst, Russell E. Lewis, Michael E. Ernst, & Pfaller, M. A. (1998). Influence of test conditions on antifungal time-kill curve results: proposal for standardized methods. *Antimicrob agents chemother. , 42*(**5**), 1207–1212.

[16] Iliopoulou-Georgoudaki, J., Pantazidou, A., & Theoulakis, P. (1993). an assessment of cleaning photoautotropic micro lora: the case of "perama" cave, ioannina greece. *Memoires e biospeologie, tome xx*, 117-120.

[17] Grobbelar, J. U. (2000). Lithophytic algae: A major threat to the karst formation of show caves. *Journal of Applied Phycology 12*, 309–315.

[18] Smith, G. M. (1950). *The fresh water algae of the United States*. New York Toronto London McGraw-Hill book company. pp 537-725.

[19] Prescott, G. W. (1962). *Algae of the western great lakes area with an lllustrated key to the genera of desmids and freshwater diatoms* U. S. A.: WM. C. Brown Company Publishers pp 932-977.

[20] Albertano, C. (1999). Structural interactions among epilithic cyanobacteria and heterotrophic microorganisms in Roman hypogea *Microbial Ecology, 38*, 244-252.

[21] Cennamo, P., Marzano, C., Ciniglia, C., Pinto, G., Cappelletti, P., Caputo, P., et al. (2011). A survey of the algal flora of anthropogenic caves of campi flegrei (naples, italy) archeological district. *Journal of cave and karst studies, 74* (**3**), 243–250.

[22] Lamprinou, V., Danielidis, D. B., Economou-Amilli, A., & Pantazidou, A. (2012). Distribution survey of Cyanobacteria in three Greek caves of Peloponnese. *International Journal of Speleology, 41*(**2**), 267-272.

[23] Hoffmann, L. (1989). Algae of terrestrial habitats. *Botanical Review, 55*, 77–105.

[24] Scheerer, S., Ortega-Morales, O., Gaylarde, C., & Ahmad, M. (2009). Microbial deterioration of stone monuments--an updated overview. *Advances in Applied Microbiology, 66*, 97-139.

[25] Marti´Nez, A., & Asencio, A. D. (2010). Distribution of cyanobacteria at the gelada cave(spain) by physical parameters. *Journal of cave and karst studies, 72*(**1**), 11–20.

[26] Czerwik-Marcinkowska, J., & Mrozińska, T. (2011). Algae and cyanobacteria in caves of the polish jura.
Polish Botanical Journal, 56(**2**), 203–243.

[27] Cennamo, P., Marzano, C., Ciniglia, C., Pinto, G., Cappelletti, P., Caputo, P., et al. (2012). A survey of the algal flora of anthropogenic caves of campi flegrei (naples, italy) archeological district.*Journal of Cave and Karst Studies 74*(**3**), 243–250.

[28] Rajendran, R., & Prasad, N. (2012). A study on the indoor and outdoor microflora associated with the biodeterioration of mural paintings at Sekharaipuram Vishnu temple, Adakkaputhur, Palakkad, Kerala, India *International Journal of Environmental Sciences and Research,1*(4), 104-108 .

[29] Mulec, J. A., & Kosi, G. (2009). lampenflora algae and methods of growth control. *Journal of cave and karst studies, 71* (**2**), 109–115.

[30] Cigna, A. A. (2011). Show cave development with special references to active caves. *Sociedade Brasileira de Espeleologia, 4*(**1**) 16.
[31] Faimon, J., Sctelcl, J., Kubesova´, S., & Zimak, J. (2003). Environmentally acceptable effect of hydrogen peroxide on cave "lamp-flora", calcite speleothems and limestones. *Environmental Pollution, 122*(**3**), 417- 422.
[32] Goldstein JI, Newbury DE, Echlin P, Joy DC, Fiori C, Lifshin E. (1981). Scanning Electron Microscopy and X-Ray Microanalysis. Plenum Press New York and London.
[33] Hayat MA. (1989). Principles and techniques of electron microscopy.Biological applications. Macmillan Press LTD Houndmills and London..
[34] Hernández Mariné M, Clavero E, Roldán M. (2004). Microscopy methods applied to research on cyanobacteria. Asociación Ibérica de Limnología, Madrid Spain. (0213-8409).
[35] Neilan BA, Jacobs D, Del Dot T, Blackall LL, Hawkins PR, Cox PT, et al. (1997). rRNA sequences and evolutionary relationships among toxic and nontoxic cyanobacteria of the genus Microcystis. International Journal of Systematic Bacteriology;47(693-697).
[36] Nubel U, Garcia-Pichel F, Muyzer G. (1997). pcr primers to amplify 16s rrna genes from cyanobacteria. applied and environmental microbiology. 63(8):3327-32.
[37] Bosshard PP, Abels S, Zbinden R, Bo¨ttger EC, Altwegg M. (2003). Ribosomal DNA Sequencing for Identification of Aerobic Gram-Positive Rods in the Clinical Laboratory (an 18-Month Evaluation). Journal Of Clinical Microbiology. 41(9):4134-40.
[38] Hebelka J. (2014). Methodology of Lampenflora removal in cave acceible for tourit. The cave adminitration of the Czech Republic. Pruhonice:15.
[39] Bertolani M, Cigna A, Maccio S, Morbidelli L, Sighinolfi G. (1991). The karst system "Grotta grande del vento – Grotta del Fiume" and the conservation of its environment. In: Sauro U. (Ed.). In: Proceedings of the International Conference on Environmental changes in karst areas. Dipartimento di Geografia UoP, editor.; p. 289-98

Table 1: Preparation of chemical concentrations (%) (sodium hypochlorite (NaOCl), calcium hypochlorite (Ca(OCl)2) and hydrogen peroxide (H_2O_2)

Chemical concentrations (%)				
0%	5%	15%	30%	60%
100 ml specific media with culture from exponential phase	5 ml chemical agents + 95 ml specific media with culture from exponential phase	15 ml chemical agents + 85 ml specific media with culture from exponential phase	30 ml chemical agents + 70 ml specific media with culture from exponential phase	60 ml chemical agents + 40 ml specific media with culture from exponential phase

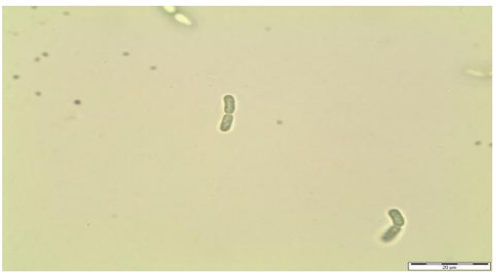

Plate 1: *Synechococcus* sp. a cylindrical oblong elliptical unicellular or sometimes 2 to 4 cells as a result of cell division.

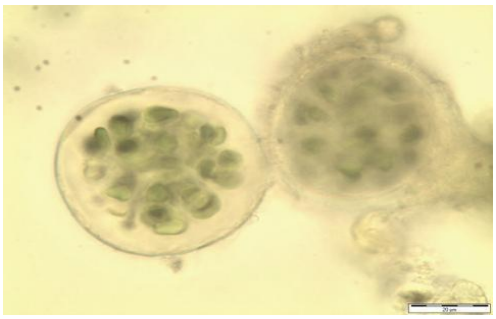

Plate 2: *Gloeocapsa. aeruginosa* cells are spherical, blue- green homogeneous contents, enclosed by wide colorless sheaths, of diameter 2- 4µm, with sheaths 4-8µm.

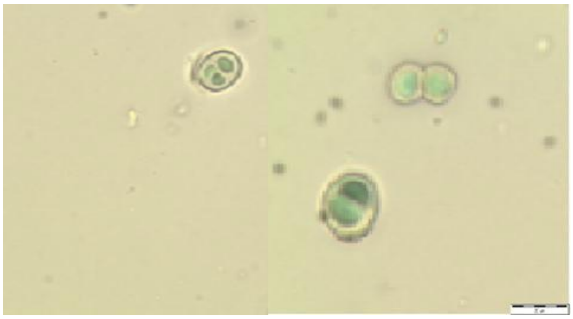

Plate 3: *Chloroococcus* sp. cells arespherical, sometimes hemispherical after division, united cell in colonies of two sheath, sheath is colorless and the individual cells were large; 20-28µm in diameter.

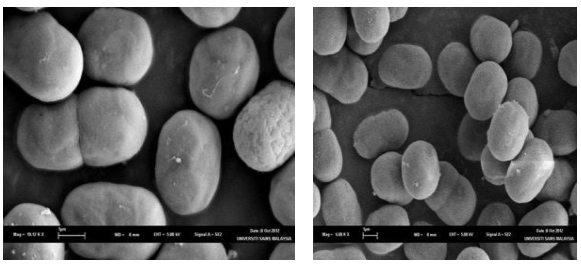

Plate 4: *Synechococcus* sp. under SEM observation

Table 2: BLAST search results of cyanobacteria strains used in this study based on 16S rRNA gene sequence analysis.

Samples	Sequence Similarities	Accession No.	Identity (% Homology)
1. cyanobacteria I	*Synechococcus* sp. TAG 16S	AF448066.1	99%
2. cyanobacteria II	*Chroococcus* sp. JJCV 16S	AM710385.1	97%
3. cyanobacteria III	*Gloeocapsa* sp. HKAR-9 16S	KJ470767.1	96%

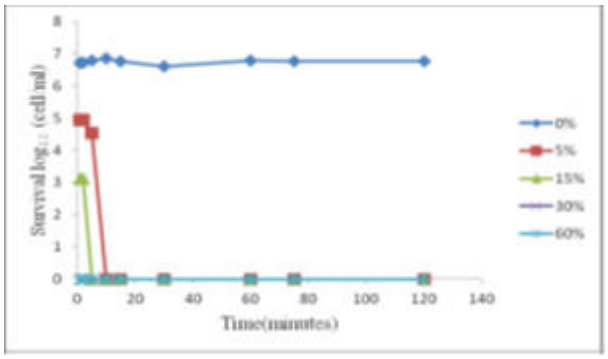

Figure 1: Treatment using sodium hypochlorite (NaOCl) to eliminate *Synechococcus* sp. Growth

Table 3: Effect of three chemical agents at 5% concentration towards cyanobacteria (percent elimination, 100%)

Experimental duration (minutes)	Elimination (%)		
	(NaOCl)	Ca(OCl)2	(H_2O2)
1	98.3	99.5	50
2	98.4	99.8	68.7
5	99.5	100	85.1
10	100	100	78.9
15	100	100	87.1
30	100	100	84.8
60	100	100	90.0
75	100	100	92.
120	100	100	92.2

Table 4: Effect of three chemical agents at 15% concentration towards cyanobacteria (percent elimination, 100%)

Experimental duration (minutes)		Elimination (%)	
	(NaOCl)	Ca(OCl)2 (%)	(H_2O2)
1	99.5	100	83.2
2	99.9	100	96.8
5	100	100	96.8
10	100	100	97.7
15	100	100	97.5
30	100	100	97.6
60	100	100	97.6
75	100	100	97.9
120	100	100	98.1

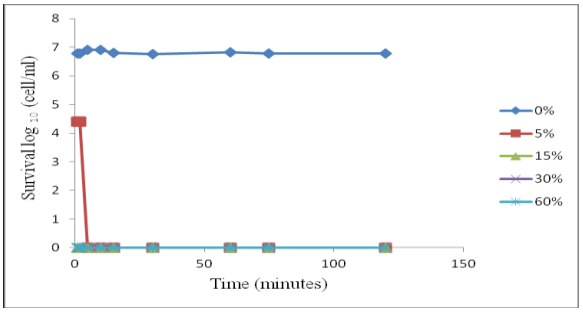

Figure 2: Treatment using calcium hypochlorite Ca(OCl)2 to eliminate *Synechococcus* sp. growth

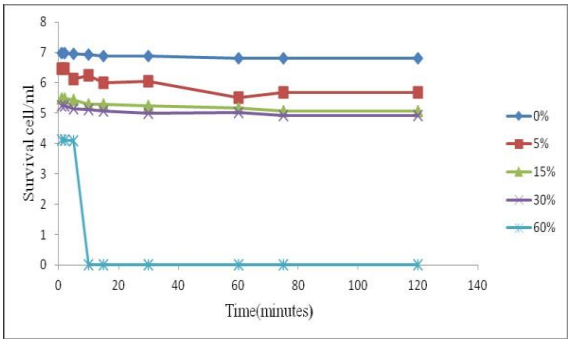

Figure 3: Treatment using hydrogen peroxide (H_2O_2) on *Synechococcus* sp. growth