CARL VON OSSIETZKY universität OLDENBURG

Rock Inhabiting and Deteriorating Fungi from Carbonate Monuments of Persepolis - Isolation, Characterization, and Inhibitory Treatment

Submitted by Parisa Mohammadi

Oldenburg, 2007

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Thesis for the awarding
of the degree of a PhD in Science
(Dr. rer. nat.)

Submitted to
the Faculty of Mathematics and Science of the
Carl von Ossietzky University, Oldenburg
by Parisa Mohammadi
born 22.05.1966
in Tehran

Supervisor and first referee: Prof. Dr. Dr. h.c. mult. Wolfgang. E. Krumbein

Second referee: Prof. Dr. Jürgen. Schneider

Date of disputation: 15 May 2007

To Reyhaneh and Hanieh

Zur Homepage der Dissertation

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1 Introduction

1.1 Aim of the Work

The problem of the deterioration of monuments made of rock is particularly relevant in countries like Iran being rich in such cultural heritage. According to UNESCO data, Iran holds the tenth rank in the list of countries that hold archaeological areas, statues, buildings, mosques, churches, museum objects and monuments belonging to the world cultural heritage which has been known up to now. The ruins of Persepolis, the sampling site for this thesis, contain an impressive display of temples and monuments, stairways and statues with engraved figures. It was the main aim of this thesis to identify biological damage functions caused by subaerial biofilms on the Persepolis stone monuments. Different biodeteriorative agents were to be identified on and in the stone materials of these monuments. To isolate the fungal rock flora of Persepolis and to characterize the isolates by molecular techniques was another aim of this thesis.

There are different techniques to control and prevent the development of biodeteriogenic organisms growing on and in monuments made of rock. Chemical inhibition agents are most frequently employed. However, diverse biodeteriorating organisms may display different reactions and susceptibility to different treatments. Therefore it is necessary to assay biocidal activity before applying this kind of products to artefacts. Several important prerequisites must be analysed before suggesting and exerting any kind of treatment. For this reason efficiency of some chemicals to inhibit biofilm growth was assayed in *in vitro* experiments. Two methods have been used for evaluation: plate counting agar and fluorescent activity stains. CFU is the classic method, which is in use to evaluate the efficiency of chemicals by counting cultivable cells. But some of the microorganisms and especially the aggressive black yeast-like microcolonial fungi (MCF) are difficult to cultivate. Some of these fungi have an extremely slow growth rate and others produce microcolonies that consist of only a few hundred of cells, which can be underestimated regarding the cytotoxicity of biocides. To overcome these problems, a fluorometry technique was further developed and improved and used to assay the efficiency of inhibiting chemicals on rock fungi. For this reason, the work concentrated also on the efficiency of fluorometric analytical techniques in the control of rock treatment success. In this study, for

the first time, viability testing by fluorescent stains on rock fungi was compared with viability of cells by the classical cultivation method.

The dramatic increase in the weathering and biodeterioration rate and the consequent damage caused to buildings and stone monuments especially in the Mediterranean and Middle East under present day climate change conditions promotes studies to introduce new methods of analysis and treatment. These can be cheaper, more reproducible and faster ones especially to judge the cytotoxicity of biocides, which are in use to treat the biofilm growth on rock monuments.

1.2 Biodeterioration and Weathering (wear down) of Building Material

Weathering is the process of change, especially decomposition, of rocks and other mineral materials due to chemical changes or mechanical processes such as the expansion of freezing water or the growth of roots into rock cracks. Weathering is often confused with erosion, which is the movement of rock or weathering products, by water, wind, ice, or gravity. Weathering is only the chemical or mechanical breakdown, not the movement. However, chemical and physical weathering often goes hand in hand. For example, cracks exploited by mechanical weathering will increase the surface area exposed to chemical action. Furthermore, the chemical action at minerals in cracks can aid the disintegration process. The breakdown product, after chemical weathering of rock and sediment minerals and the leaching out (loss) of the more soluble parts, when combined with decaying organic material, is called soil (http://www.e-paranoids.com/w/we/weathering.html).

What is bioweathering? Bioweathering, biocorrosion or biodeterioration is an exchange or biotransfer of material (atoms) and energy between two heterogenous open systems: the (solid) substrate (rock, brick, concrete etc.) and gaseous or liquid (sometimes solid) environment. Both systems are defined by their physical, chemical and living properties. This interaction of all components and processes leads to a more or less complete turnover of the initial material at the border zone between the two systems (Krumbein et al. 1991; Krumbein et al. 1996). Biodeterioration can be defined as the irreversible loss of value and/or information of an object of art following the attack by living organisms (Urzi and Krumbein 1994). Biodeterioration of stone monuments is one of the principal fields of interest of researchers. However, other groups are also concerned with the biodeterioration

of other materials such as book, glass, wood, and textile (http://www.arcchip.cz/w08/w08_de_leo.pdf).

1.2.1 Diversity of Biodeterioration and Bioweathering Agents

Each monument or building located in a given climatic area can be considered as a specific habitat. In addition, in each monument or building, different micro-niches also occur considering outdoor or indoor environments or different expositions. In these positions different group of organisms can be settled. Biodeterioration research has focused chiefly on lichens, fungi, algae and bacteria. Microorganisms living on inorganic substrate form more or less complex communities structured in biofilms, or microbial mats (Saiz -Jimenez 2003). Recently the term biofilm was split into two considerably different systems and systemic approaches namely the study of subaquatic and subaerial biofilms sensu (Gorbushina personal communication). Among macroorganisms, lichens and among microorganisms fungi, which were both found in Persepolis stones as subject of this thesis will be considered to some more detail. Because the composition and structure as well as antifouling treatment are the main topics in this work a closer glance on characteristic subaerial biofilms as a weathering agent is given.

1.2.1.1 Lichens

Lichens are symbiotic associations made up by microscopic green algae or cyanobacteria (photobiont) and filamentous fungi (mycobiont). There is evidence that lichens might involve a controlled form of parasitism or exploitation of the algal cells (Ahmadjian 1993) . In laboratory settings, algae grow faster when they are alone rather than when they are part of lichen. This evidence suggests that the lichen symbiosis is parasitic or exploitive from the side of the fungus involved rather than mutualistic. Another view is that of domestication of algae by fungi since some algae would not be able to independent metabolism in environments where they survive jointly with the fungal partner. The photosynthetic partner can also exist in nature independently of the fungal partner, but rarely vice versa. Furthermore, photobiont cells are routinely destroyed in the course of nutrient exchange. The association is able to continue because photobiont cells reproduce faster than they are destroyed (Ahmadjian 1993; Brodo et al. 2001).

Lichens take the external shape of the fungal partner and hence are named based on the fungus. Although the form of lichen is determined by the genetic material of the fungal partner, association with a photobiont is required for the development of that form. When grown in the laboratory in the absence of its photobiont, a lichen fungus develops as an undifferentiated mass of hyphae. If combined with its photobiont under appropriate conditions, the morphogenesis of the lichen occurs and its characteristic form emerges. The lichen fungus is typically a member of the Ascomycota rarely a member of the Basidiomycota. Some lichen taxonomists place lichens in their own division, the Mycophycophyta, but this practice ignores the fact that the components belong to separate lineages (Brodo et al. 2001).

The algal cells contain chlorophyll, permitting them to live in a purely mineral environment by producing their own organic compounds, a significant portion of which is transferred to the mycobiont. The fungus protects the alga against drying out and, in some cases, provides it with minerals obtained from the substratum and regulates the quality and quantity of light reaching the photobiont, promotes gas exchange, chemically controls herbivores, and effectively neutralizes some groups of potentially toxic airborne elements that the lichen may accumulate from atmospheric outwash. If a cyanobacterium is present, as in certain terricolous lichens, this can fix atmospheric nitrogen, complementing the activities of the green alga.

Lichens live on various surfaces: soil, trees, rocks, and walls. They are often the first to settle in places lacking soil, constituting the sole vegetation in some extreme environments such as found at high mountain elevations and at high latitudes. Some survive in the tough conditions of deserts, and others on frozen soil of the arctic regions. Recently the European Space Agency (ESA) research shows that lichens can even endure extended exposure to space (http://www.sfam.org.uk December 2003). The ESA has discovered that lichens can survive unprotected in space. In an experiment led by Leopoldo Sancho from the Complutense University of Madrid, two species of lichen – *Rhizocarpon geographicum* and *Xanthoria elegans* – were sealed in a capsule and launched on a Russian Soyuz rocket on 31 May 2005. The Lichens were exposed to temperatures ranging from -20°C to 20°C, the vacuum of space and radiation for 15 days. Afterward, they were found to be in the same state as at the start of the experiment (http://www.newscientistspace.com/article/dn8297/ *Hardy lichen shown to survive in space*).

Some lichens have the aspect of leaves (foliose lichens); others cover the substratum like a crust (crustose lichens); others adopt shrubby forms (fruticose lichens); and there are

gelatinous lichens in which the cyanobacteria produce a polysaccharide that absorbs and retains water. A group of lichens very important for rock wear down are crustose endolithic lichens.

Lichens most frequently reproduce asexually, either by vegetative reproduction or through the dispersal of diaspores containing algal and fungal cells. Soredia are small groups of algal cells surrounded by fungal filaments that form in cavities called soralia, which open when the lichen dries or surrounding tissues die and release the soredia to be dispersed by wind. Another form of diaspore is isidia, elongated outgrowths from the thallus that breaks off for dispersal. Fruticose lichens in particular can easily fragment. Due to the relative lack of differentiation in the thallus, the line between diaspore formation and vegetative reproduction is often blurred. Many lichens break up into fragments when they dry, dispersing themselves to resume growth when moisture returns.

Lichens also reproduce sexually in a manner typical of fungi, forming fungal and algal "propagules" that, following germination, must meet with a compatible partner before functional lichen can form. This is generally not a common means of reproduction for most lichens, though it is more common in basidiomycetous lichens since they appear to lack structures specifically designed for asexual reproduction. Spores are produced in spore-producing bodies; the three most common spore body types are the apothecia, perithecia, cleistothecium and the pycnidia.

Because lichens are morphologically small relative to most terrestrial plants, yet require access to sunlight in order to grow, most forms are attached to either large boulders, other inert surfaces, or woody plants in somewhat to completely open or exposed situations. However, where adequate moisture exists, lichens develop on surfaces (particularly those of slow-growing trees) in forests as part of an epiphyte community. Stability of a surface is a common characteristic of most lichen habitats.

Lichens are a part of the food available for many animals, such as reindeer, living in arctic regions. Although lichens typically grow in harsh environments in nature, many lichens are sensitive to man-made pollutants. Hence, they have potential as pollution indicator organisms. When growing on mineral surfaces, some lichens slowly degrade their substrate by secreting acids that dissolve the minerals, contributing to the process of weathering by which rocks are gradually turned into soil (Brodo et al. 2001) as it is coming in next part.

1.2.1.2 Fungi

Until recently, six fungal divisions were recognized. Based on molecular phylogenic data, some groups are no longer accepted as fungi, while, in contrast, other organisms appear to have unexpected relationships to the fungi. The *Mycetozoa* and the *Mesomycetozoa* are purported members of the kingdom *Protista*; the *Oomycota* belong to the kingdom *Chromista*. Because of their fungus-like appearance, they are indicated as 'Pseudofungi'. *Microsporidium* was supposed to have close affinity to the fungi (De Hoog et al. 2000; James et al. 2006). Members of the kingdom fungi (*Eumycota*) are currently restricted to five divisions: *Chytridiomycota*, *Zygomycota*, *Glomeromycota*, *Ascomycota* and *Basidiomycota*.

Chytridiomycota have small, often unicellular thallus. Most species live in an aquatic environment. Gametes and asexual spores are motile, having single flagella at their ends. After copulation, a new thallus is formed which produces either zoospores or motile gametes (De Hoog et al. 2000).

Zygomycota have basically coenocytic thallus; the cell walls are composed of chitosan and chitin. Asexual reproduction is by non-motile spores. Sporangia contain numerous nuclei, which develop into sporangiospores after cleavage of the cytoplasm. Sexual reproduction is isogamic, i.e., with gametangial cells which are indistinguishable from each other. Cellular fusion leads to a zygospore with a thickened, characteristically ornamented wall.

Glomeromycota include those species that form arbuscular mycorrhizae with plants. They are essential for terrestrial ecosystem function. Originally placed in the order Glomales of division Zygomycota, these fungi were elevated to their own division in 2002 by Walker and Schüßler. *Glomeromycota* are characterized by the formation of dichotomously-branching arbuscules in the roots of angiosperms, in an obligate symbiotic relationship known as a mycorrhiza (*pl.* mycorrhizae or mycorrhizas). All species in the division are known to reproduce asexually by the formation of blastospores. The large (40-800 µm) spores with layered walls, containing several hundreds to thousands of nuclei. Spores may be formed singly, in clusters or in morphologically distinct "fruit bodies" called sporocarps. Similar to most Zygomycota, the cellular filaments (hyphae) of glomeromycotan fungi lack regular septae. Blastospore development and morphology and DNA sequence data are used to separate groups within the Division (http://tolweb.org/tree?group=Glomeromycota).

Ascomycota thallus is septate; septa mostly have simple pores with Woronin bodies, or they have micropores. Asexual propagules are of widely divergent morphology. Most species

reproduce with an asexual thallus, which in culture grows as yeast, as a coelomycete or as a hyphomycetes. The telemorphic and anamorphic cycles may occur in this division.

The thallus of *Basidiomycota* consists of a septate, dikaryotic mycelium, often provided with clamp connections; these are bridges between adjacent cells to provide each of them with a nucleus derived from one of the parent strains. The septa are perforated by a single, central pore. The wall of the pore canal is often characteristically swollen; such a structure is called a dolipore. Spores produce a short-lived, haploid mycelium. Cells of suitable mating type show plasmogamy, but karyogamy is postponed. Consequently a heterokaryon with clamp connections is formed. This condition is maintained during the major part of the life cycle, including fruit body production. Karyogamy, immediately followed by meiosis, takes place in the basidium, which produces meiospores exogenously, the spores often being forcibly discharged (De Hoog et al. 2000).

The anamorphic fungi are artificially classified according to their form of growth and the production of asexual fruit bodies, as follows: Hyphomycetes, Coelomycetes, and Yeast. Hyphomycetes consist of septate hyphae; variously shaped conidia are produced on more or less differentiated branches. Conidial fruit bodies are absent. Criteria for identification of hyphomycetes are details in the formation of conidia (conidiogenesis). Sometimes, several types of asexual propagation can be found next to each other in the same strain. In nature, the various types of conidiation have different ecological roles in dispersing the fungus through different microhabitats. As it was mentioned the great majority of Hyphomycetes are of ascomycetous affinity (De Hoog et al. 2000).

Coelomycetes consist of septate hyphae; conidia are produced in fruit bodies, while the rest of the mycelium remains sterile. Fruit bodies are either spherical with an apical opening (pycnidia), or flat, cup-shaped (acervuli). Except for morphological characters of fruit bodies and conidia, the process of conidiogenesis is also of significance for the taxonomy of Coelomycetes. Nearly all members of Coelomycetes are of ascomycetous affinity (De Hoog et al. 2000).

The term of ‘yeast’ is a descriptive term and stands for any fungus, which reproduces by budding. Criteria for identification of yeast are fundamentally different from those of hyphomycetes, physiologically and morphologically, regardless of their biological relationship. Yeast-like fungi with melanized cell walls are often designated as black yeast. These are anamorphs of several orders of the Euascomycetes (*Chaetothyriales* and *Dothideales*) and some Basidiomycetes. They are nearly always able to produce in addition true mycelium and therefore treated under the Hyphomycetes. Occasionally black yeast-like

fungi reproduce by isodiametric enlargement and subsequent meristematic development. Melanized asexual fungi more in general are frequently referred to as *Dematiaceae* (De Hoog et al. 2000).

1.2.1.2.1 Rock fungi

Rock is very hostile environment, where organisms encounter extreme fluctuations of moisture and temperature and limited nutrient availability and high electrolyte concentration and is exposed to high solar radiation. Nevertheless, biological activity is abundant in and on rocks that have been exposed to the atmosphere for some time (Gorbushina and Krumbein 2000). Free-living fungi and lichens establish on bare rocks. Below the rock surface, endolithic fungal communities may also accelerate weathering. Saprotrophic fungi can accelerate the decay of sandstone and limestone statues, but may also slow down weathering rates (Mottershead et al. 2003).

The major fungi involved in weathering in taxonomic and ecological perspectives belong to *Ascomycota, Basidiomycota, Zygomycota* and *Glomeromycota*. Five important functional groups of fungi with potential weathering capabilities can be recognized:

1) Lichen-forming fungi. As mentioned, lichens are associations between fungi and algae or cyanobacteria. Because the associated fungus is closer to the mineral interface than the phycobiont, the weathering ability of lichens is most probably due mainly to the fungus (Chen et al. 2000).

2) Mycorrhizal fungi, especially ectomycorrhizal (sheathing mycorrhizal) and ericoid mycorrhizal fungi, which were recognized recently as potentially important for mineral weathering in soils (Landeweert et al. 2001).

3) Saprotrophic fungi, such as *Aspergillus niger,* which use relatively simple carbohydrate sources and quickly proliferate under favourable conditions like weeds with many aggressive secondary metabolic products excreted to the immediate environment.

4) Meristematic black yeasts and other stress-tolerant saprotrophic ascomycetes that use a range of carbohydrate sources and effect chemically and physically on immediate contact surface of their environment (Staley et al. 1982; Sterflinger and Krumbein 1997).

5) Saprotrophic organisms that preferentially use the lignocellulose complex. White rot and brown rot fungi produce large amounts of organic anions that they use for generating hydrogen peroxide and for the production of Mn chelates that can oxidize a range of

phenolic compounds and may therefore be potential weathering agents which is discussed in more details in the next part.

Group 1 and 2, through their mutualistic association with a photosynthetic organism, are likely to possess sufficient carbohydrate sources to be responsible for appreciable amounts of bioweathering. Species of group 3 and 4 are restricted to a few habitats and their contribution to weathering is limited by the scarcity of assimilable carbon or by their very slow growth. Species in group 5 have the potential to wear down rock, but are normally active on dead organic matter, where degradable minerals are absent or very scarce (Hoffland et al. 2004).

1.2.1.2.2 Molecular Identification of Fungi

Unfortunately, only few culture-independent approaches exist, which would allow the detection of diverse groups of fungi present in a given ecosystem. In fact, historically important fungal metabolites, like muscarine, lysergic acid and ergotomine were discovered and elucidated before their source organisms were cultivated *in vitro* (Ruibal 2004). Traditionally taxonomic classification of fungi has been made based on classic morphometric criteria, growth parameters, sexual compatibility and/or biochemical characteristics, and ultrastructure. On the natural substrate fugal colonies show very little expansion growth and these fungi have been terminated microcolonial fungi (Staley et al. 1982). Sometimes such rock inhabiting fungi which are predominantly found on and in rock morphologically are similar, but belong to unrelated fungi in a single anamorph genus (Sterflinger et al. 1999). Furthermore many hyphomycete genera with isodiametrically enlarging cells were reported from epilithic lichens which are able to grow on bare rock surfaces (Hawksworth 1979). In addition some meristematic fungi show hyphal morphology in the culture whereas they display isodiametric enlargement by growth at very low pH (Sterflinger and Krumbein 1995). For this reason sometimes it is impossible to match morphological circumscriptions of genera due to insufficient rates of differentiation and several molecular approaches are applied for the identification and clarification of phylogenic relationships. In fungi, nuclear genome rDNA is organized in a repetitive transcription unit, in a number of approximately 200 per cell. This unit includes three genes for the 18S, 5.8S and 28S rRNA genes. The internal spacer (ITS1 and ITS2) in each transcription unit separates these genes, and these units are separated from the next by the intergenic spacers (IGS1 and IGS2) and the 5S rRNA gene. The Internal Transcribed Spacer

of fungal rDNA is highly variable sequences of great importance in distinguish fungal species by PCR analysis.

DNA sequencing involves the comparison of the actual sequences of bases in particular parts of DNA (or RNA) molecules; the ribosomal gene clusters have been most used in phylogenetic studies by mycologists, particularly the genes coding for 5.8S, 18S, and 28S rRNA genes and adjacent non-coding spacers (e.g. ITS); the method involves the use of the PCR method, and the base sequences are determined by automated sequencing machines (Ruibal 2004).

1.2.1.3 Biofilms

During the millions of years when bacteria were the only form of life on Earth, the prevailing aquatic environment was extremely oligotrophic. The niches permissive for life were limited by hostile environmental factors like UV radiation, heat and acidity (Costerton et al. 1995). At that time the purpose of the planktonic, free-living mode of bacterial growth was to enable them to move from one habitat to another until a niche permissive for growth was found. Biofilm formation allowed these first sessile organisms to remain in place, and to trap and utilize the scarce organic compounds. The development of co-operation among microbial groups permitted the use of more complex or more refractory nutrients. Biofilm formation also changed the environment of the colonized surface and made it more suitable for the bacteria growing there. Biofilm formation has therefore been a means of survival for microorganisms, and is the reason why microbial biofilms are characteristic of the life forms found in extreme environments like stone surfaces in hot springs or the surface of stones in cold, oligotrophic mountain streams, and in deserts or desert like rock surfaces (Costerton 1995). Nowadays this survival strategy is successfully used by bacteria in medical, dental, agricultural, environmental, industrial systems. In technical systems, biofilm formation can cause problems like the contamination of drinking or process water by microorganisms. The presence of pathogenic bacteria in biofilms in drinking water distribution systems is a health risk (Szewzyk et al. 2000). Common primary colonizers on all subaeric stone surfaces are microorganisms (fungi, algae and bacteria), followed up by lichens or mosses and later higher plants, whereby the latter are especially found in wall joints (Warscheid and Braams 2000). Organisms from all phyla may be colonizers of stone and other building material and are therefore potentially involved in biogenic stone weathering processes. Despite the frequent problems caused by biofilms, there are industrial systems where biofilm growth is

a beneficial phenomenon. The proper functioning of activated sludge plants, trickling filters or anaerobic digesters is based on functional biofilms (Mattila 2002). Furthermore, biofilms could be probably used as a plugging agent in microcracks. Suchomel et al. (1998) and Charbonneau et al. (2006) investigated alteration of solute diffusion in fractured rock and showed that biomass accumulation could plug the pores and then decrease permeability and porosity of rock on the reservoir surfaces. In this thesis, one of the focuses is to remove undesirable colonies and subaerial biofilms which can deteriorate building stones.

1.2.2 Mechanisms of Biological Weathering (wear down)

Weathering of natural rocks and stones is a general phenomenon, which can be observed all around us. It leads to the formation of gravel, sand and eventually even clay minerals. However, weathering of rock surfaces, of building stones and prehistoric/historic monuments has accelerated in recent times, and it has now become important to understand the reasons for this and to understand the actual weathering processes. For this reason the activities of lichens, fungi and biofilms which were of interest for this work were reviewed briefly.

1.2.2.1 Weathering Activities of Lichens

Much of the recent research about biodeterioration has been centred on algae, lichens, fungi, and bacteria. Jones and Wilson (1985) and Bjelland et al. (2002) have reviewed the action of lichens, confirming that the effects of lichens on sandstone are both physical and chemical. One estimate places biological weathering at 100 to 1000 times greater than the inorganic weathering (Aghamiri and Schwartzman 2002). Weathering under lichens is nearly proportional to the square of rainfall (Brady et al. 1999).

Mechanical damage is caused by penetration of the hyphae into the stone, and by the expansion and contraction of the thallus under changes of humidity. Swelling action of organic salts produced by lichens and fracturing and incorporation of mineral fragments must be considered (Bjelland et al. 2002). Chemical damage, however, is important, and may arise in three ways: by the secretion of oxalic acid, by the generation of carbonic acid, and by the generation of other acids capable of chelating metal ions such as calcium and magnesium (Brady et al. 1999; Adamo and Violante 2000). Biochemical weathering by lichens generally takes the form of surface etching on minerals, leaching or replacement of

minerals, and the production of weathering compounds (Lee and Parsons 1999). Etch marks visible with SEM appear on minerals beneath lichen; in time the rock crumbles or becomes completely devoid of all minerals other than silicon (Aghamiri and Schwartzman 2002). Lichen-secreted compounds, including oxalic, citric, gluconic, and lactic acids, can induce a charge transfer that reduces mineral strength (Adamo and Violante 2000). The secretion of oxalic acid, which reacts with a calcareous stone also produce calcium oxalate, is of particular interest. A number of authors have noted the presence of calcium oxalate on the surface of stone monuments, where it can form part of a coherent and seemingly protective layer known as *scialbatura*. Del Monte and Sabbioni (1987), for example, have argued that *scialbatura* is caused solely by lichen activity, whereas Lazzarini and Salvadori (1989) have enumerated other possible causes, including the deliberate application of a protective coating. Gehrmann et al. (1988) suggested that the oxalic acid secreted by the mycobiont is the chemical substance probably involved in production of whewellite found in all interfaces. The chemicals commonly called lichen acids are actually polyphenols that can chelate and complex with structural metal ions in the rock, leading to the decomposition of the minerals (Adamo and Violante 2000). The rate of lichen-induced disaggregation of a rock substrate depends on both the species of lichen and on the hardness, porosity, lamination, and induration of the rock (Adamo and Violante 2000). Bjelland et al. (2002) studied the weathering rinds on arkosic metasandstone beneath 4 lichen species in Vingen, Norway. The rind exhibited varying degrees of porosity, mineral dissolution depth, and mineral composition. They note that different thalli of the same species may produce distinct effects and that within a single thallus the compounds may be concentrated in certain regions.

Do lichens amplify or reduce the effect of acidifying substances in weathering? Lichens show both amplification and reduction of this effect. Cracks and weak spots can become sites for preferential colonization, which then amplify the effect of acid rain. However, the presence of lichens also prevents the entry of diluted sulphuric acid into the interior of such objects, thereby decelerating weathering (Hoffland et al. 2004).

1.2.2.2 Weathering Activities of Fungi

Fungi have fewer geochemical capabilities because of redox reactions than bacteria. However, because of their ubiquitous presence and their ability to bridge distances by means of mycelial growth, fungi do have opportunities for weathering as well. Some of the fungal activities are common with lichens due to the mycobiont partners.

Fungi can penetrate solid material using both physical and chemical tools. The mycelial growth form allows fungi to exploit weak spots on the rock or grain surface (Jongmans et al. 1997). The hyphae respond to surface by following scratches, ridges, and groove, and by penetrating pores or tunnels. These irregularities may result from previous abiotic weathering. At such spots, the fungi may further weather the mineral surface using an array of physical and chemical tools. By preventing rapid diffusion of exudates into the soil solution, these irregularities may help increase fungus-mediated weathering, and may lead to the formation of tunnels (Hoffland et al. 2002). Using osmotic pressure, fungal appressoria (infection organs) produce pressure of up to 10-20 $\mu N/\mu m^2$, sufficient to penetrate inert material (Howard et al. 1991). However, such forces alone are probably not great enough to allow the hyphae tip to penetrate a rock, making chemical tools essential. Fungi most likely do accelerate physical weathering, by sending hyphae penetration into cracks, spaces, and mineral fragments in the fungal thallus, by forming secondary minerals that further disrupt the rocks, and by expanding and contracting hyphae during cycles of wetting and drying and freezing and thawing. Simultaneously, fungi can decelerate physical weathering where mycelium provides a cover that reduces temperature fluctuations, wind abrasion, or rain drop impact (Arino et al. 1995).

Biogeochemical mechanisms of weathering are produced by two ways: proton-based and ligand-based agents. Proton-based agents include respiratory CO_2 /carbonic acid and other acids produced in the areas directly surrounding the tips of fungal hyphae. Ligand-based weathering agents include organic anions, siderophores, and phenolic acids and other acids. Another aspect of weathering is formation of secondary minerals, which are produced by fungi. Biogenic minerals may be present in the patina, can decelerate weathering under humid conditions because it reduces the impact of rain. Fungi amplify and reduce the effect of acidifying substances in weathering. Microcolonial Ascomycota show both amplification and reduction of this effect as it mentioned for lichens. Cracks and weak spots can become sites for preferential colonization, which they amplify the effect of acid rain. However, the presence of black yeasts also prevents the entry of diluted sulphuric acid into the interior of

such objects, thereby decelerating weathering as it was resulted for lichens (Hoffland et al. 2004).

1.2.2.3 Weathering Activities of Biofilms

Microbial biofilms are ubiquitous in aquatic and terrestric ecosystems as well as on man-made material. Microbial colonizers have several features in common. In most habitats, where a primary energy source (visible light) is readily available and organic carbon is limited, microbial biofilms contain photoautotrophic primary producers (algae and cyanobacteria). A sufficient concentration of reduced inorganic compounds, especially ammonia or sulphide, allows the upcoming of chemolithoautotrophic ammonia or sulphide oxidizers. Due to the production of mineral acids, these organisms are known to be most deleterious agents to concrete and other building material (Mansch and Beck 1998). Whereas the amount of reduced sulphur compounds is too low for the development of sulphur oxidizers in subaerial biofilms, ammonia and nitrite oxidizers have been frequently isolated from building material, especially in urban environments.
In spite of the diversity of the primary microbial colonizers and their different effects on stone surfaces (excretion of acids, remineralization, formation of patinas etc.), most of these films has one feature in common: the organisms excrete extracellular polymers (mostly extracellular polysaccharides, EPS) of varied compositions. Together with monument components, mineral particles and residual decomposed cellular material, the extracellular polymers build up a biofilm matrix. The biofilm matrix rather than the organisms is in immediate contact to the surface of the building material (substratum). The biofilm matrix transfers all agents to the substratum. Thus, the important reactive interface for biogenic weathering is the interface between the matrix and the building material (Kemmling et al. 2004).

1.2.3 Biodeterioration at Persepolis; a Special Site of World Cultural Heritage

The magnificent ruins of Persepolis lie at the foot of Kuh-i-Rahmat, or "Mountain of Mercy," on the plain of Marv Dasht about 400 miles south of the present capital city of Teheran. Persepolis is registered as a World Heritage Site by UNESCO.

The exact date of the founding of Persepolis is unknown. It is assumed that Darius I initiated the work on the platform and its structures between 518 and 516 B.C., visualizing Persepolis as a show place and the seat of his vast Achaemenian Empire. However, the security and splendour of Persepolis lasted only two centuries. Its majestic audience halls and residential palaces perished in flames when Alexander conquered and looted Persepolis in 330 B.C., and according to Plutarch, carried away its treasures on 20,000 mules and 5,000 camels (Langsdorff and McCown 1942).

Fig.1 shows a portion of the ruins at Persepolis

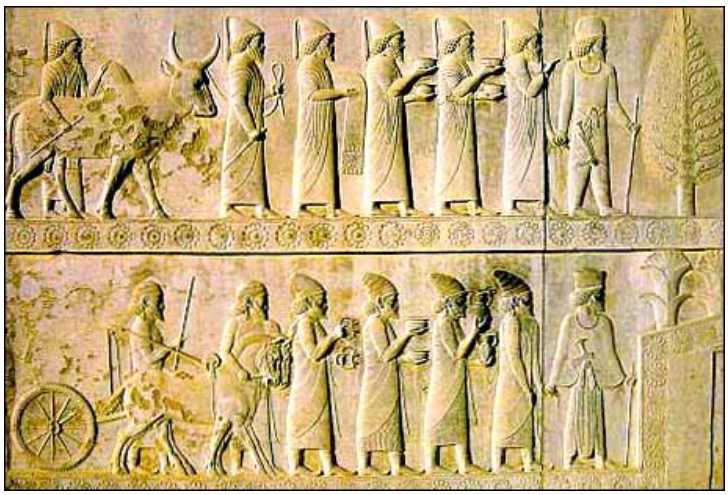

Fig.2 Biodeteriorated antique engraving figure showing typical fungal/lichenic colonies especially in the left side

From the time of its barbaric destruction until A.D. 1620, when its site was first identified, Persepolis lay buried under its own ruins. During the following centuries many people travelled to and described Persepolis and the ruins of its Achaemenid palaces. Many of their

observations were later condensed and published by Curzon in *Persia and the Persian Question* (1892). However, scholarly and scientifically planned work was not undertaken until 1931. Then Ernst Herzfeld, at that time Professor of Oriental Archaeology in Berlin, was commissioned by James H. Breasted, Director of the Oriental Institute of the University of Chicago, to undertake a thorough exploration, excavation and, if possible, restoration of the remains of Persepolis. In 1931-34, assisted by his architect, Fritz Krefter, he uncovered on the Persepolis Terrace the beautiful Eastern Stairway of the Apadana and the small stairs of the Council Hall. He also excavated the Harem of Xerxes. When Herzfeld left in 1934, Erich F. Schmidt took charge. He continued the large-scale excavations of the Persepolis complex and its environs until the end of 1939, when the onset of the war in Europe put an end to his archaeological work in Iran. During the last years of excavating, the University Museum in Philadelphia and the Museum of Fine Arts in Boston had joined the Oriental Institute in order to cope with the tremendous work at hand (Tilia 1972). After the departure of the Persepolis Expedition, the Iranian Antiquity Service continued excavating on the Persepolis Terrace. The structures they found, prior to 1953, are included on the reconstructed plan of the Persepolis Terrace, and are indicated by broken lines. In 1964, preserving and restoring of the monuments were entrusted to the Italian Institute of the Middle and Far East, Rome. A report of their work (Tilia 1972), [Rome, 1972]) adds significantly to our knowledge of the structures on the Persepolis Terrace (http://oi.uchicago.edu/OI/MUS/PA/IRAN/PAAI/PAAI_Introduction.html/).

Persepolis is 125 thousands sq.m. in area, and is composed of several sections. The various temples and monuments are located upon a vast platform, some 450 metres by 300 metres and 20 metres in height. At the head of the ceremonial staircase leading to the terrace is the Gateway of All Nations built by Xerxes I. Beyond the Gate lie the Hall of 100 Columns (Xerxes' throne room), the Apadana Palace (the Great Hall of Audience of Darius the Great) with its spellbinding display of bas-reliefs, the Tachara (or Winter Palace) of Darius, the unfinished palace of Artaxerxes III, the Palace of Xerxes' and the Tripylon Palace. Banked against the mountainside are the royal stables, guard rooms and domestic quarters. The rock tombs cut into the mountain are those of Artaxerxes II and Artaxerxes III. After Alexander attack only ruins have remained. As an important outcome of this brief historical review on the archaeology of Persepolis it may be stated, that the biodeterioration patterns and products in terms of material losses from rock surfaces stems mainly from the past 75 years.

1.3 The Chemical Control of Biodeteriorating and Biological Wear down Agents

The main effort in the field of biodeterioration has been to develop methods for preventing the biodeterioration of materials and thus preserve their value and usefulness for as long as possible. It is worth noting that no control methods are perfect. Often these methods are attempting only to control or reduce the level of growth in or on a material at a minimum level. Chemical preservatives are variously referred to as biocides, bactericides, fungicides, fungistates, antifouling compounds, and material protectants. To be effective, the ideal biocide should have the characteristics described in the following paragraph (Price 1996; Allsopp et al. 2004).

It must be toxic to the target biodeteriorants. For this reason it is important to identify the causative organisms so that the correct type of biocide is used. The chemicals must be non-toxic to human and non-target animal and plant life. On the other hand the biocides must have low risk of environmental pollution. It must be compatible with the product and not impart any unwanted colour or alter the properties of the material. It should be stable in both the concentration and diluted form, and finally it should have a low cost. Major chemical agents include the oxidizing agents, aldehydes and alcohols, phenolic compounds, organic acids and their esters, quaternary ammonium and phosphonium compounds, isothiazolinones, and other organohalogen-containing biocides (Allsopp et al. 2004).

1.3.1 Modes of Chemical Action

Biocides exert their effect on the target organism in a number of ways, including oxidation, hydrolysis, denaturation, cell lysis, metabolic inhibition, and alteration of membrane permeability (Loughlin et al. 2002; Trosken et al. 2006). Oxidising agents such as hypochlorite cause lysis of cell wall and cell constituents. Quaternary ammonium compounds and alcohols may affect active transport mechanisms or disrupt membrane integrity. Compounds such as phenols and aldehydes can denaturate proteins. Protein synthesis inhibitors such as isothiazolinones bind with thiol groups in cell affecting enzyme activity. Nuclear division inhibitors can inhibit DNA synthesis. The synthesis of ergosterol in fungi can be inhibited by the membrane synthesis inhibitors such as tebuconazole, and photosynthesis inhibition affects electron transport. Their efficacies, often quoted as

minimum inhibitory concentrations, will vary from less than 10 ppm up to several thousand. In use their activities are affected by both the ingredients in the product and the environment in which they have to work (Allsopp et al. 2004). For example, a concentration of biocide which is used to control planktonic cells and biofilm structure of the same organisms may considerably differ (Brown et al. 1988). Therefore to eradicate well-protected biofilm structures it is necessary to use higher concentration of biocide. It has been suggested that EPS which is secreted by the microorganisms in the film, acts as an ion-exchange resin, limiting the penetration of charged molecules as well as altering physiological state of sessile cells. It has shown that the preventing the formation of a normal biofilm, by manipulating the quorum sensing behaviour of the bacterial cells, results in increased sensitivity to the sodium dodecyl sulphate (Allesen-Holm et al. 2006). Further research in this area may help to destroy deteriogenic biofilms without increasing biocide levels. No single biocide in use today is capable of solving all biodeteroration problems.

1.3.2 Chemical Selection against Biodeteriorants

As it mentioned before in choosing chemicals some prerequisites must be considered: high efficiency, harmless on the physico-chemical characteristics of materials, low toxicity for the operators and animal-plant life, low risk of environmental pollution. Any treatments should be recommended only after accurate diagnosis to detect the colonizing organisms and the effectiveness of treatments. Many generic tests for cell viability are used as cytotoxicity tests because they actually measure a particular process that only takes place in viable cells. For example, the neutral red uptake (NRU) assay measures the ability of lysosomes to take up waste materials in the cytoplasm. Another popular assay looks at how well mitochondrial membranes can reduce substances such as the tetrazolium salt MTT. When cell membranes are compromised they become porous and allow that stable macromolecules to leak out and be quantitated using a variety of fluorescent, luminescent, and colorimetric assays (Warscheid 1990; Kepner and Pratt 1994; Breeuwer and Abee 2000).

1.3.3 Fluorometry as a Tool for Biocide Selection

Advances in technology during the past few years have led to the emergence of a variety of detection techniques for use in screening assays. In particular, fluorescence detection is more and more exploited as assay readouts, although problems are also related to the detection of fluorescence signals. The most important of these problems are: the signal can be subject to quenching by compounds, plastics or media, fluorescence emissions can be scattered by particulates, or the signal can be masked by autofluorescence (from protein or mineral compounds) or by high background fluorescence from unbound labelled probes (Rogers 1997). The presence of rock and mineral particles in the materials analysed may further complicate the procedure. The following part will give short descriptions of this category.

Fluorometric detection

Fluorescence occurs when photons of light absorbed by a molecule cause electrons to become excited to a higher electronic state and to return to the ground state with emission of light energy. The light emitted has a longer wavelength than that absorbed (the Stokes shift) as a result of a small dissipation of energy during the excited state (Emptage 2001). Fluorescence can be schematically illustrated by a Jablonski diagram, first proposed by Jablonski (1933) to describe the absorption and emission of light.

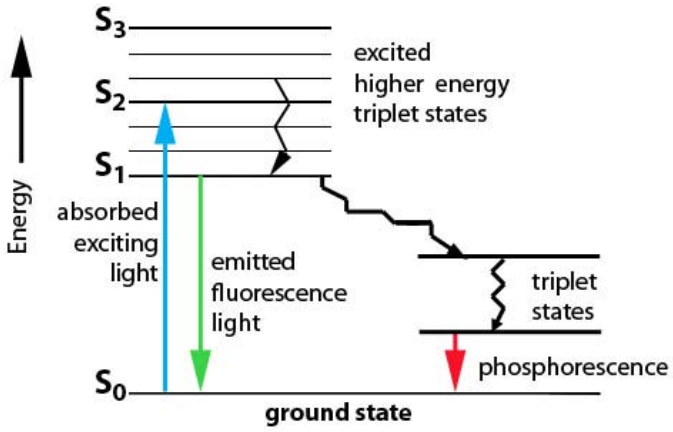

Fig.4 The Jablonski diagram (from Jablonski 1933)

Prior to excitation, the electronic configuration of a molecule is defined as being the ground state. Once a molecule has been excited to a higher energy and vibration state, there are a number of routes by which the electron can return to the ground state: if the photon emission occurs between the same electron spin states this is termed fluorescence, and if the spin states of the initial and final energy levels are different, the emission is called phosphorescence.

In an attempt to improve the characteristics of fluorometric detection, multiple modifications have been created and used for diverse assay formats including homogeneous and cell based assays. These modifications can be categorised as 1) fluorescence intensity (or prompt fluorescence, FI), 2) fluorescence polarisation/anisotropy (FP), 3) fluorescence resonance energy transfer (FRET), 4) fluorescence life time (FL), 5) time resolved fluorescence (TRF) and 6) single molecule detection methods, such as fluorescence intensity distribution analysis (FIDA) and fluorescence correlation spectroscopy (FCS) (Hemmilea and Hurskainen 2002; Gribbon and Sewing 2003). FI will be explained in more detail, because it was the main tool of this study.

The simplest form of fluorescence, FI, typically utilises fluorescent enzyme substrates and indicators loaded into membranes or compartments that alter their intensities due to environmental change (Gonzalez and Negulescu 1998; Pope et al. 1999). The measured steady state FI is linearly related to changes in fluorophore concentrations. Owing to the simple methodology, FI is widely used, although it is also very much affected by compound quenching, autofluorescence effects and inner filter phenomena (Pope et al. 1999; Gribbon and Sewing 2003). In this work, FI was used as a detection method in cytotoxicity of chemicals on fungi cells in microscopic visualisation and fluorometry tests. Furthermore the quenching effect and the presence of autofluoresence compounds insde the rock inhabiting fungi was measured.

Fluorescein diacetate (FDA), Calcein AM (CAL AM), Propidium Iodide (PI) are commonly used fluorescent molecules for staining live and dead eukaryotic cells. Rotman and Papermas (1966) used FDA and derivatives on mammalian cells and proposed a model for cell permeability to these kind of stains. In metabolically active cells, the nonfluorescent FDA molecule is cleaved by esterase into a green fluorescent product, whereas inactive cells remain non-fluorescent (Rotman and Papermas 1966; Larsson and Nygren 1989).

The acetomethoxy derivate of Calcein (CAL AM) is highly lipid soluble; it rapidly penetrates the plasma membrane of cells and is practically non-fluorescent. By cleavage of the ester bonds, cytosolic esterase quickly and irreversibly converts CAL AM into the

hydrophobic, non-permeable and fluorescent-free acid form, Calcein. The acetomethoxy group of molecule chelates calcium. As dead cells lack this enzyme, only live cells are marked.

Propidium Iodide (PI) does not penetrate into cells with an intact plasma membrane, but complexes with the DNA of cells with a damaged membrane, which result in cells with a highly fluorescent nucleus (Vornov et al. 1991). Propidium iodide is marketed with a variety of vital dyes as “live/dead” assays.

2 Material and Methods

2.1 Sampling in Persepolis

Geographic position and climate of Persepolis

The climate on Persepolis is alike the climate of the Shiraz in general, although the existence of sub-climatic variations should not be forgotten. The geographic position of the area is 29° 55’ 60” North, 52° 54’ 00” East (Fars Province). The altitude in the area is 1.740 m.

Table.1 Geographic data of Persepolis and Shiraz was compared

Persepolis Geographic Data		Shiraz Geographic Data	
Height	1740 m	Height	1488 m
Geographic Position	29°, 55’, 60” North 52°, 54’, 00” East	Geographic Position	29°, 36’ North 52°, 32’ East
Weather	Semiarid - Semitropic	Weather	Semiarid - Semitropic

The climate is Semiarid – Semitropic with a seasonal contrast greater than in the Mediterranean area described in many biodeterioration studies (Krumbein and Urzi 1991). The mean annual temperature is 15.8 °C. The mean annual precipitation is 341.1 mm with about 47.8 rainy days per year. Average relative humidity is 41%. Number of days with snow or sleet, and sunshine are 2.6 and 139.96 days per year, respectively. Another important phenomenon is the enormously high mean of annual dust days (64.1 days per year). Kondratyeva et al. (2006) recently hinted at the traditional and continuous spreading of microorganisms with aerial dust. The number of cloudy and thunderstorm days is 36.2 and 11.3 days per year, respectively (http://www.weather.ir/; http://www.farsmet.ir/).

Eight samples of three areas (Gate of Xerxes or International Gate, Throne Hall or Hundred Column Hall, Xerxes Palace) located at different places and heights of the antique site of Persepolis (Iran) were taken aseptically. Samples were immediately placed into sterile Petri dishes and taken to the laboratory for studying biofilms, and rock/biofilm interaction, and for the isolation of fungi by using selective culture media for fungi.

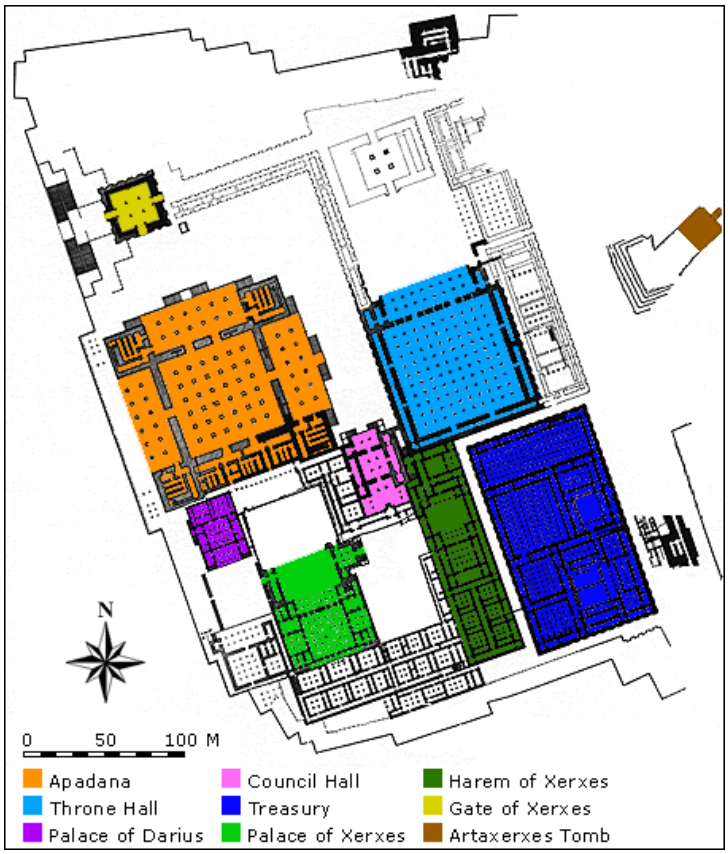

Fig. 3 Map of Persepolis

2.2 Light and Electron Microscopic Study of Rock Biofilms in Persepolis Samples

2.2.1 Optical Microscopic and Stereoscopic Observation

An optical microscope (Axioscope II Zeiss) was used to detect and study lichens and biofilms on the stone surfaces. Digital photos were taken using a digital camera Olympus 3030 with analytical software analySIS (Soft Imaging Systems GmbH, Münster). A dissecting stereomicroscope (Zeiss equipped with a Winder M35 camera) was used to study alterations of stone surfaces and biofilms, which were present on the stones. Photos were taken for documentation.

2.2.2 Scanning Electron Microscopic Observation

SEM was used to study biofilm characteristics and structures. With this aim, samples were initially fixed in 4% glutaraldehyde buffer solution, later washed with 0.1 M phosphate buffer, dehydrated through an ethanol series (30, 50, 70, 80, 90, and 100%) and finally critical point dried. Before observing the samples, they were gold coated at 10^{-3} mm Hg in a sputtering apparatus (Balzers). Observations were done using an Environmental Hitachi scanning electron microscope S-3200.

2.2.3 Thin Sections

Marble and limestone samples, which were taken at Persepolis, were used to demonstrate the main lines of biogenic formation of pitting and other wear down structures on the stone surface through the action of fruiting bodies and fungal hyphae and algal and bacterial cells. This analysis was achieved by the preparation and study of numerous thin sections. Small pieces of stones encrusted with biofilm were fixed in 4% glutaraldehyde for one hour. Vacuum treatment was also done. The samples were then washed 3 times in 0.1 M phosphate buffer. Dehydration was accomplished in a graded ethanol series (30, 50, 70, 80, 90 and 100%) modified after Golubic et al. (1970).

The specimen were embedded in a low-viscosity resin according to Spurr (1969). With infiltration of resin vacuum treatment was applied. Finally, the samples were embedded in beamer capsules and polymerized. For the preparation of thin sections the hardened blocks were cut by using a saw (Leitz model 1600) in sections vertical to the stone surface, washed in ethanol, mounted on ground (600 grit) microscope slides (46x27) embedded in Spurr's resin and polymerized. The samples were polished with silicon carbide powder in a series of 320, 400, 600 and 1000 grit. Photo-micrographs of the sections were made using a Zeiss Axioscope II.

2.2.4 PAS Staining

The Periodic acid Schiff (PAS) reaction was used for staining rock biofilms, the mycobiont of lichens or free living fungi to visualize the microbial penetration inside the rock. The PAS technique (Whitlatch and Johnson 1974) was performed on the biofilms on

the stone surfaces as well as on thin sections. Schiff's reagent containing hydrochloric acid (HCl) will dissolve calcium carbonate to some extent, thus the hyphae will be easily visible microscopically.

2.2.5 Maceration Technique

In order to study features of corrosion on the stone surfaces, it is necessary to remove biofilms from the surfaces without disturbing them. For this reason, seven samples, which showed different kinds of biofilms, were chosen. They were immersed in ''EAU DE JAVELLE''. This solution can dissolve away the organic material. The remaining cells easily detach from the surfaces without any mechanical pressure applied. After maceration the remaining stones were washed several times in distilled water. After drying in the air they were coated with gold and examined under the scanning electron microscope. EAU DE JAVELLE comprises 4 g $CaOCl_2$ in 20ml H_2O with 10 g K_2CO_3 in 100 ml H_2O (Schneider 1976).

2.3 Isolation of Rock Inhabiting Fungi from Persepolis Samples

Fungal colonies were aseptically isolated under the dissecting microscope by using a sterile needle and then cultured on Petri dishes containing DRBC medium comprising 10 g glucose; 5 g pepton; 0.5 g $MgSO_4$ * 7 H_2O; 1 g K_2HPO_4; 15 g agar; 0.002 g dichloran; 0.025 g Rose Bengal; 1.0 ml chloramphenicol (1.0 g/100 ml ethanol); DW 1 litre; and malt extract agar (MEA) included; 20 g malt extract; 20 g dextrose; 1 g peptone; 15 g agar; DW 1 litre; pH 7.0. The isolates were incubated at 25°C until visible fungal colonies were observed and then sub-cultured onto malt extract agar (MEA) for microscopic examination and macroscopic morphology studies. Each agar plate was inoculated with 5 colonies. For maintaining fungal pure cultures, slant agar of MEA was inoculated with a small fragment of mycelium or spore suspension. The cultures were kept at 4°C and transferred to the fresh medium every six months.

2.4 Morphologic, Microscopic and Molecular Characterization of Fungal Isolates

2.4.1 Macroscopic Study

The shape, colour, and the size of colonies were documented by using a stereomicroscope and compared using “Introduction to food and airborne fungi” (Samson et al. 2000) and “Atlas of clinical fungi” (De Hoog et al. 2000). Photographs were taken for documentation.

2.4.2 Microscopic Study

For light microscopy, a piece of mycelium grown on an agar plate was mounted in a drop of lactic acid (for darkly pigmented fungi) or lactophenol cotton blue (for hyaline, pale fungi). Squash mounts for fungi with fruit bodies were used.

Slide Cultures

A square block of MEA was placed on a sterile microscope slide supported on a bent glass rod in a Petri dish which served as a damp chamber. The four sides of the block are inoculated with the fungus and a sterile cover slip was lowered on the block. Sterile water was then added to the plate. The slides were controlled continuously. After growth of fungi occurred, the cover slip was removed from the slide, leaving mycelium attached to the glass to be stained.

2.4.3 Molecular Diagnosis

One of the aims of the molecular method is to identify fungal isolates according to the information contained in their nuclear genome. In fungi, nuclear genome rDNA includes three genes for the 18S, 5.8S and 28S rRNA genes. Transcribed Spacer of fungal rDNA is highly variable sequences of great importance in distinguish fungal species by PCR analysis. DNA extraction, PCR amplification of rDNA accompanying classical techniques would be used to identify fungi (Ruibal et al. 2005).

DNA extraction, PCR amplification of DNA, Sequencing

Total DNA was extracted using the UltraClean™ Soil DNA Isolation Kit (MO BIO Laboratories). The manufacturer's protocol was followed, except using liquid nitrogen to grind the fungi, and adding 10 times freeze-thaw cycles after the 10 minutes vortexing step to lyse cells more effectively. Spin filters were air dried. All together 5 fungal isolates from Persepolis were analysed. 50-230 mg of fungal colonies was weighted for extraction. Each sample was subjected to PCR amplification of the complete nuclear ITS1-5.8S–ITS2 rDNA, which was performed using the primers ITS1F (Gardes and Bruns 1993) and ITS4 (White et al. 1990). The internal transcribed spacer (ITS) region of the nuclear ribosomal DNA was selected because it is variable enough in sequence and in length to separate even closely related species. The sequences of the upstream primer ITS1F is: 5'-CTT GGT CAT TTA GAG GAA GTA A-3'. The sequence of the downstream primer ITS4 is: 5'-TCC TCC GCT TAT TGA TAT GC-3'.

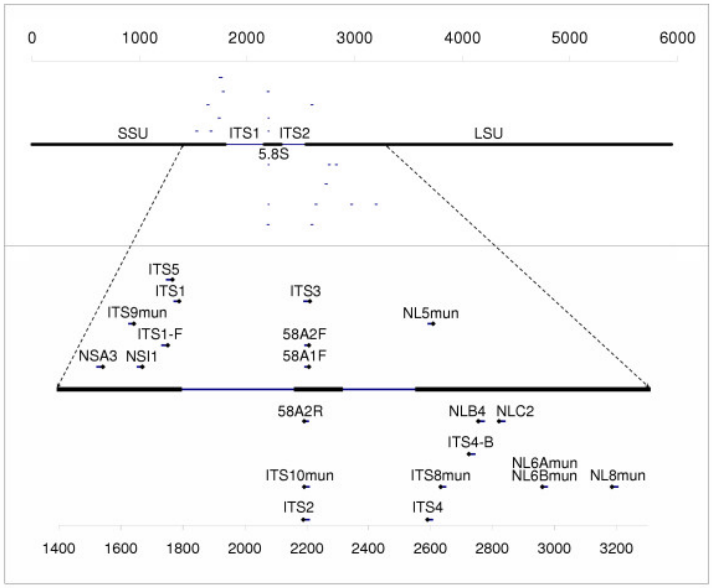

Fig.5 Diagram of primer locations in the ribosomal cassette consisting of SSU, ITS1, 5.8S, ITS2, and LSU rDNA (Martin *et al. BM Microbiology* 2005 **5**:28)

The PCR mixture contained 50 µl Promega Master Mix 2x (50 units/ml of Taq DNA polymerase, 400 µM of each of the four dNTPs, 3 mM $MgCl_2$), 50 µl H_2O, 0.5 µl of each primer, and 0.5 µl of DNA extraction. The following PCR cycling parameters were used: a four minute hold at 94°C, followed by 30 cycles consisting of denaturation at 94°C for 60 s, annealing at 52°C for 60 s, and extension at 72°C for 60 s, and a final 10 minute hold at

72°C, after which the reaction was cooled to a constant temperature of 4°C. PCR products were electrophorized in a 1 % agarose gel and visualized using ethidium bromide. Single PCR products were cleaned using the PCR Purification Kit (QIAGEN). Cleaned PCR products were sent for sequencing to a professional sequencing laboratory. Best database matches with ITS1-5.8S-ITS2 fragments of the sequences were identified with Blast (NCBI) and probable identity was assigned using the highest scoring matches.

2.5 *In Vitro* Tests of Chemical Inhibitors against Fungal Biofilm

2.5.1 Chemical Inhibitors against Fungal Biofilm

Different chemical compounds were used to inhibit biofilm growth. The compounds are classified according to their mechanisms of action as a biocide [Preventol A8 (PV) and Alkyl benzyl dimethyl ammonium chloride (BAC)], melanin inhibitor (Tricyclazole), EPS inhibitor [Bismuth-2, 3-dimercaptopropanol (BisBAL)] and permeabilizer [Polyethyleneimine (PEI)]. More details are given in the next part.

2.5.1.1 Biocides

Antibiotics are used as chemotherapeutic drugs, and biocides are used as antiseptics, disinfectants, and preservatives. They may serve to inhibit metabolic activity while present, with effective metabolism being restored upon removal, or cause irreparable damage to the target cells which results in cell death (Denyer 1990). Several factors affect biocidal activity, notably concentration, period of contact, pH, temperature, the presence of interfering material, and the types, numbers, location, and condition of microorganisms. Assessment of biocidal activity by microbiocidal testing is more relevant than by determination of minimum inhibitory concentrations. Laboratory studies are useful for examining responses to antimicrobial agents. Translation of such findings to clinical and environmental situations is needed to provide evidence of a possible relation between biocide usage and their activity in the natural situation.

2.5.1.1.1 Preventol A8 (PV)

Phenolic derivatives have been used as fungicide in paper conversation for almost 60 years. Then this chemical as a fungicidal agent was evaluated and used. This substance has also high toxicity in mammals. Preventol A8 (2 phenyl phenol or ortho-phenyl phenol, OPP) as a biocide is registered in many countries for use as a disinfectant, antimicrobial, preservative, antioxidant and sanitizing solution in various industries (www.fao.org and http://www.irg-wp.com/). PV 1%, 0.1%, 0.01% was prepared in Isopropanol 70% and used for experiments.

2.5.1.1.2 Alkyl benzyl dimethyl ammonium chloride (BAC)

Quaternary ammonium compounds are surface active substances and are used both as detergents and disinfectants. These compounds are hazardous substances in high concentration for many living beings for example *Aspergillus* sp., *Bacillus* sp., *bird* sp., *Drosophila melanogaster* and it causes DNA damages in the cells (http://physchem.ox.ac.uk/MSDS/AL/alkyl_dimethyl_ethylbenzyl_ammonium_chloride.html). BAC 1%, 0.1%, 0.01% was prepared in dH_2O and used for treatments.

2.5.1.2 Melanin inhibitor (Tricyclazol)

Tricyclazole (5-methyl-1, 2, 4-triazolo [3, 4-*b*] benzothiazole), a specific inhibitor of pentaketide melanin biosynthesis, inhibits synthesis of the pigment and inhibits the reductase activity and the synthesis of other non melanin poliketide pigments (Butler and Day 1998). 30 µg/ml of this substance was prepared in 70% Ethanol and used in assays.

2.5.1.3 EPS inhibitor (Bismuth-2, 3-dimercaptopropanol BisBAL)

EPS inhibitors could be used to minimize attachment of microorganisms to the stone surfaces, thus reducing the biodeterioration processes on stone as it was reported to decrease pathogens attachment to the cells. There are many of reports to use different kinds of natural products in medicine to reduce infections. At least as long ago as the ancient Egyptians, sulfur-bearing natural products have been used for their potent medicinal properties (Heldreth and Turos 2005). Bismuth thiols are antibiofilm agents

with up to 1000-fold-greater antibacterial activity than other inorganic bismuth salts (Domenico et al. 1999; Huang and Stewart 1999). BisBAL was prepared by dissolving bismuth nitrate in dimercaprol containing 5 mM BisBAL as a stock solution.

2.5.1.4 Permeabilizer (Polyethyleneimine PEI)

Certain external agents that either release some components from the membrane or intercalate in the membrane can abolish the integrity of the membrane. Such agents are called permeabilizers (Alakomi et al. 2000).

Polyethyleneimine (PEI), a polycationic polymer substance used in various bioprocesses as a flocculating agent and to immobilize enzymes, bind and precipitate DNA and probably to use for purification of DNA binding protein, was recently shown to make microorganisms permeable to hydrophobic antibiotics and to detergents (Helander et al. 1997). Without releasing LPS or chelating effect, PEI damage wall. PEI in combination with other biocides would be ideal to inhibit microorganism growths. 30 µg/ml of this substance was dissolved in distil water and used in assays.

2.5.2 Fluorometry Technique

2.5.2.1 Strains

Fluorometry tests were performed with the three isolates A18, J26, and PF6. A18 (*Trimmatostroma abietis*) was isolated from an Ancient Greek marble monument in Chersonesus (Ukraine). J26 (*Exophilia jeanselmei*) was isolated from a sandstone monument in Northern Germany. These two fungi were also used in the BIODAM project of the EU. PF6 (*Sarcinomyces sp.*) was isolated from Persepolis stones in the course of this work.

To obtain cells for fluorometry tests, the isolates were pre-cultures on malt agar and incubated at 28 °C. The fungal cells were resuspended in PBS. Microscopic observation of fungi revealed that sufficient amounts of cells were present in the aggregates. Aggregates were partially disintegrated by using a homogenizer (Ultra-Turrax T25). Ultra-sonicator (Bandelin Sonorex RK100) was then used for 5, 10, 15, 30 minutes to get homogenously dispersed cells. By using sterile normal gauze as a filter (4 layers), a homogeneous suspension of cells was prepared. A Thoma slide chamber was used to count the cells and

suspensions with 10^5cells/ml were used for tests. The cells were stained with FDA and observed in a fluorescence microscope Zeiss Axioscop II equipped with a 450-490 nm and 510-560 nm band-pass filter and 515 nm and 590 nm long pass filter for FDA and CAL AM, and PI, respectively. The fluorometry method and CFU was done to measure the alterations of cell viability after treatment by homogenizer and sonicator.

2.5.2.2 Stains

A working solution of FDA-stain (Sigma) was prepared in Tris-HCL (20µl/ml) by using a stock solution (0.05% FDA in acetone). Solution containing more than 1 µg/ml tend to flocculate (Rotman and Papermas 1966).

A working solution of CAL AM (Sigma) was prepared in dH_2O (3µl/ml).

A staining solution of Propidium Iodide (Sigma) was prepared by diluting the 1mg/ml stock solution 1:1000 in PBS.

These working solutions were stored in the dark at 4°C.

2.5.2.3 Cell Staining Method

For this step, it was necessary to have dead cells. Living cells were killed by incubation of a part of suspension of cells in glutaraldehyde (final concentration 8%) for 1 hour. Cells were washed several times in PBS to remove glutaraldehyde. The equal volume of killed cells was mixed with untreated cells and used to evaluate the staining technique and in the fluorometric assay.

100 µl of cells were mixed with the same volume of FDA-stain, CAL AM-stain and PI and incubated in the dark at 28 °C for 1, 5, 10, 15, 30, 45, 60 minutes. Cells were then observed by fluorescent microscope equipped by different filters. CAL AM, and FDA excitation and emission wavelength of filters were 450-490 nm and 515 nm, respectively. The amount of excitation and emission of wavelength for PI was 515-560 nm and 590 nm, respectively. For fluorometric assay stained cells were washed by twice centrifugation in PBS at 6000 rpm, 4 °C and kept in ice until doing fluorometric assays.

Staining with a combination of FDA and PI were done in two ways. First, the same volume of FDA and PI was mixed and this mixture was used to dye cells. In a second approach, preliminarily cells were stained with FDA and then cells were washed twice in PBS by centrifugation. Supernatant was discarded and the cells re-suspended in PBS. The

same volume of PI was used as a counterstaining to dye cells at 28°C. For both FDA and CAL AM fluorescence was measured with a Fluoroscan-spectrofluorometer Model FLUOstar OPTIMA by using a filter pair at excitation wavelength 485 nm; emission wavelength 520 nm in a black 96-well microtiter plate (Nunc). Fluorometry PI was done in 544 nm excitation wavelength and 590 nm emission wavelength.

The procedure which was used to test efficiency of chemicals was to prepare the YNB medium (Difco). The YNB medium with 5 g glucose was re-suspended in distilled water 10X concentration and filtered. The volume was brought to 1 litre. 10^5 cells/ml was suspended in YNB medium and incubated overnight at 28 °C. Different concentrations and different kind of chemicals were used in this step as follows:

Table.2 Different chemicals and different concentrations tested in this study

Chemicals	BAC	BAC	BAC	PV	PV	PV	BB	TC	PEI
Concentration	1%	0.1%	0.01%	1%	0.1%	0.01 %	5mM	30 µg/ml	30 µg/ml

20µl of biocide was added to 100µl of 10^5 cells/ml suspended in YNB medium and filled up to 200µl with sterile water. To assay simultaneous cytotoxicity of chemicals, mixtures of these chemicals were prepared and tested as follows:

Table.3 Combinations of chemicals used in this study

Chemicals	PV (0.1%)	BB (5mM)	TC (30µg/ml)	PEI (30µg/ml)	YNB culture	Sterile water
1thCombination	20µl	20µl	_	_	100µl	60µl
2thCombination	20µl	_	20µl	_	100µl	60µl
3thCombination	20µl	_	_	20µl	100µl	60µl
4thCombination	20µl	20µl	_	20µl	100µl	40µl
5thCombination	20µl	_	20µl	20µl	100µl	40µl
6thCombination	20µl	20µl	20µl	20µl	100µl	20µl

All test were performed in quadruplicate and experiments repeated at least twice. The cultures were incubated at 28 °C over night. 20 hours incubation periods were used for TC. The cells were twice washed in PBS and centrifuged at 6000 rpm. The washed cells were stained with the same volume of FDA-stain. After incubation in the dark at 28 C° for 30 minutes the cells were washed by twice centrifugation in PBS in 4C° in 6000 rpm. Then 100µl of the cells were transferred in a black microtitre plate and fluorescence

intensities were measured. 100µl of cells without any chemicals (sterile water was added instead of treatment) used as a control. PBS as a blank in the Fluorometer was used. Fluorescent intensity of the YNB medium was measured as well. Autofluorescence of fungi was measured by pouring 100 µl of 10^5 cells into the wells to be sure, that fluorescence was only caused by staining.

H_2O_2 treatment

The effect of H_2O_2, which could increase the staining capacity of melanized rock fungi, was tested. One volume of H_2O_2 (3%) was added to the same volume of cell suspension and incubated at 28 °C for 1, 5, and 10 minutes. Cells were washed two times in PBS and stained by FDA and CAL AM. The cells were washed twice in PBS and then the stained cells were observed through the fluorescence microscope.

2.5.3 Plate Count Agar

Viable microorganisms capable of growth under the prescribed conditions (medium, atmosphere, time and temperature) develop into visible colonies (colony forming units) which are counted. The term colony forming unit (CFU) is used because a colony may result from a single microorganism or from a clump / cluster of microorganisms. This is a classic tools to assay chemical efficiency that are used in medicine, food microbiology, industerial microbiology as well as biodeterioration studies. To compare fluorometry results with the cultivation technique, the same procedure, which was used for the fluorescence technique was applied. 100µl of treated and washed cells were transferred to MEA and spread on MEA by using a Drigalski glass axle. The plates were incubated at 28 °C and kept in the incubator until visible colonies appeared. After 24 h, 48 h, one week and one month the plates were checked.

3 Results

3.1 Analysis of Macroscopic and Microscopic Structures on and inside the Stone Surfaces

3.1.1 Analysis of Macroscopic Structures on Persepolis Samples

Table.4 summarizes the macroscopic description of alterations observed on the Persepolis samples. The surface colour of all samples analyzed differs between the freshly broken and naturally exposed stone. Most samples were found to be extensively covered by epilithic and endolithic lichens and tiny black-brown spots. On sample 8 no lichens were observed.

Table.4 Macroscopic description of alterations observed on samples taken from Persepolis

Sample	Position and quality of stones	Description
1	Gate of Xerxes or International Gate (Army street), limestone	Pitted sample shows some holes inhabited by lichens, different kind of lichen colonies and algal or cyanobacteria colonies were visible
2	Gate of Xerxes or International Gate, marble	Black spots, lichen colonies and crystallization phenomena were visible
3	Gate of Xerxes or International Gate, limestone	Intensive homogenous as well as heterogeneous lichenized areas, black spots and green biological layer were obvious
4	Gate of Xerxes or International Gate, limestone	Heterogeneous lichenized stone, green layer was obvious under the lichens
5	Throne Hall, gypsum	Strong biopitting produced by lichens, subaerial surface shows green colonies, freshly deposited crystals are on the stone and under the green layer
6	Throne Hall, gypsum and $CaCO_3$	Biodeteriorated stone shows a thick layer of orange crystallization over gypsum and calcite layer as well cracks under this layer
7	Xerxes palace, limestone	The sample was heavily colonized by lichens
8	Xerxes palace, dark and hard limestone	Small and new crystallizations show recent colonization, no lichen colonies are visible

The density and distribution of these crusts were heterogeneous as well as homogenous. Many of tiny black or brown spots are easily confused with dust particles or metal deposits. The surface of some samples showed sugar-like or crumbling structures. Some of the stone surface exhibited exfoliation patterns. The most important phenomena were pitting or biopitting described in another part. On the crumbling samples fewer pits were seen in macroscopic observation. In addition to biopitting other alterations were also evidenced in Persepolis stones. Green patinas or discolouration in the subsurface parts of stones were frequent. The presence of photosynthetic microorganisms was seen in these extreme light protected deeper layers. Furthermore, soil and dust deposition layers on the main surface of one sample were observed.

3.1.2 Analysis of Thin Sections and PAS stained Stone Samples

Petrographic thin sections were used to document the biogenic developments of the pits and other biodeteriorative phenomena inside the stones induced by the penetration of lichens and fungi. On the other hand, the objective of thin section analyses was to demonstrate and document hyphae penetration into deeper rock layers. For this reason small pieces of stone encrusted with microflora were selected, hydrated, fixed and cut. Fruiting body of lichen (Fig.6A, 7A, 8B and 9A), individual hyphae and bundles of hyphae (Fig. 6B, 7B, 8E and 8F) are growing on and inside the stones and inter-crystalline space, and clusters of fungi were found inside the stones (Fig.6E, 7D and 9E). Thin sections of stone sample show mesopit lining of endolithic fruiting bodies (Fig.8D). In vertical sections of the stone as well as SEM micrographs algal cells and bacterial cells were revealed in large invasive clusters (Fig.6F and 7D).

Examination after the PAS reaction visualized that the extent of hyphae penetration into the stone by epilithic and endolithic lichens was considerable. The highest depth was up to 2 mm and sometimes even deeper penetration was seen (Fig.6B, 7C, 8E and 9B). The abundance of medullar hyphae was found in the upper area of stone revealing an extensive network of hyphae branching in all directions (Fig.9B). With increasing distance from the stone surface hyphae became narrower. A perithecium of an endolithic lichen was seen in the petrographic stained thin section and shows semi-spherical pits (Fig.8E). Black fungal cells and photobiont cells are present both on the surface and in the inner layer of samples (Fig.7D).

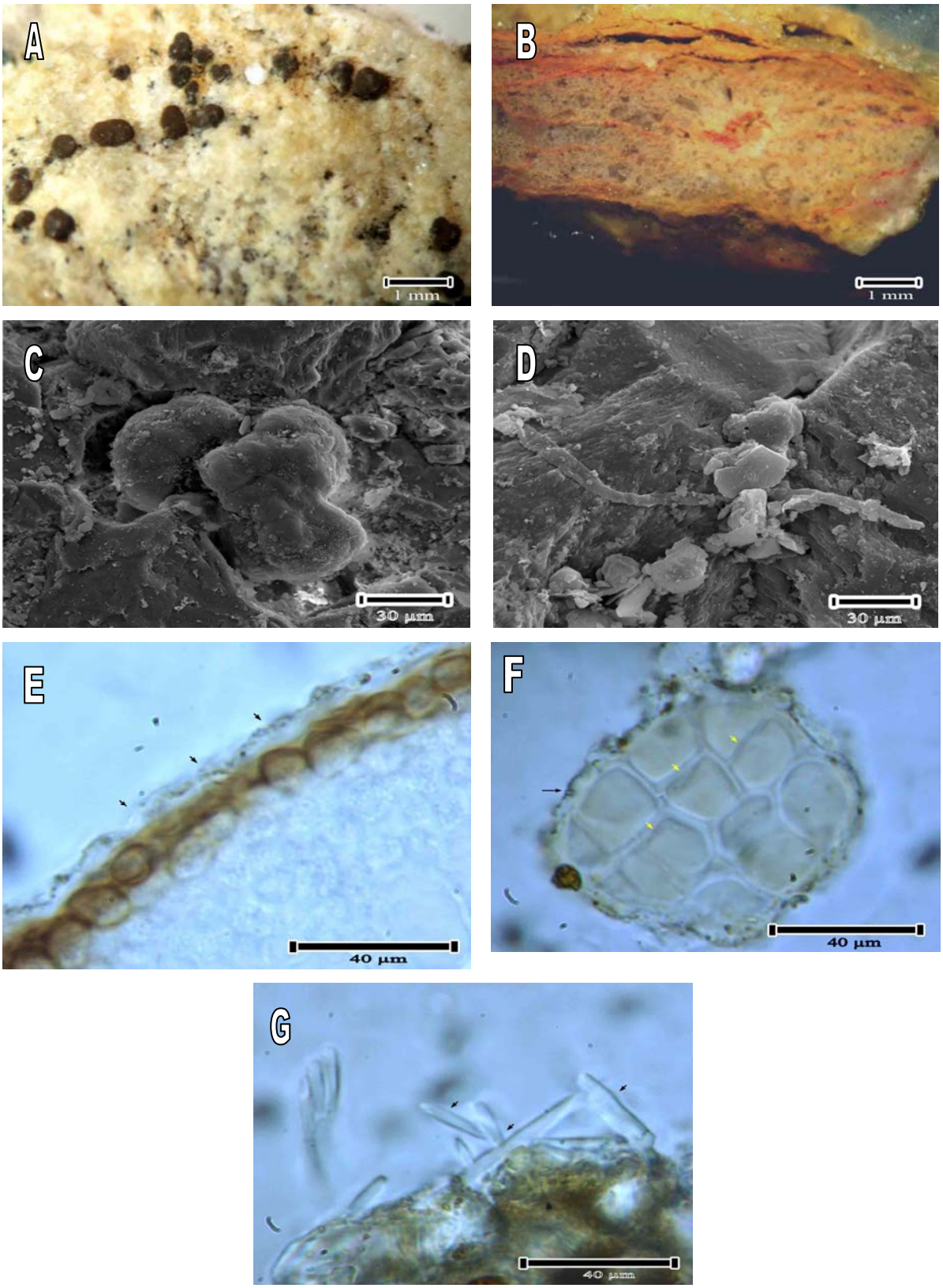

Fig.6 shows sample 2; **A.** Fruiting bodies of lichen was shown on the surface of sample 2, **B.** PAS-stained petrologic thin section showing penetration (growth) of hyphae into (inside) the stone. Penetrations of hyphae are parallel to the surface. This phenomenon causes an extensive exfoliation and flaking of stone, **C.** The cluster of cells covered with slime and embedded in a cavity. Minerals were seen in SEM graph, **D.** Fungal hyphae are going through the stone, **E.** Beneath the slime layer on the lichen surface an upper melanised layer of fungal cells and a protected inner parts of fungal fruit body are visible, **F.** A cluster of cyanobacteria, **G.** Mineral deposits might be gypsum needles on the fruiting body of epilithic lichens were shown.

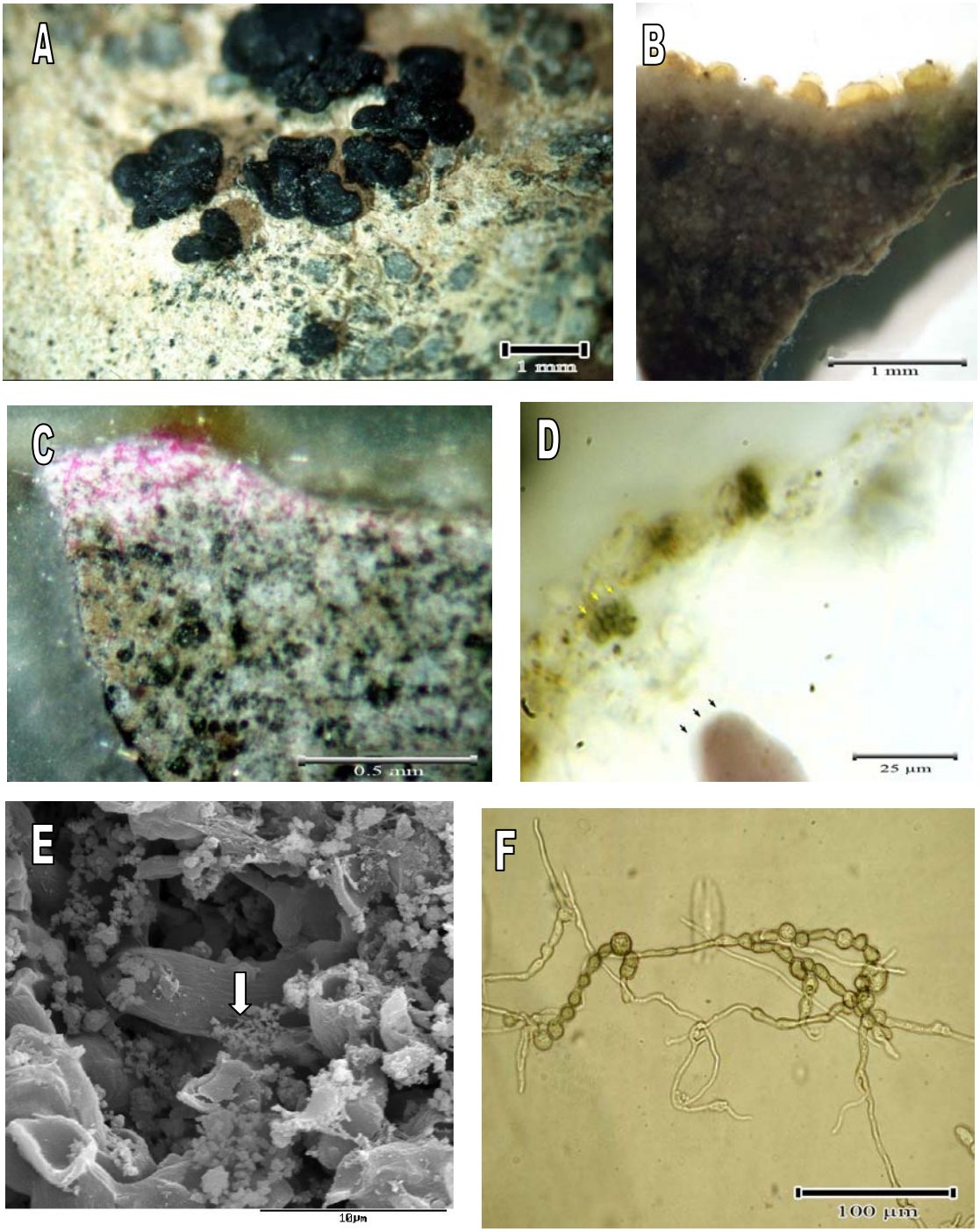

Fig.7 shows sample 3, **A.** Overview of sample 3 shows lichenized surface of stone, **B.** Petrologic thin section through a limestone surface shows lichen fruit bodies and an extensive compact network of hyphae penetrating and altering in minerals, **C.** PAS-stained petrological thin section showing penetrating hyphae into the stone substrate. Stone material has been dissolved or eliminated where the growth occurs, **D.** Micrograph showing a thallus of a lichen, yellow arrows hint cluster of fungi and black arrows refer to algal cell, **E.** Cross section of fungal hyphae with coagulated proteins was shown in SEM graph, **F.** Dimorphic mycelium of rock inhabiting fungi which was isolated from this sample.

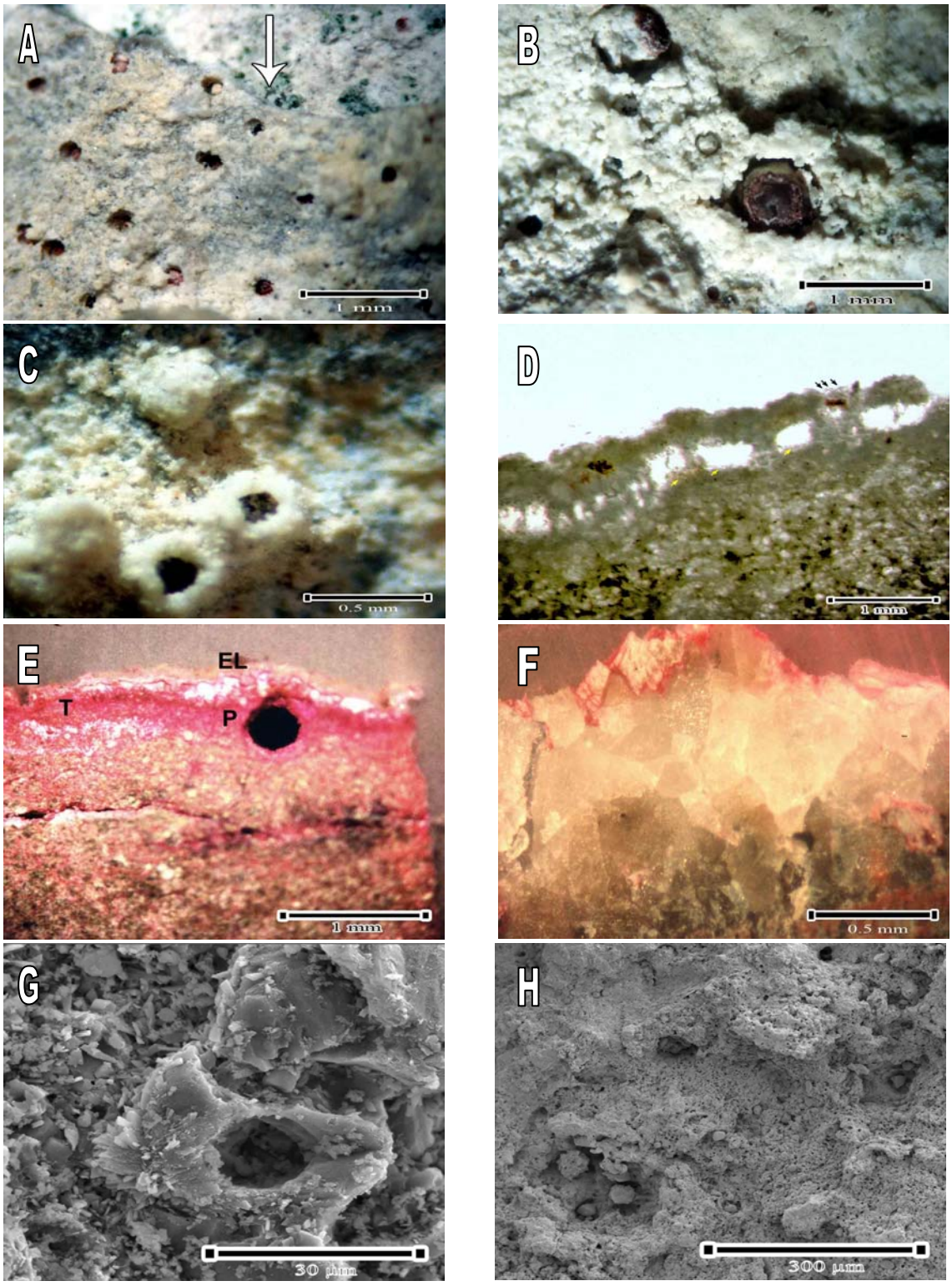

Fig.8 shows sample 5, **A.** Endolithic thalli on the sample 5 created biopitting. On the upper side, right corner photobionts are visible, **B.** High magnification of this sample revealed a lichen fruiting body embedded inside the stone, **C.** In this graph casts were made by the lichens, **D.** Thin section of a stone sample 5 shows pits lining, yellow arrows show mesopits and black arrows show a cluster of cells, **E.** PAS-stained thin section of the sample5. Endolithic lichen perforated the stone with a thallus (T) and a perithecium (P) fruiting body which created a mesopit. White material situated on top of the thallus presents an external layer (EL) of minerals, formed by lichen activity, **F.** Thin section of the stone sample 5 treated by PAS-stain. Biomineralization products have been formed and fungi hyphae has been showed upper and below these mineral layers (calcite), **G.** Pitting (10 µm) with deposite layer of minerals, **H.** Macerated sample revealed leftover cells inside the pits.

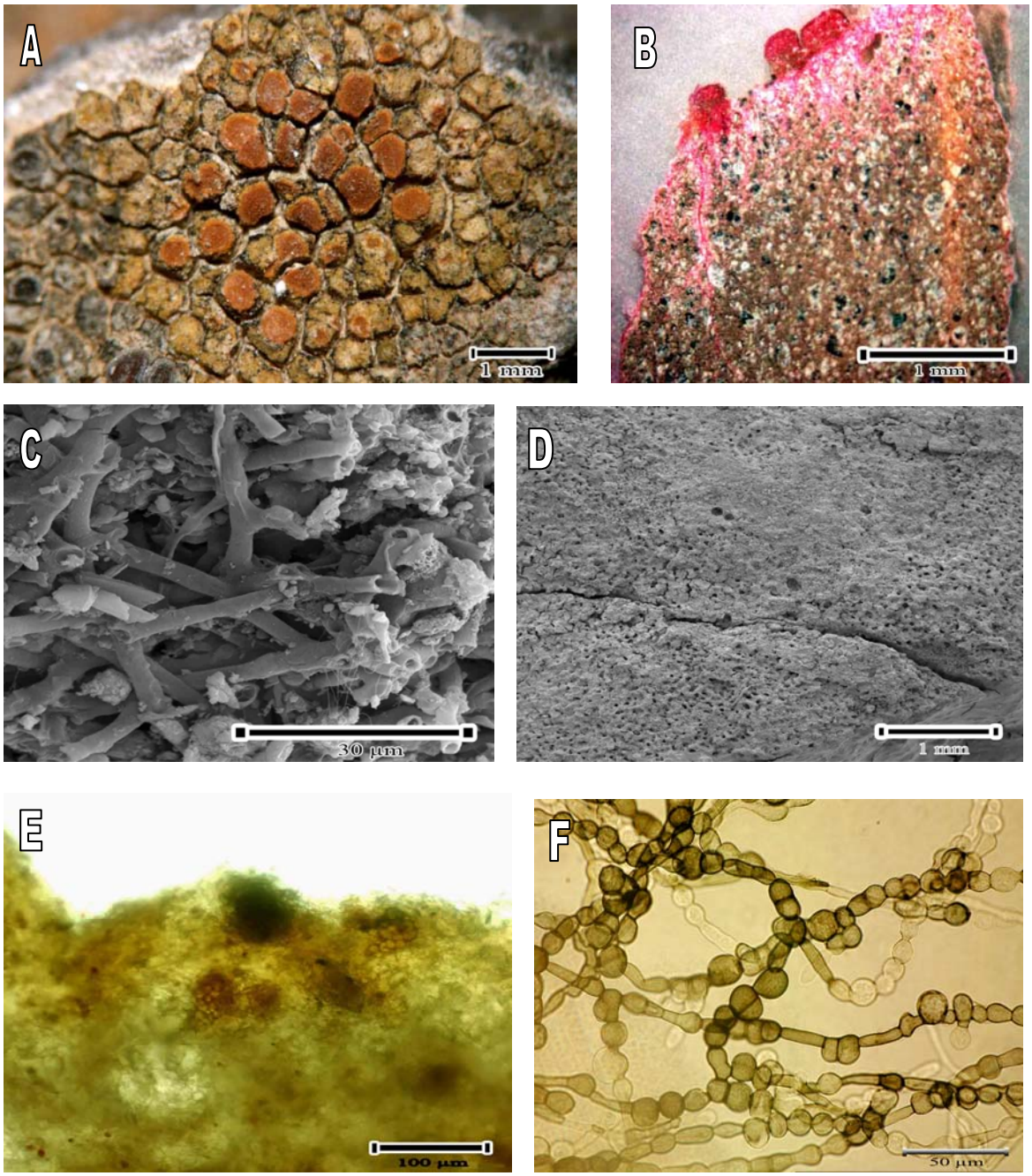

Fig.9 shows sample 6, **A.** Macrograph showing extensive coverage of lichens on the sample 6, **B.** Vertical thin section of the stone 6 treated by PAS reveals fruiting bodies and an extensive network of fungal hyphae penetrating the deeper stone layers. The picture demonstrates the destructive activity of microflora in separating the mineral parts, **C.** A bundle of branching fungal hyphae was accompanied with EPS, **D.** Overview of pitted surface. Micro- and mesopits shows visible typical clusters associated to fungal hyphae as well as fruiting bodies of lichens, **E.** Melanised fungal cell clusters in the deeper layers of the stone, **F.** Dimorphic rock inhabiting fungi which was isolated from this sample.

In some samples bundles of fungal hyphae are growing in a certain depth are arranged parallel to the surfaces (Fig.6B). They seem to arrange at a layer of optimum humidity. External polysaccharide material is covering the cortical and medullar hyphae as shown in Figure 6E and 8E. As a result of reaction between metabolites excreted by lichens and the minerals of the Persepolis samples, appreciable amounts of biomineralization products have been formed and deposited on lichen thalli and on the surface of stones (Fig.8F). Those are usually calcium carbonate with considerable amounts of oxalate and gypsum admixed. Casts of lichens on Persepolis stone surfaces are readily visible, morphological shape of them is correlated to the individual constituents of the endolithic lichens (Fig.8C). Table.5 summarized the presence of different biota on Persepolis stones through the thin section, light and scanning electron microscope investigations.

Table.5 Presence of different biota on Persepolis stones through the thin section, light and scanning electron microscope investigations.

Sample	Lichens	Algal cells	Filamentous hyphae	Fungi cells	Actinomycetes	Bacteria
1	+	+	+	-	-	+
2	+	+	+	-	-	+
3	+	+	+	+	-	+
4	+	+	+	-	+	+
5	+	+	+	+	+	+
6	-	+	+	+	-	+
7	+	+	+	-	-	+
8	-	-	+	-	-	+

3.1.3 Analysis of Scanning Electron Microscopic (SEM) Observations

SEM micrographs are excellent tools to document the biodeteriorated stone surface beneath the microbiota. In the microscopic analysis the fungal mycelia on and within the rock are clearly established (Fig.6D and 7E). The structure of colonies in SEM micrographs shows that fungi are producing hyphal extensions, protruding on the stone surfaces or penetrating deeper in the stones often as mycobiont forms of lichen. Lichen thalli accompanying the biomineralization phenomena were seen in these samples (Fig.10, 11 and 12). In some samples encrusted with epiliths enhanced decomposition of minerals in form of crumbling known as “sugar-like” weathering was visible (Fig.27).

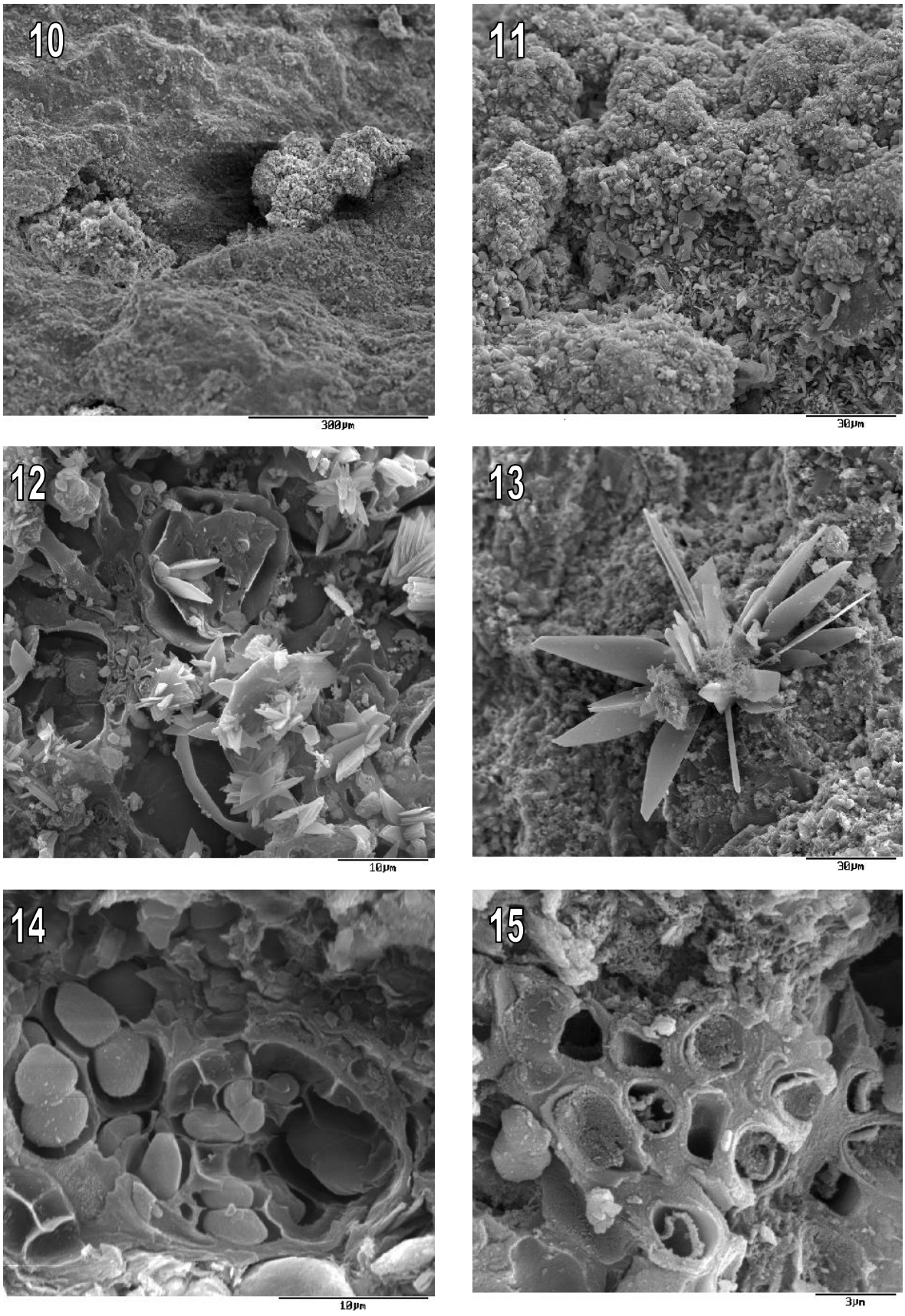

Fig.10 Lichen in a cavity revealing mesopitting, **Fig.11** Different size of crystals cover the lichens as showing in this micrograph, **Fig.12** Biofilm and mineralization of lichens by Apatite or phosphate deposit was shown, **Fig.13** Organic material on the rossette crystal was shown, **Fig.14** Lichen thallus showing cell division, **Fig.15** Photobiont cluster on the surface of stone was revealed, some of the holes without cells was visible.

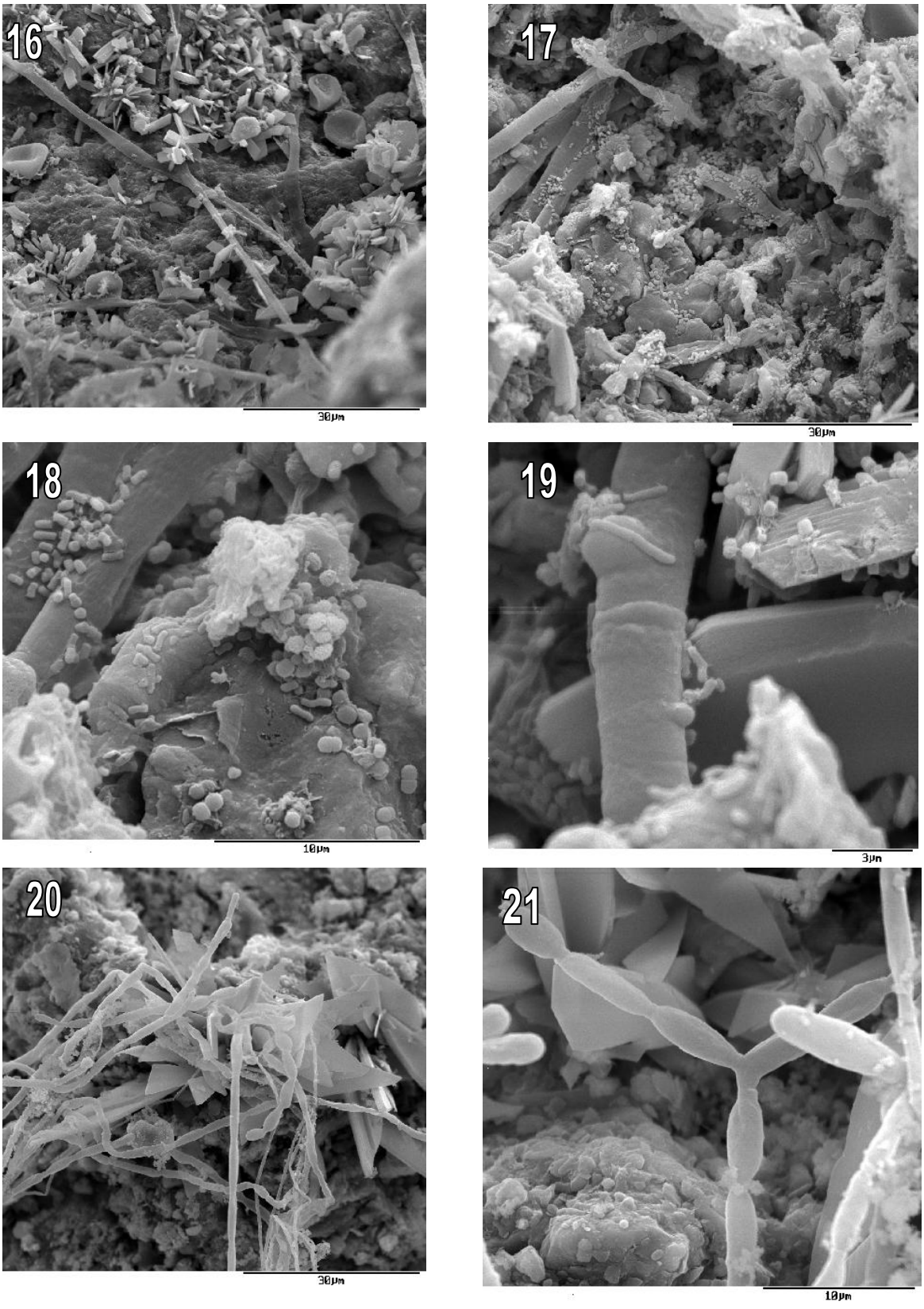

Fig.16 Secondary mineral deposit on some flattened hyphae was shown, some cells are visible, **Fig.17** Different size as well as different shape of microorganisms on the stone surfaces, **Fig.18** Epifungal bacteria were shown on and around the wound, rough and smooth bacteria as well as different size and shape of bacteria is visible, **Fig.19** Fungal hyphae, bacterial cells, actinomycetes, slimes, and solution pattern on a mineral crystal was shown in this micrograph, **Fig.20** Fungal growth (mycelium and conidiophores) on the rosette minerals, **Fig.21** Branched fungal hyphae looks like *cladosporium* sp. and bacterial cells, fresh crystallization (needle forms) was revealed.

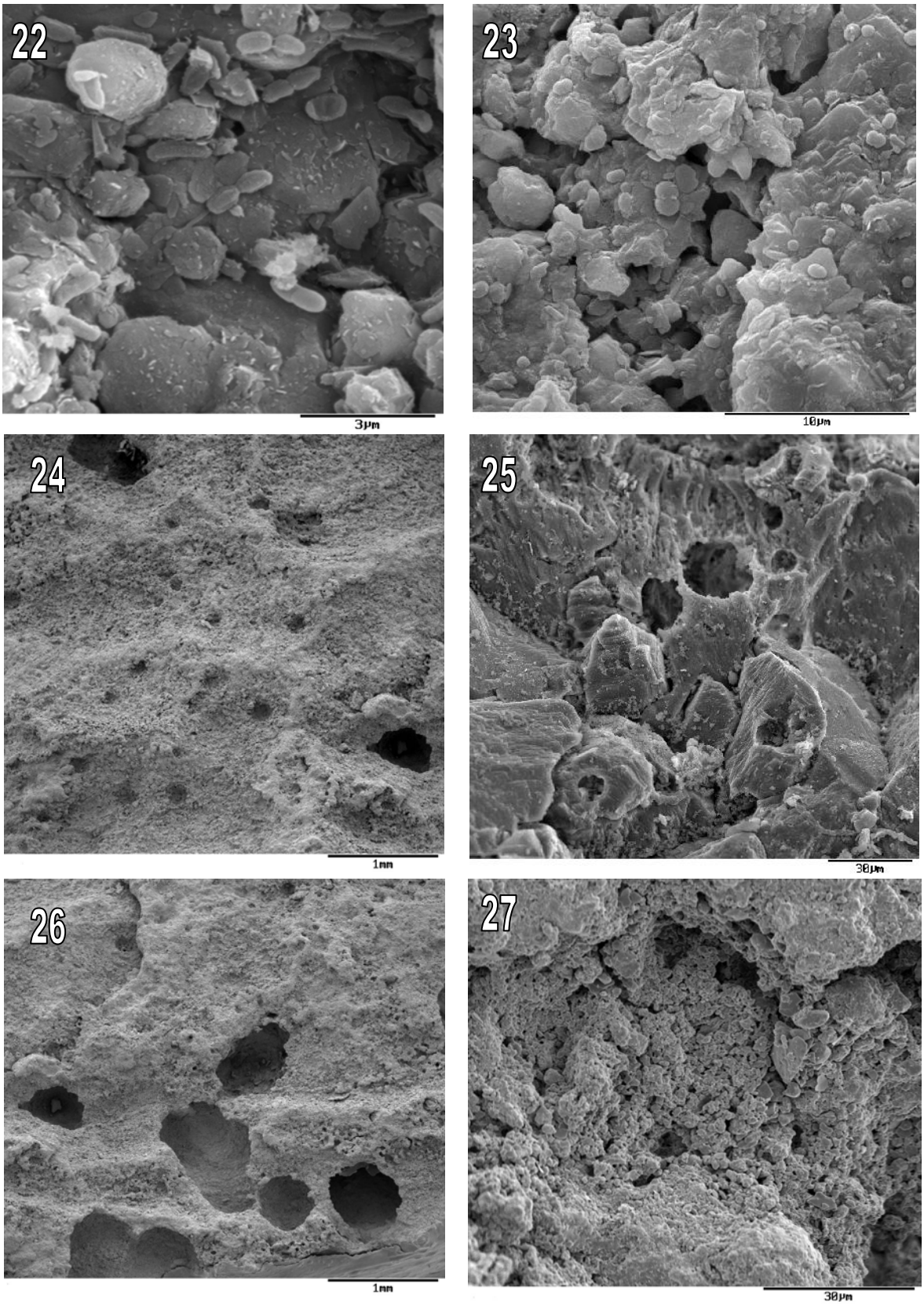

Fig.22 The micrograph revealed EPS around the double divided bacteria in the centre, **Fig.23** Etching pattern is clear, dividing bacterial cells are visible, **Fig.24** Typical structure of pitting after maceration revealing granular rock debris, micropits and mesopits and imprint of fruiting bodies, **Fig.25** Different size of pitting, triangle pitting without any cells documented, organic material in chain less than 1 µm shown, **Fig.26** Macerated stone sample revealing mesopits and micropits, in the upper left side borderline of young lichen thallus was shown, **Fig.27** The biodeteriorated zone made a channel from left to right with very granular loose particles, while smooth and unovergrown surface has intact cemented rock structure.

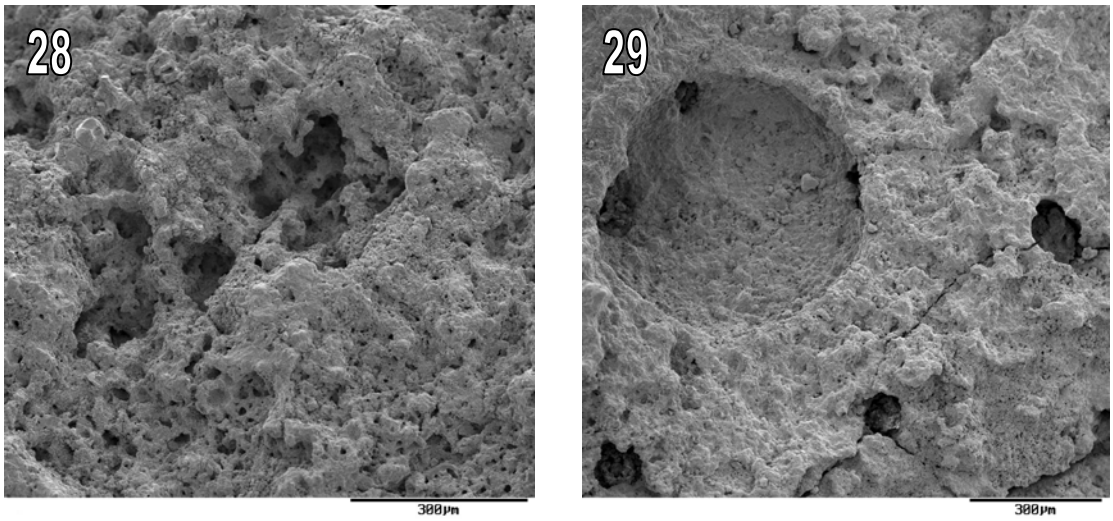

Fig.28 Overview of fungal pitting was shown, mesopit related to thallus also shown, **Fig.29** A typical mesopit left after the fruiting body was removed by maceration, in the lower right side a hiding fruiting body of endolithic lichens and micropits inside of the pits are visible.

Fresh and biogenic biomineralization of surface was shown in some samples and the depth of these coverages was on average 200 to 800 µm. Furthermore, the biomineralized cracks inside the stone produced bio-exfoliation patterns on the Persepolis stones. Different size and different shape of bacterial and algal cells were present in some samples (Fig.15 and 23) and bacterial cells also form considerable etching phenomena on the surface of stones (Fig.18, 19, 22 and 23).

As a result many rock surfaces of the antique monuments at Persepolis are intensively inhabited by lichen colonies as well as by fungal, algal, and bacterial cells. All organism types contribute considerably to the rock wear down patterns with changing morphological patterns.

Macerated Persepolis stone samples often show pitting. These pits were brought about by penetration of bundles of hyphae or penetration of fruiting bodies of lichen thalli inside the stone. These samples are revealing a homogenously pitted area as well as heterogeneously pitted regions. Two shapes of pits were abundant in Persepolis samples. Round to ellipsoidal pits with sharp margin and rough bottom were observed. The second shape is forming a meandric pattern on the samples (Fig.28) and can be compared to the biocorrosion fronts described already by Krumbein (1969). The roundish pits were found to branch inside so that under one large superficial hole several smaller internal holes are found (Fig.29). The diameter of the larger pits was about 800 µm (mesopit). The smallest pit is about 10 µm (micropit). Continued solution of vulnerable sites causes fusion of biopits. Grooves or channels are forming (Fig.27). Pits inside of a pit could also be observed that indicating the recolonization of these pits by algal cells or individual hyphae. Similar observations were

made with the macro/ and mesopitting structures on the monuments in Rome. Another biodeterioration pattern produces etching figures in the form of shallow imprints on the stone surfaces resulting from the activity of the thallus of lichen or imprints of bacterial cells and colonies (Fig.23 and 24). These patterns could only be seen in connection with these organisms. Macropits were not seen in Persepolis stones. Detailed descriptions of pitting structures and classes are given by Gehrmann et al (1992) and Sterflinger and Krumbein (1997).

3.2 Morphologic, Microscopic and Molecular Characterization of Fungal Isolates

3.2.1 Morphologic and Microscopic Characterization of Fungal Isolates

From all Persepolis samples 3-5 sub-samples were taken to culture using the needle isolation technique described by Wollenzien et al. (1995). Black colonies of fungi were visible on the surface of the stone from which the strain was isolated. Seven fungal strains with different morphologies were isolated. No typical fungal strains were isolated from sample 2 of Persepolis. Figure 30-36 show the fungal morphologic and microscopic photographs which was isolated from stone samples.

The colonies of PF1 are growing moderately slowly on DRBC medium with a pale aerial mycelium. Colonies exhibit white to olive green colour and are round, the reverse side is pink. Colonies on MEA attain a diameter of 2.5-3.0 cm in about one week, appearing blackish-brown and powdery due to the conidial masses. The reverse is light brown coloration. Conidiophores are simple or branched; hyaline or greyish at first, becoming brown to black and roughened towards the apex, bearing clusters of conidiogenous cells. Phialides abovate, hyaline at first, are becoming dark to olivaceous-gray. The strain was identified as *Stachybotrys chartarum* which has world-wide distribution. This fungus can produce very toxic secondary metabolites such as satratoxin G and H (De Hoog et al. 2000).
The colonies of PF3 are growing moderately slowly on DRBC. Restricted colonies are woolly and their coloration is olive-green. The reverse is nearly black and attached to the substrate by rhizoid hyphae. The appearance of colonies on MEA is grey-blackish.

Microscopic view shows moniliform melanized hyphae, mid to dark olive brown, ellipsoidal.

PF4 colonies are growing very slowly on DRBC. Restricted, projecting and woolly colonies have pink-red coloration on DRBC. The appearance of colonies on MEA is white-pink. The reverse is pink-redish. Aerial hyphae are regularly seen on the colonies. Septated hyaline hyphae and moniliform cells are seen. This fungus could not be cultivated any more after reculturing in MEA.

PF5 colonies are growing very slowly on DRBC medium. Restricted colonies, which are wet and exudates drops of liquid have dark olive to black coloration, the reverse nearly black. Blackish restricted colony was seen on MEA. They form an irregular, folded, friable, mulberry like mass. Hyphae are absent. The thallus is initially yeast like, converted into aggregates of sclerotic cells. Cells clumps are densely melanized, thick-walled muriform, spherical in shape, budding occurs multilateral.

The colonies of PF6 grow slowly and they have projecting and restricted colonies, pink to black, reverse nearly black. They have a pale aerial mycelium and punctuated hyphae. PF6 has grey-black colonies on MEA and attached to the substrate by rhizoid hyphae. One-celled conidia are formed in long strongly coherent chains, and are undifferentiated and no specialized structure as sexual or asexual sporulating states was seen. Some of the meristematic cells show swelling of hyphal cells becomes multiseptate. Dark olivaceous brown, broadly roundish to ellipsoidal conidia was visible.

The colonies of PF7 grow slowly. They have restricted and projecting colonies. Fresh aerial hyphae are regularly seen on the colonies. Reverse is nearly black. Septae are abundant, brown hyphae and irregular appressoria were seen. Sexual and asexual sporulating structures and tissues were not seen.

PF8 colonies are expanding, velvety to powdery, olivaceous green to olivaceous brown, reverse grey to blackish. Aged hyphae are more brownish. Conidiophores of variable length, without swelling, bearing branched conidial chains, pale olivaceous brown conidia, ellipsoidal to limon form were observed.

Fig. 30 Morphology and microscopy of PF1; **A.** The green-black colony of PF1, **B.** The colony become darkly pigmented with age, **C.** Cluster of colourless to brown swollen phialides at the tips of colourless to brown sometimes branched, conidiophores. The dark brown 1-celled conidia are produced successively from the tips of the phialides.

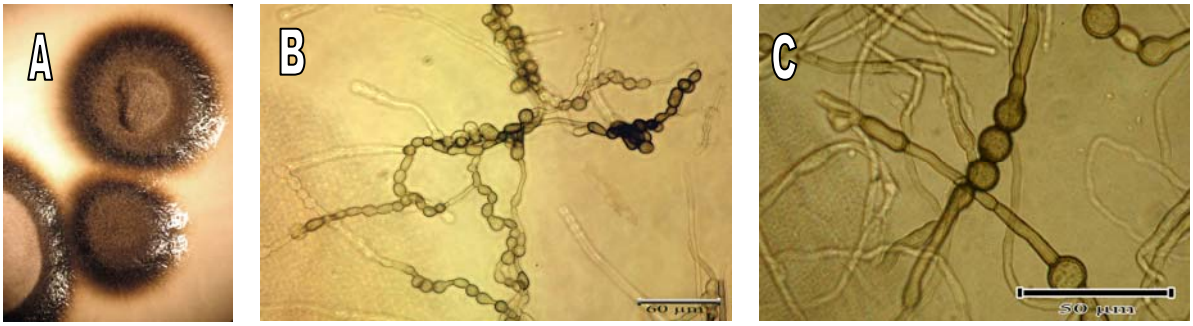

Fig. 31 Morphology and microscopy of PF3; **A.** Morphology of melanized, rock-inhabiting fungi colony, **B.** Fertile and vegetative hyphae, **C.** Melanized moniliform fungal hyphae was shown.

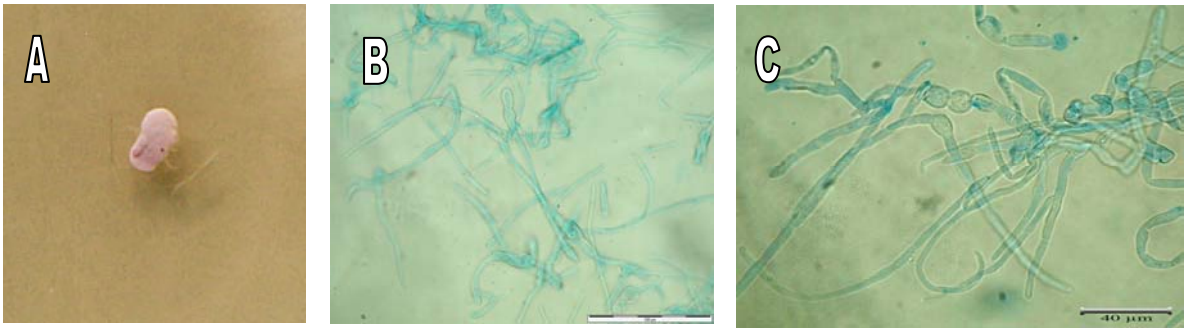

Fig. 32 Morphology and microscopy of PF4; **A.** Morpholgy of PF4, **B.** Hyaline septate hyphae shows blastic development of fertile mycelia, **C.** Moniliform fungal structure.

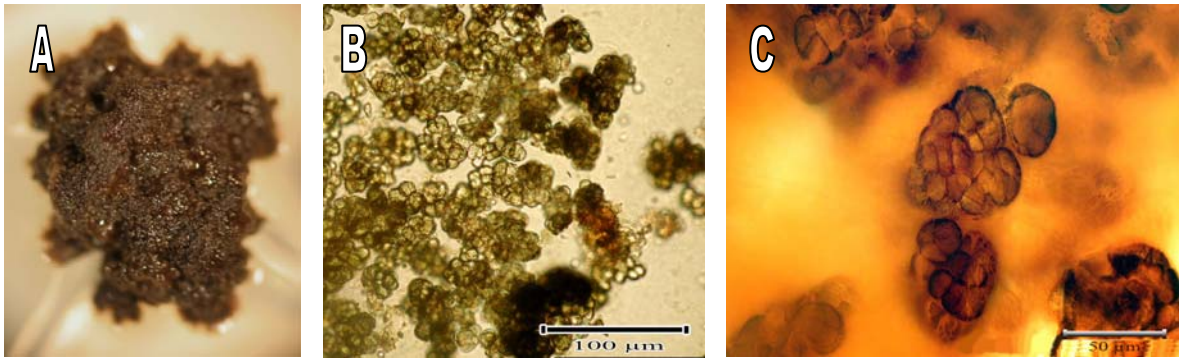

Fig. 33 Morphology and microscopy of PF5; **A.** Exudated colony of PF5, **B.** Clusters of meristematic cells and budding cells , **C.** Mature meristematic cells growth with higher magnification shows isodiametrically swelling of hyphal cells which become multiseptate.

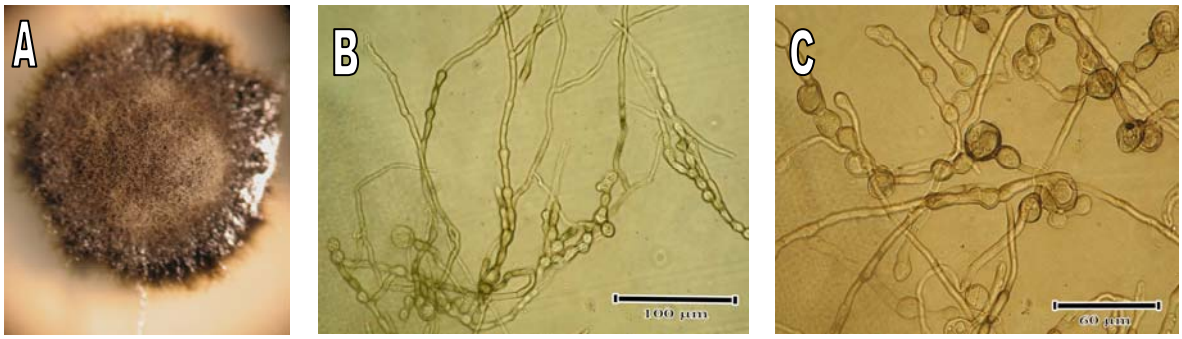

Fig.34 Morphology and microscopy of PF6; **A.** Melanized colony of PF6, **B.** Conidiogenous cells and conidia on MA, **C.** Meristematic cells show swelling of hyphal cells become multiseptate.

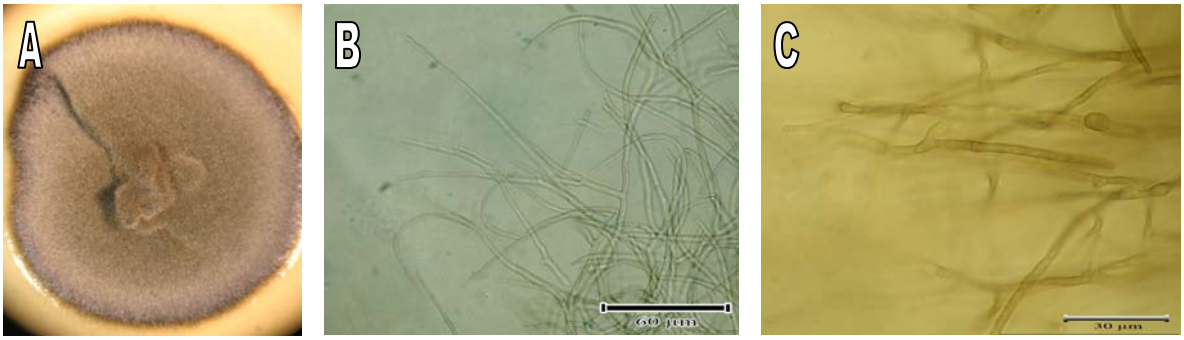

Fig.35 Morphology and microscopy of PF7; **A.** Morphology of PF7, **B.** Branched and septated hyphae was shown, **C.** Brown hyphae and appressorium cells was shown in this figure.

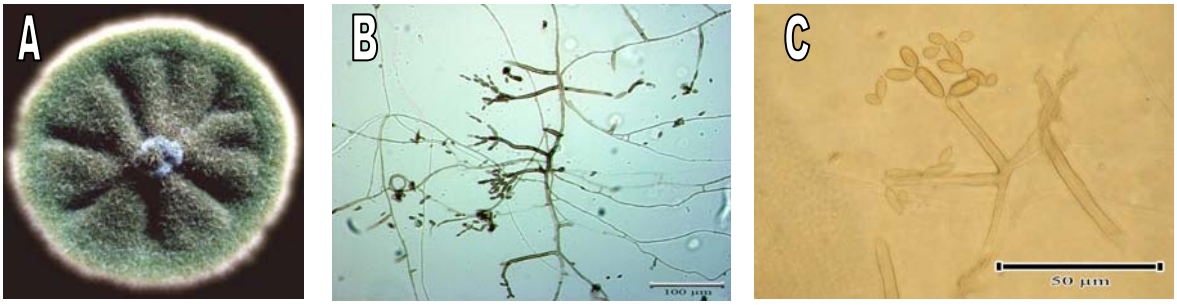

Fig.36 Morphology and microscopy of PF8; **A.** Olivaceous-black colony of PF8, **B.** Pigmented fungal hyphae shows elongated conidiophores producing branched chains of conidia and liberated conidia; **C.** Elongated conidiophores of PF8 produce branched acropetal chains of conidia.

3.2.2 Molecular Characterization of Fungal Isolates

3.2.2.1 DNA Extraction

Two DNA extraction methods were used for all isolates. Total DNA extracted by using the Ultra clean ^{TM} Soil DNA isolation kit and CTAB/chloroform method and the results were evaluated and compared. Using Ultraclean ^{TM} Soil DNA kit more clear bands were shown in 1% agarose gel, whereas the CTAB method revealed only a weak band for PF7 and no

bands were detectable for other DNA extractions of fungi. Figure 37 compares two different methods to extract DNA from the PF5, PF6, PF7 isolates.

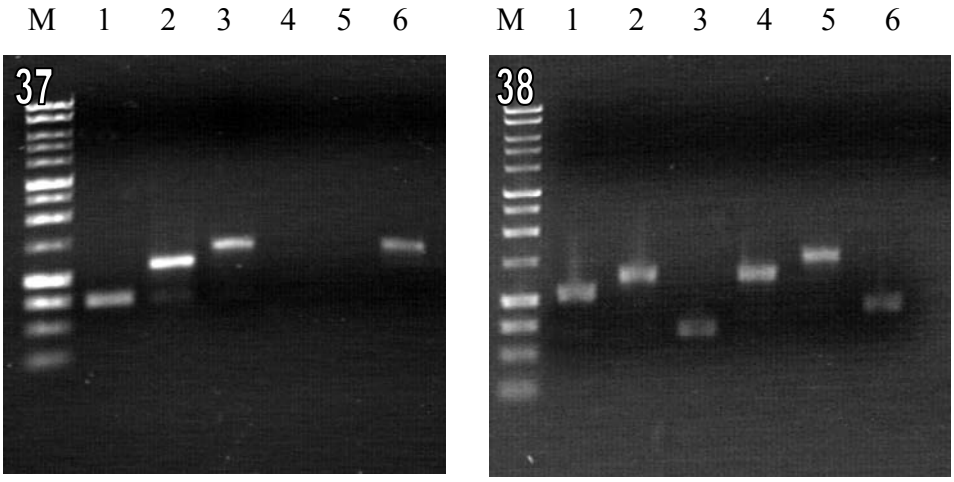

Fig.37 Stained gel shows two different method to extract DNA, M-marker; 1- PF5; 2-PF6; 3- PF7 DNA which extracted by using kit; 4- PF5; 5-PF6 and 6- PF7 DNA which extracted by using CTAB method, **Fig.38** Stained gel shows comparing of two pair primer; 1-PF6; 2-PF7; 3-positive control which amplified by ITS1F/ITS4 and 4-PF6; 5-PF7and 6-positive control which amplified by ITS1_for/ITS4

3.2.2.2 Sequencing- Identification

Fungal DNA was amplified with ITS1F/ITS4 and ITS1 _for/ITS4 primer pair. The primer ITS1F and ITS1_for bind near the 3' end of the 18S rRNA gene. The primer ITS4 binds near the 5' end of the 28S rRNA gene. These probes capture the end of the 18S rRNA gene subunit→ ITS region1→ 5.8S rRNA gene→ ITS region2→ lastly the beginning of the 28S rRNA gene. The ITS regions are available which allows for identification. After PCR a staining procedure with ethidium bromide was done. For this reason the gels were stained by ethidium bromide. Purification of PCR products was done using QIAquick PCR purification kit. For five fungi, ITS1_for/ITS4 had very visible and sharp bands in agarose gel and ITS1F/ITS4 had wider bands in the gel. The first combination of primers was chosen for next step. Using the first combination of primers PF6 and PF7 showed two bands on the gel, in this case, PF3, PF5 and PF8 had single sharp bands. In order to gain purified DNA of PF6 and PF7, 45µl of PCR products were isolated in the gel and cleaned up using QIAquick gel extraction kit. DNA was sent out for sequencing. The results of the sequencing analysis analyzed through Blast matching are shown.

PF3ITSfb

```
AGGCTTCGGACTGGCCCCAGGAGGTCGGCAACGACCACCAGGGGCCGGAAAGTTGGTCAA
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AACACTGGAGAGCCCCTGAAATGCTAGTCTGCATGCACACTGCAGGCAACGCTGTCAAAT
TGCGGGAACACCCTAAAGACCTCAACACCAAGCGCCCGCCGGAaACGGCGGCGTGGCCGA
GCTAGCAGCCCTGGGTACGGTAACAGCTTGAGGTATAAAGCCCCTTAAAAGGGGCAGAAA
TGGACAATCCGCAGCCAAGTCCTACAGCCCTTAGAGGGCCACGGATGCCGTTCACAGGCC
AAATGATAGCGGGTGGCTCATGCAGCCGCTTAAGATATGGTCGGGCTCCCGCAGAGATGC
GGGAGATAAGCGTTACGCTATAAACCGTTCCGTAGGTGAACCTGCGGAAGGATCATTACC
GAGTCAGGGTGTAACAGCCCGCACTCCAACCCTCTGTGTACCATCTCTCTGTTGCTTTGG
CGGGCCCGCGGTGTCATACCGCCGCCGGCTTCGGCTGGTCAGCGCCCGCCAGAGGATTTT
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CAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAaATGCGATAAGTAAT
GTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCTTTGGTAT
TCCGAAGGGCATGCCTGTTCGAGCGTCATTACAACCCTCAAGCTCTGCTTGGTATTGGGC
TTCGCCGGAGCGATCCGACGGGCCTCAAAGTCATAGGCGGCGGTGCCCCTGCTTTAAGCG
TAGTAAAACAATCTTTCGCTTAGGAGTAGAGGCGTCGCTTGCCGAACAACCCGGGCTCTG
CCCACTT
```

PF3ITSfb: gi|66990764|emb|AJ972815.1| Sarcinomyces sp. MA 4787 18S rRNA gene, 5.8S rRNA gene, ITS1 and ITS2, isolate MA 4787 Length=656

Score = 833 bits (420), Expect = 0.0 Identities = 471/487 (96%), Gaps = 2/487 (0%)

Strand=Plus/Plus

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Sbjct  145  CCGAGTCAGGGTGTAACAGCCCGCACTCCAACCCTCTGTGTACTATCTCT--GTTGCTTT  202

Query  61   GGCGGGCCCGCGGTGTCATACCGCCGCCGGCTTCGGCTGGTCAGCGCCCGCCAGAGGATT  120
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Sbjct  203  GGCGGGCCCGCGGTGTCATGCCGCCGCCGGCTTCGGCTGGTCAGCGCCCGCCAGAGGATT  262

Query  121  TTCAAACCAAACTCCTGTCACATCAGTGATTGTCTGAGCAAAAATCAATTAATCAAAACT  180
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Sbjct  323  TTCAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTA  382

Query  241  ATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCTTTGGT  300
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Query  301  ATTCCGAAGGGCATGCCTGTTCGAGCGTCATTACAACCCTCAAGCTCTGCTTGGTATTGG  360
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Query  361  GCTTCGCCGGAGCGATCCGACGGGCCTCAAAGTCATAGGCGGCGGTGCCCCTGCTTTAAG  420
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Sbjct  563  CGTAGTAAAACAATCTTTCGCTTAGAAGCAGAGGTGTTGCTTGCCGAATAACCCGGGCTA  622

Query  481  TGCCCAC  487
            |||||||
Sbjct  623  TGCCCAC  629
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PF5ITSfb

CACACCGCCCGTCGCTACTACCGATTGAATGGCTCAGTGAGGCCTTCGGACTGGCTCAGG
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TCTCTGGCCCGACCTCCAACCCTGTGTGTACCTAACCTCGTTGCTTTGGCGGGCCGCCAG
GTGTCAACCTGCCGTGGGCTTTCGGGCCTGCGAGAGCCCGTCAGAGGACCTTTGAAAACC
CGTCTCAACCGTACCGTCTGAGTGTGATTTTAACCATATAAAACTTTCAACAACGGATCT
CTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAATTGCAGAA
TTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCCTCGGTATTCCGGAGGGCATG
CCTGTTCGAGCGTCATTTCACCACTCAAGCGCCAGCTTGGTGTTGGGCCTACGTCCCTAG
GGGCGGGCCTTAAAGATCGCGGCGGCGTTGCCCGGCTCCAAGCGTATCGGAACAATCCTC
TCGTCTTGGCAGCTTGTGGCAGTGTCGGCCAGGCCCTTTGACGGCCCAATGAACCACCTC
AAGGTTGACCTCGGATCAGGTAGGGATACCCGCTGAACTTAAGCATATCAATAAGC

PF5ITSfb only ITS: gi|15787946|gb|AF332117.1|AF332117 Bellemerea alpina clone J3 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence; and 26S ribosomal RNA gene, partial sequence Length=562 Score = 50.1 bits (25), Expect = 0.004 Identities = 34/37 (91%), Gaps = 0/37 (0%) Strand=Plus/Plus

Score = 50.1 bits (25), Expect = 0.004
Identities = 34/37 (91%), Gaps = 0/37 (0%)
Strand=Plus/Plus

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Query  30  ACCCTGTGTGTACCTAACCTCGTTGCTTTGGCGGGCC  66
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Score = 40.1 bits (20), Expect = 3.9
Identities = 23/24 (95%), Gaps = 0/24 (0%)
Strand=Plus/Plus

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Query  190  GCTTGGTGTTGGGCCTACGTCCCT  213
            ||||||||||||||| |||||||
Sbjct  382  GCTTGGTGTTGGGCCTCCGTCCCT  405
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PF6ITS4kor_reverse_complement

TGCTTGGTATtGGGNTTnGCCGGAGCGATCCGGCGGGCCTnAAAGTCATAGGCGGnGGTG
CCCnnNCTNTAAGCGTAGTAAAACAATCTTTCGCTTAGGAGTAGAGGCGTCGCTTGCCGA
ACAA

PF6ITS4kor_reverse_complement: gi|66990761|emb|AJ972812.1| Sarcinomyces sp. MA 4660 5.8S rRNA gene, ITS1 and ITS2, isolate MA 4660 Length=498

Score = 168 bits (85), Expect = 2e-39 Identities = 110/121 (90%), Gaps = 0/121 (0%) Strand=Plus/Plus

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Sbjct  341  CCCCTGCTTTAAGCGTAGTAAAACAATCTTTCGCTTAGAAGTAGAGGTGTTGCTTGCCGA  400

Query  121  A  121
            |
Sbjct  401  A  401
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PF7ITS4kor_reverse_complement
TTGGTGTtGGGnTGGATTTGTCCAAAAATCGGCCTCAAAGATAGTGACGGCGTTCCAGAT
GGAACCGGTACGAGGAGCTTTTAACCAAGCATGTACGCGGCGTGAATCTGTGGGCGGCCA
TCTTCAACCACTAcTCTTCTGGAGTATC

PF7ITS4kor_reverse_complement: gi|66990748|emb|AJ972799.1|Rhinocladiella sp. MA 4765 18S rRNA gene, 5.8S rRNA gene, ITS1 and ITS2, isolate MA 4765 Length=615 Score = 165 bits (83), Expect = 4e-38 Identities = 89/91 (97%), Gaps = 0/91 (0%) Strand=Plus/Plus

Score = 165 bits (83), Expect = 4e-38
Identities = 89/91 (97%), Gaps = 0/91 (0%)
Strand=Plus/Plus

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Query  28   ATCGGCCTCAAAGATAGTGACGGCGTTCCAGATGGAACCGGTACGAGGAGCTTTTAACCA  87
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Sbjct  424  ATCGGCCTCAAAGATAGTGACGGCGTCCCAGATGGAACCGGTACGAGGAGCTTTTAACCA  483

Query  88   AGCATGTACGCGGCGTGAATCTGTGGGCGGC  118
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Sbjct  484  AGCATGTACGCGGCGTGAATCTGCGGGCGGC  514
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PF8ITSfb
CCTGCCCTTTGTACACACCGCCCGTCGCTACTACCGATTGAATGGCTCGGTGAGGCCTTC
GGACTGGCCCAGGGAGGTCGGCAACGACCACCCAGGGCCGGAAAGTTGGTCAAACCCGGT
CATTTAGAGGAAGTAAAAGTCGTAACAAGGTCTCCGTAGGTGAACCTGCGGAGGGATCAT
TACAAGAACGCCCGGGCTTCGGCCTGGTTATTCATAACCCTTTGTTGTCCGACTCTGTTG
CCTCCGGGGCGACCCTGCCTTCGGGCGGGGGCTCCGGGTGGACACTTCAAACTCTTGCGT
AACTTTGCAGTCTGAGTAAACTTAATTAATAAATTAAAACTTTTAACAACGGATCTCTTG
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GCCTCAAATCGTCCGGCTGGGTCTTCTGTCCCCTAAGCGTTGTGGAAACTATTCGCTAAA
GGGTGTTCGGGAGGCTACGCCGTAAAACAACCCCATTTCTAA

PF8ITSfb: gi|33591049|gb|AF393716.3| Cladophialophora minourae strain ATCC 56961 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence Length=590 Score = 309 bits (156), Expect = 2e-81 Identities = 156/156 (100%), Gaps = 0/156 (0%) Strand=Plus/Plus

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Query  61   TCCGGGGCGACCCTGCCTTCGGGCGGGGGCTCCGGGTGGACACTTCAAACTCTTGCGTAA  120
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Sbjct  138  TCCGGGGCGACCCTGCCTTCGGGCGGGGGCTCCGGGTGGACACTTCAAACTCTTGCGTAA  197

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Sbjct  198  CTTTGCAGTCTGAGTAAACTTAATTAATAAATTAAA  233
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Score = 293 bits (148), Expect = 1e-76
Identities = 148/148 (100%), Gaps = 0/148 (0%)
Strand=Plus/Plus

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Sbjct  390  CACCACTCAAGCCTCGCTTGGTATTGGGCAACGCGGTCCGCCGCGTGCCTCAAATCGTCC  449

Query  217  GGCTGGGTCTTCTGTCCCCTAAGCGTTGTGGAAACTATTCGCTAAAGGGTGTTCGGGAGG  276
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Sbjct  450  GGCTGGGTCTTCTGTCCCCTAAGCGTTGTGGAAACTATTCGCTAAAGGGTGTTCGGGAGG  509

Query  277  CTACGCCGTAAAACAACCCCATTTCTAA  304
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Sbjct  510  CTACGCCGTAAAACAACCCCATTTCTAA  537
```

Table.6 The results of molecular biological characterization of fungal isolates

Fungus	Length of sequenced ITS fragment1	Best Blast match (accession numbers)2	Similarity1	Family
PF3	489 nt	*Sarcinomyces* sp. (AJ972815)	96 % (487 nt)	Chaetothyriomycetes
PF5	491 nt	*Acarospora sinopica* (DQ374139)	96 % (198 nt) 90 % (49 nt) 79 % (491 nt)3	Lecanoromycetes
PF6	124 nt	*Sarcinomyces* sp. (AJ972812)	90 % (121 nt)	Chaetothyriomycetes
PF7	148 nt	*Rhinocladiella* sp. (AJ972799)	97 % (90 nt)	Chaetothyriomycetes
PF8	450 nt	*Cladophialophora minourae* (AF393716)	100 % (450 nt)	Chaetothyriomycetes

[1]ITS1-5.8S-ITS2
[2]only ITS1 and ITS2 considered
[3]calculated with ClustalW

Applying molecular techniques such as genomic DNA extraction and PCR amplification and sequencing of the intragenic transcribed spacers (ITS) between the small and large subunit ribosomal DNA, one isolate (PF8) can be identified as *Cladophialophora* sp. Recently, some species of *Cladosporium* have been reclassified in *Cladophialophora* (De Hoog et al. 2000). *Cladosporium minourae* is the synonym of *Cladophialophora minourae.* Two others (PF3, PF6) had sequences quite similar to database entries for *Sarcinomyces* sp. and one (PF7) to an entry for *Rhinocladiella* sp. For another isolate no well-corresponding ITS sequences were found in the GeneBank database and further characterization of this novel strain is carried out. By caculating with ClustalW the best hit for this isolate (PF5) was *Acarospora sinopica*. All fungal isolates investigated are microcolonial members of the *Ascomycota,* and four of five belong to the interesting ecological group of stress-tolerant melanized *Chaetothyriomycetes* and the other to the *Lecanoromycetes.*

3.3 The Results of *In Vitro* Tests for Chemical Selection

3.3.1 The Results of Fluorometry

3.3.1.1 The Results of Physical treatment on Fungal Cells

One minute homogenization and ten minutes sonication were the best time which could separate the cells and there were not observed big disturbances or wrinkled cells when they checked microscopically. Fluorometry of milling fungi by using the homogenizer did not drastic effect on A18 (11 % reduction) and PF6 showed reduction of fluorescence intensity due to prepare separated cells (41% reduction). Sonication after milling rose up the fluorescence intensity for A18 (25% increase), but fluorescence intensity of sonicated PF6 fell down (10% reduction). In figure 39 fluorescence of PBS, FDA, YNB and cell autofluorescence were measured as well to check fluorescence of compounds.

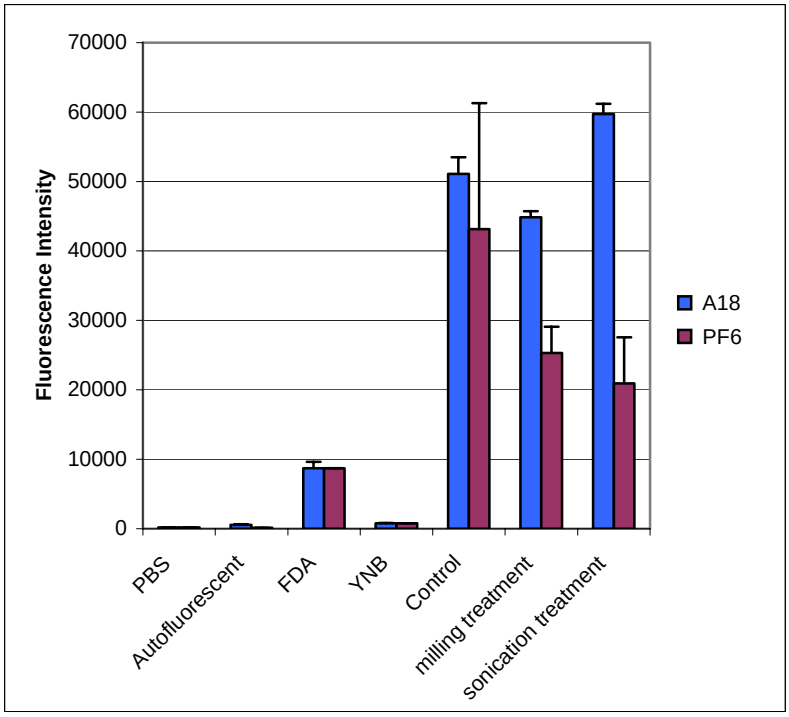

Fig.39 Effect of physical treatment on viability of A18 and PF6; fluorescence of buffer, FDA, YNB, and autofluorescence of cells were measured parallel with the treated cells by using the fluorometric technique. The experiments were performed as described in Material and Method and are presented as mean of triplicate of trials and as one typical experiment of four.

3.3.1.2 The Results of Stained Cells Fluorometry by Using Different Stains

Metabolically active fungal cells produced bright green fluorescence when stained with FDA and CAL AM, unlike killed cells, which did not fluoresce; a completely black picture was obtained for them. The incubation time of the staining procedure was optimized during the experiments. The response time of fungi to FDA, Calcein AM, and PI were assayed. FDA staining-cells performed a strong staining. When cells were incubated with CAL AM, and fluorescence of individual cells was examined under the fluorescence microscopic and using the fluorometric method qualitatively and quantitatively, a weak fluorescence was observed in microscopic analysis especially for strongly melanized cells, and fluorescence intensity was low for A18. In this experiment J26 was better stained than A18 by the CAL AM concentration used in this study when it was observed through a microscope, whereas fluorometric measurement showed as well as A18 low intensity for J26.

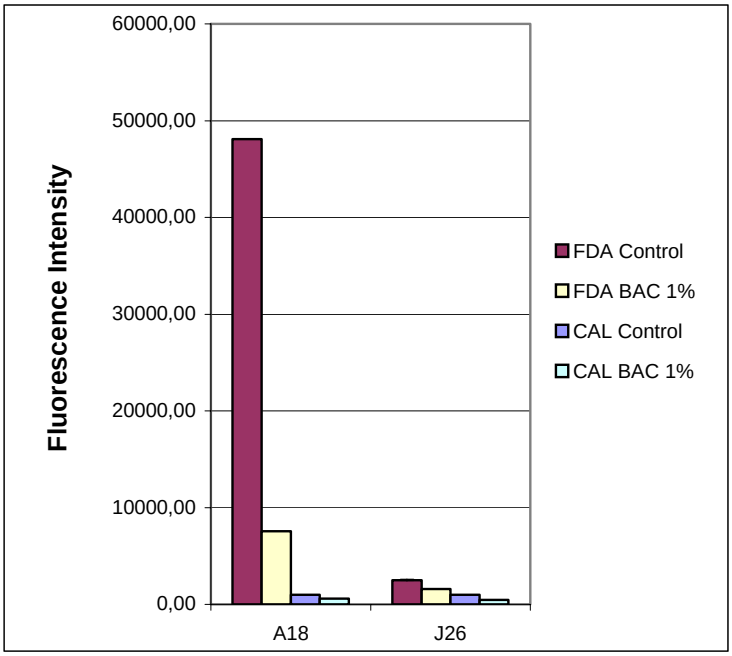

Fig.40 Fluorescence intensity of A18 and J26 with different stains was measured. The fluorescence intensity of treated cells by BAC 1% was measured parallel the experiments. The experiments were performed as described in Material and Method and are presented as mean of quadruplicate of trials.

Treating the cells with H_2O_2 was done for A18 and J26. Results showed that no big strong differences were microscopically observed in the fluorescence of A18 by the expected better penetration of FDA into the cells (Fig.41), although J26 was shown better staining than A18.

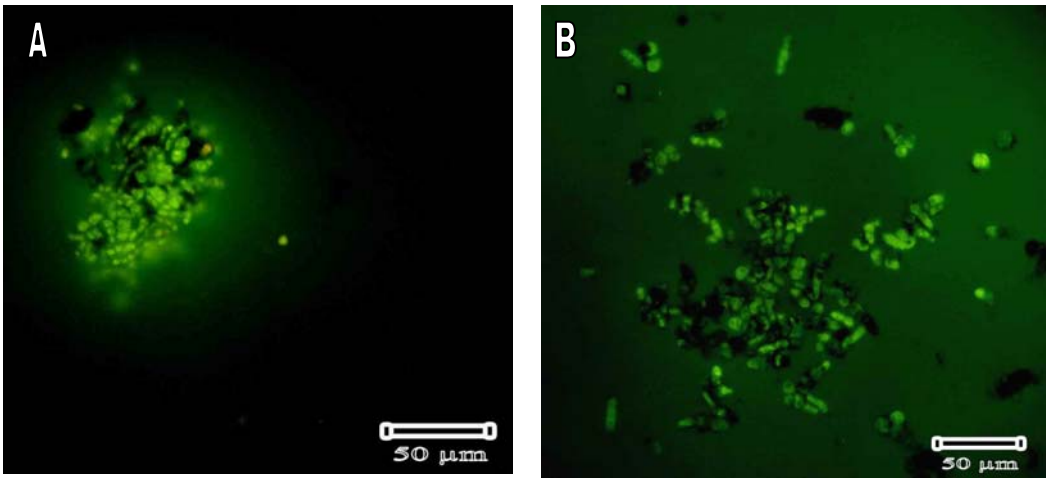

Fig.41 A18 cells was stained by FDA as it mentioned in Material and Methods, **A.** The cell was stained by FDA without H_2O_2 treatment. **B.** The distributed cells was shown after 5' treating in H_2O_2 and then stained.

Furthermore, A18 and J26 shown more fluorescence when they objected to H_2O_2 and stained by CAL AM. But sedimentation of A18 and J26, that was important step to do fluorometry, was disturbed. To neutralize this problem it was necessary to use some

treatment such as using catalase. To minimize the effect of other substances to assay chemical effect, using of H_2O_2 was abandoned.

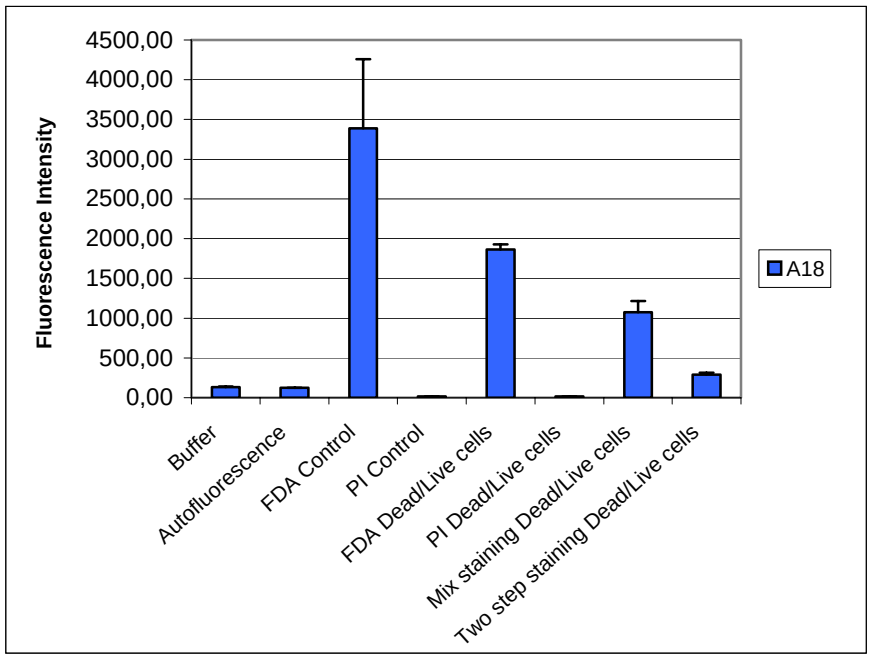

Fig.42 Fluorescence intensity of different stains on A18, a part of cell was killed as described in Material and Method and a 50/50 mixture of live and dead cells used to evaluate the efficiency of staining by different stains and different staining method. The experiments are presented as mean of quadruplicate of trials.

After that, the fluorescence intensity of the fungal cells, based on the combined use of FDA and PI respectively, and a mixture of FDA and PI, and FDA and PI alone were measured. Fungi stained by PI concentration used in this test, were not visible in any microscopic experiments. Furthermore, fluorometric results did not show any considerable fluorescence intensity. On the other hand, estimate of viability of cells by PI had no very successful results. Staining resulted when using the mixture of two stains FDA and PI and to stain the cells in two steps by FDA and PI, had not useful estimate and also the fluorescence intensity of cells was decreased when fluorometric measurement was done. On the contrary, when FDA was used alone, strong fluorescence intensity was shown and it could well be used for chemical treatment assays.

3.3.1.3 The Results of Time Effect on Fluorometry

Results related to compound quenching showed that the cells not only keep the fluorescence but also increase the fluorescence intensity with time. This assay was done for A18 and PF6

and the same pattern was observed for two strains. As shown in the figure 41 and 42 A18 demonstrated a higher intensity than PF6. Fluorescence intensity of A18 brought to 35000 after 30 hours. For PF6 intensity of fluorescence increased slowly and brought to 29000 after 30 hours. After one week of staining, fluorescence of cells was checked through the fluoromicroscope and cells absolutely shown fluorescence. The other phenomenon was observed in these two graphs is increasing the fluorometry of treated cells by BAC 0.01%. The increasing pattern was similar for two fungi. Although this alteration was seen after 30 h for A18, PF6 showed fluorometry increase from the first measurement.

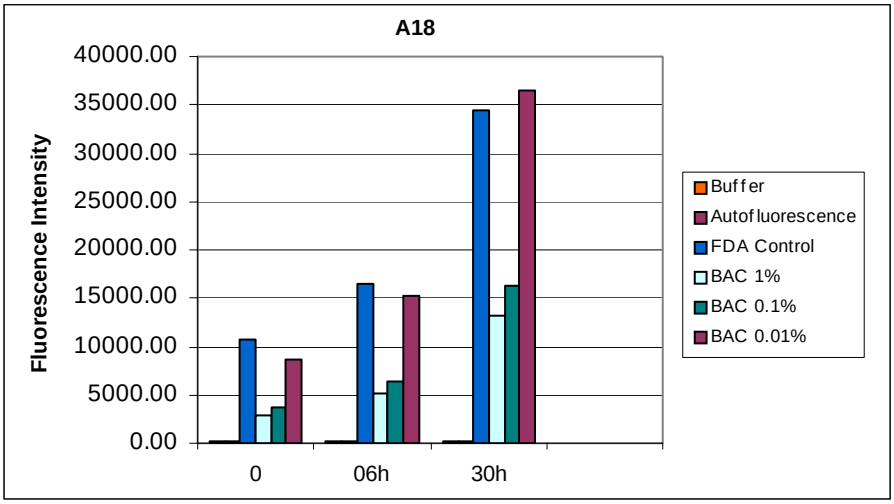

Fig.43 Time effect on fluorescence intensity of A18; fluorescence intensity of FDA control and buffer, as well as autofluorescence and chemical treating was measured. The experiments were performed as described in Material and Method and are presented as mean of quadruplicate of trials.

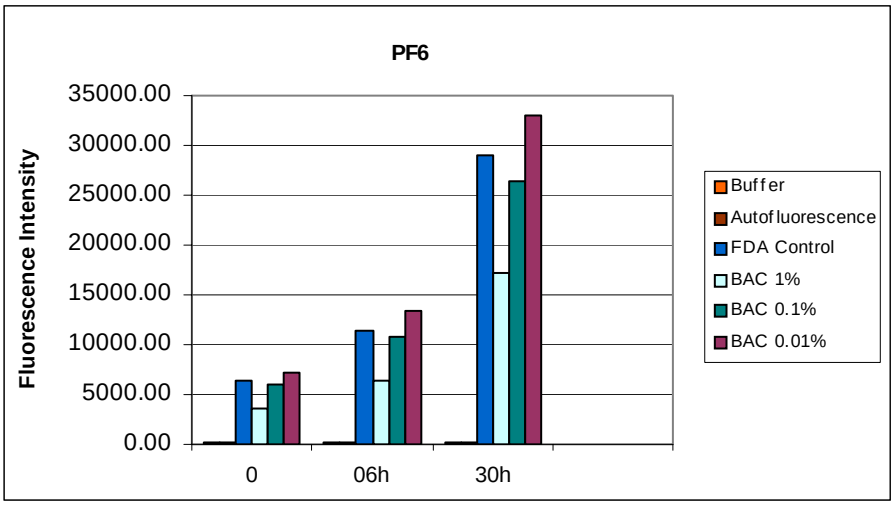

Fig.44 Time effect on fluorescence intensity of PF6; fluorescence intensity of FDA control and buffer, as well as autofluorescence and chemical treating was measured. The experiments were performed as described in Material and Method and are presented as mean of quadruplicate of trials.

3.3.1.4 The Results of Chemical Treatments

It was investigated the effect of three concentration of two biocide on FDA fluorescence in the cell suspensions of A18, PF6, J26. Fluorescence intensity of PBS as a blank and autofluorescence of cells was measured as well in parallel with tests. As shown in figure 45 the amount of fluorescence of PBS and cells showed low fluorescence intensity during the trials. These results showed that the methyl esterase activity of fungi was reduced but not eliminated on the used concentrations. Addition of 1% PV to the three cell suspensions reduced the fluorescence intensity, but a prominent increase in fluorescent intensity was observed after using 0.1% for A18, whereas fluorescence of PF6 was low in this concentration and J26 was shown a little decrease in fluorescence. For 0.01% PV the fluorescence intensity rose slightly up for PF6 and J26. This concentration of PV increased severely fluorescence intensity of A18.

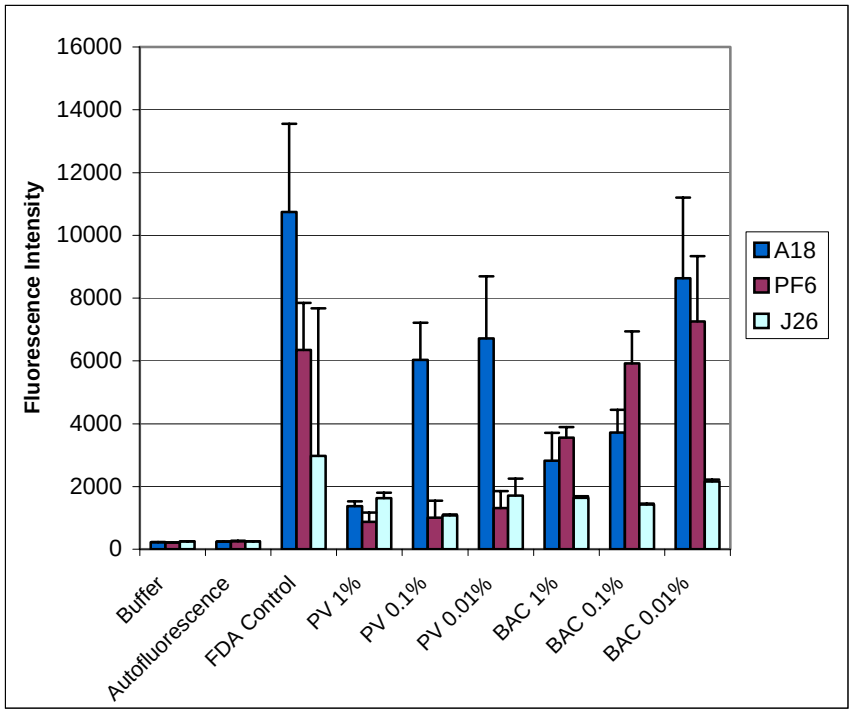

Fig.45 Comparison of effect of three concentrations of two biocides PV and BAC on A18, PF6 and J26 was shown. The fungal cells were stained by FDA. The experiments were performed as described in Material and Method and are presented as mean of quadruplicate of trials as one typical experiment of three.

The well- known biocide BAC was tested in parallel with PV. With each of the three fungi cells mentioned above, BAC caused fluorescence reduction that differed in different concentrations, indicating that this substance has potency to kill cells in some conditions

employed. A18 showed more susceptibility than two others of fungi to 1% and 0.1% BAC, whereas, less fluorescence was observed for J26 in concentration of 1% and 0.1% BAC than PF6. The fluorescence intensity 0.01% BAC rose up for all fungi.

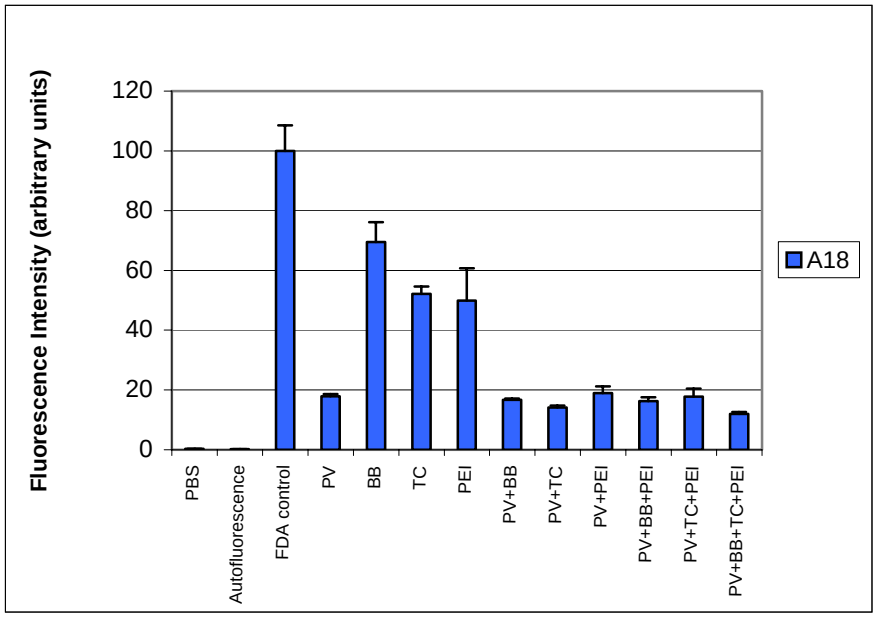

Fig.46 Results of fluorometry A18 in different chemical treatments and combination of them was shown. The results are expressed in arbitrary units and presented as mean values of four individual trials of one typical experiment of three. The concentrations of chemicals were 0.1% PV; 5 mM BB; 30 µg/ml TC and 30 µg/ml PEI.

Later a sensitizing effect of BB, TC, and PEI on PV was assayed. It was tested the susceptibility of A18 and PF6 to a set of chemicals and combination of them. The analysis of data was done by significant difference using *t-test*. The significant difference is done with pair comparison test. In the significant difference analysis, if *p*-value is less than 0.05 there is significant difference between two variables. The results were shown in figure 46 for A18 which indicates that 0.1% PV reduced the fluorescence of cells (82%). BB (5mM), TC (30µg/ml) and PEI (30µg/ml) show 31%, 48% and 51% reduction of fluorescence, respectively. Analysis of results by using *t-test* shows when different combination of chemicals was used significant differences was observed for A18. Exceptions were for the combination of PV and PEI, and PV, TC and PEI. In this case fluorescence intensity of cells was a little increased in these combinations.

The effect of chemicals on PF6 was also tested. In this experiment, 0.1% PV alone brought about only 15% decrease in fluorescence of PF6 cells. Reduction of fluorescence for BB, TC and PEI was 8%, 41% and 64%, respectively. The result of *t-test* showed that combination of PV with BB, TC and PEI increased the efficiency of chemicals and

fluorescence rose slightly down. Similar results were obtained for PF6 with combinations of three and four chemicals and fluorescence intensity reduction was observed.

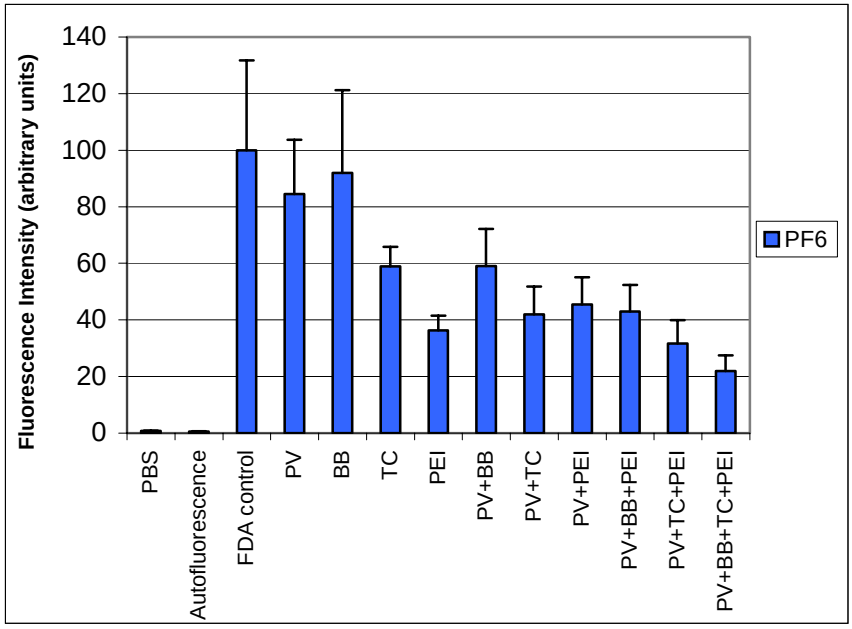

Fig. 47 Results of fluorometry PF6 with different chemical treatments and combination of them was shown. The results are expressed in arbitrary units and presented as mean values of four individual trials of one typical experiment of three. The concentrations of chemicals were 0.1% PV; 5 mM BB; 30 µg/ml TC and 30 µg/ml PEI.

3.3.2 The Results of Plate Count Agar

CFU is one of the well known and widely-used procedures to estimate microbial growth. In this experiment, the inoculum, which was used for CFU and fluorometry was quantitatively the same, i.e. the same suspensions treated with different treatments used in this assay. As shown in figure 48 after treatment by PV, BB, TC and PEI visible A18 colonies were seen on MEA as well as were treated by combination of PV and BB. For others no visible A18 colonies were seen. When homogenizing treatment was used in order to separate individual cells, the number of cultivable A18 sharply decreased (72% reduction). This reduction was not very big when sonication treatment was used. The results were expressed as mean ± SD CFU. Figure 49 shows CFU of PF6 cells after physical and chemical treatments. Homogenizing treatment decreased 15% reduction in PF6 CFU whereas, sonication had little effect on PF6 CFU. After treatment by BB, TC and PEI visible PF6 colonies were seen on MEA. Combination of PV with TC and PEI had less than 30 colonies and was ignored.

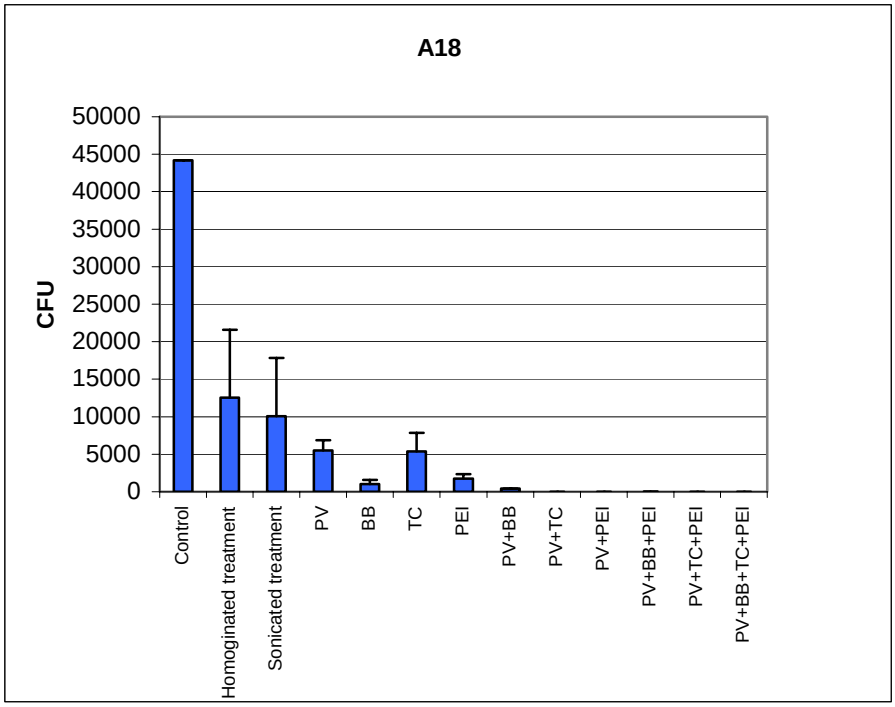

Fig.48 Results of different chemical and physical treatments on A18 CFU. These results are presented as mean values of four individual trials and were done twice. The concentrations of chemicals were 0.1% PV; 5 mM BB; 30 µg/ml TC and 30 µg/ml PEI.

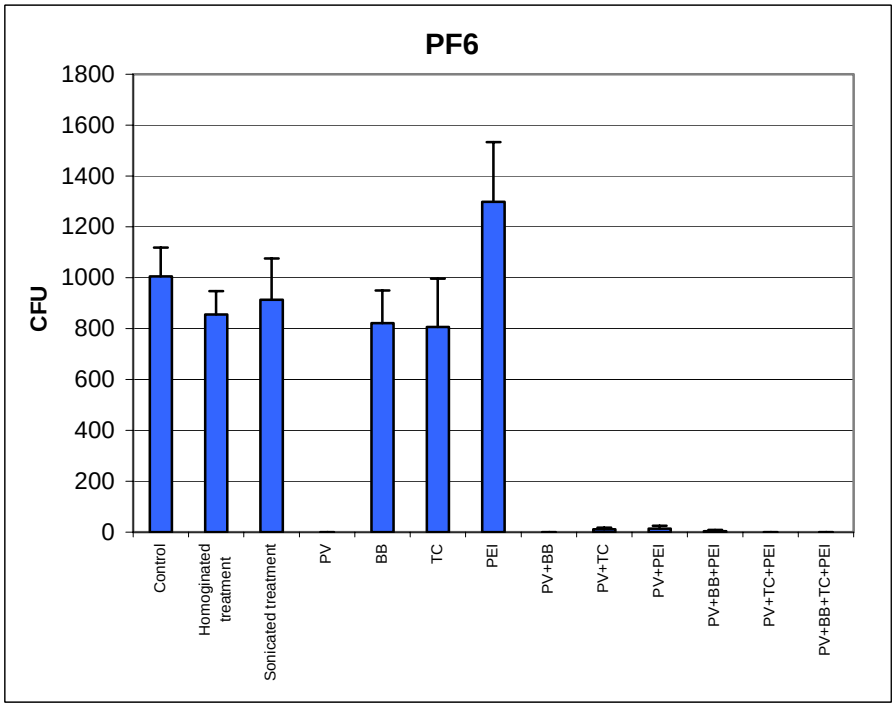

Fig.49 Results of different chemical and physical treatments on PF6 CFU was shown. These results are presented as mean values of four individual trials and were done twice. The concentrations of chemicals were 0.1% PV; 5 mM BB; 30 µg/ml TC and 30 µg/ml PEI.

For others no visible colonies were seen. The results were expressed as mean ± SD CFU per millilitre.

For both fungi correlation between fluorescence intensity methods versus CFU method were calculated. The correlation test was done using the Spearman test. In the Spearman correlation test when significant level is less than 0.05 (p-value≤0.05) the correlation is significant. In this analysis p-value is more than 0.05 and shows that the correlation is not significant. As shown in table 7, these correlations between the two methods (plate counting agar and fluorometry) were not significant.

Table.7 Correlation between Viability Measurements with FDA staining (FI) and Culturability Measurement (CFU); p-value≥0.05 so corrolation is not significant, N indicated the number of test. The analysis was done by SPSS software.

Calculation	AVG CFU A18	AVG CFU PF6
Pearson Correlation AVG FI	-0,193	-0,039
Significant p-value (2-tailed)	0,527	0,899
N	13	13

4 Discussion

4.1 Biodeterioration Phenomena and Fungal Diversity on Persepolis Monuments

The deterioration of stones in buildings and monuments through the action of living organisms has been recognized over a long period, and the topic has received increasing attention over the past few years. Some of the literature is primarily concerned with the influence of organisms on the appearance of stone surfaces, while other deal primarily with the deterioration of the stone itself. Microbial covering alters the original colour and aspect by development of populations with variously pigmented cells of heterotrophic and phototrophic microorganisms (Urzi and Krumbein 1994), by entrapment of dust, soot and particulate matter into the EPS, or by oxidation processes of substrate elements and cell excretion products (Saiz -Jimenez 2003). Schaffer (1932) wrote: “Living organisms also contribute to the decay of stone and similar materials and, although their action is, generally of somewhat less importance than certain of the other deleterious agencies which have been considered, their study presents numerous features of interest”. The effect of certain organisms, such as bacteria, is still a matter of controversy, but the effect of others, such as the growth of ivy, is generally considered to be detrimental (Price 1996). Danin and Caneva (1990) wrote that cyanobacteria cells and lichens can absorb water especially in the extra cellular polymeric substances (EPS) and increase their size by 170-300%, and lichens even 1400% to 3500% due to similar EPS in photobiont and mycobiont. An excellent review is provided by Griffin et al (1991). Another useful overview is given by Caneva et al. (1991). Krumbein and Dyer (1985), Krumbein and Urzi (1991) set out a comprehensive terminology for describing aspects of biodeterioration on stone.

The presence of biological agents in Persepolis stones was studied using light microscopy and scanning electron microscopy as Brock (1991) has stressed that any study in microbial ecology must begin with the microscope. Penetration of mycobiont hyphae and rhizines into occurring crevices and cracks in the stone and on the surfaces were shown by the vertical and inclined thin section preparations.

Maceration techniques were used to reveal the different sizes and morphotypes of biopitting patterns on the stone surfaces beneath the lichens and fungal cells. Lichens play an important role in the process of biopitting of Persepolis stones. These microorganisms are among the first living beings to occupy freshly exposed rock surfaces after archeological excavations have taken place. They also play an important role in pedogenesis (Brodo et al. 2001). The extent of the biopitting corrosion process on the stones depends on the morphology and physiology of lichens and fungi involved, as well as on the physical and chemical nature of the mineral substrate and environmental conditions (microclimate) in which the microorganisms grow. In Persepolis stone materials biopits formed by the joint (cooperative) penetration of bundles of hyphae or individual fungal hyphae of endolithic lichens were found at more or less regular distances from each other, whereas biopits caused by penetration of bundles of hyphea of epilithic lichens in general are heterogeneously distributed. The ecology of rock inhabiting free living bacteria and fungi and of lichen as related to biodeterioration and especially biopitting is, however, need more scientific works. Biodeterioration by lichen has already been reported by 19th Century naturalists. Julien (1883) mentioned the influence of lichens as an organic agent in deterioration of stone material, concerning the thallus nature of crustose lichens coming into direct contact with the stones and closely related to it by fungal hyphae penetration into the stones. But the definition of biopits as a biocorrosive crater erosion was first introduced into literature by Krumbein in 1969 (Clair and Seaward 2004). During field studies on weathering and crust formation in Negev and Sinai deserts, Krumbein and Jens (1981) found that lichens had colonized and destroyed rock varnish formed on rock surfaces. Gehrmann (1995) reported that the formation of whewellite and weddellite seems to be correlated to the specific microclimatic conditions in which the lichens grow. It was shown that epilithic lichens seem to be more aggressive than endolithic lichens based on the higher biomass of the thallus. Furthermore, endolithic lichen growth can be used to describe the ecophysiological adaptations of them to the environmental extremes of the rock (Bungartz et al. 2004). Gorbushina et al. (2002) have described the highly adaptive potential of micropit forming free living black fungi and Gorbushina and Krumbein (2000) described individual life styles and adaptation patterns for the microcolonial fungi. From Italy Nimis and Monte (1988) described pitted surfaces of carbonate rocks. From Spain, Saiz-Jimenez (1981); Saiz-Jimenez and Garcia-Rowe (1991); Saiz-Jimenez et al. (1991); Saiz-Jimenez et al. (1995) and Arino et al. (1997) reported biodeterioration of polychrome Roman mosaics and

Spanish romanesque and Gothic cathedrals and building materials which are pitted from colonization by crustose lichen species and mosses.

Another factor observed in lichenic weathering of Persepolis stones is external polysaccharide substances covering cortical and medullary hyphae in a kind of biofilm material. It was proved in dry state; it can produce high adhesion strengths, leading to a reduction of cohesion and adhesion between the structural components. Many of these substances are aggressive or active on the surface or degraded by acid producing bacteria (Krumbein and Schnborn-Krumbein 1987). These materials also reduce the physiological demand for water (Gorbushina et al. 1999). Furthermore, these materials can catch particulate aerosols and contribute to accelerated biodeterioration of rocks.

Why were green patinas in the subsurface of Persepolis stones also observed? The presence of photosynthetic bacteria has been reported from many parts of the world such as hot and cold deserts (Friedmann 1980). It was mentioned that a response to a harsh environment conditions such as temperature and UV-radiation is rock penetration. Some species of cyanobacteria can be stimulated by higher light intensity to change from epilithic to endolithic growth (Wessels and Budel 1995). The pitting of rock or stone surfaces by microorganisms provide better microenvironmental habitats protected from extreme living condition as it was reported by Sterflinger and Krumbein (1997).

As a result of extensive penetration of fungal hyphae and colony propagules inside the Persepolis stone including chemical activities is alteration and crumbling the stone which known as sugar-like weathering. This weathering structure is related to the connected pores which weathering agents can penetrate, reducing original mechanical strength of the grains which lead to a loosing and separating of the grains from each other (Poschlod 1990). In very beginning state this structure is distinguished in the cortex and algal layer which is amorphous. With increasing distance from this area, the stone appears compact and solid. The deteriorative effect of hyphae penetration brings mechanical disruption of crystals and is drilled channels by micropits and mesopits. These drilling effects of lichens and fungi increase the surface area of the minerals and stone. As a result promote a change in water-holding capacity and humidity in the stones. Göppert (1860) was the first one who discussed about the biodecaying of rock by mechanical action of fungal penetration inside the rock (Gehrmann 1995). Fry (1924) stressed that mechanical etching on rock surfaces is brought by thallus expansion and contraction through the wetting and drying of gelatinous and mucilaginous substances covering fungal hyphae (Gehrmann 1995). Biotransfer of cations

by fungal isolates resulting in crust formation and exfoliation in sandstone monuments have been reported by Petersen et al. (1988).

A large number of fungi are reported to cause deterioration of stones. Pitzurra et al. (2003) reported that different fungal communities were observed in urban polluted and less polluted area in carbonate rock weathering in Italy by using molecular techniques, but there was no big difference in the total levels of microbial contamination. Sorlini et al. (1982) and Karpovichtate and Rebrikova (1991) reported that fungi can only be secondary colonisers, getting their nutrition from dead cells of other microorganisms or their metabolisms and the alteration to the surfaces are aesthetic in nature. Nevertheless, the increased deposition of airborne organic pollutants can support their growth and they behave as primary colonisers (Prenafeta-Boldu et al. 2006). Sterflinger and Krumbein (1997) proved that the dematiaceous fungi are an important agent to produce biodeterioration and biopitting on marbles and limestones. These organisms synthesize melanin-like pigments, which confer more resistance to the attack of other microbes and protect against high UV radiation. Jongmans and et al. (1997) showed fungal hyphae tips producing millimolar concentrations of organic acids that could dissolve cations to form pores at rates of 0.3-30 μm/yr. Duane (2006) proposed a model, firstly indicating early-stage biochemical weathering, followed by biophysical weathering. He wrote that minerals respond differently to chemical weathering by producing peeling structures and microbrecciation features. The dissolution of these crystals appears to be a surface reaction controlled process mediated by microbial microfilaments and nanofilaments. Disintegration of the rock outcrops is caused by a complex interplay of several events, probably beginning at the nanoscale with penetration of sites on crystal faces. However, mechanical damage through microorganisms, especially poikilotroph microorganisms maybe more important for the conservation status in time and space; than environmental and biochemical induced damage. Ruibal (2004) wrote that rock surfaces may merely provide mechanical support and protection, and the mineral composition may produce minimal effect on fungal nutritional metabolism. These rock inhabiting fungi would use exogenous sources of nutrients only when available, in a classic oligotrophic pattern. That is, water from rain, fog, and surface run off carry dissolved or suspended organic and/or inorganic nutrients to the vicinity of fungal cells.

The isolation technique used to isolate microcolonial fungi (MCF) from Persepolis stones was similar to those used by others studying microcolonial fungi in rock surfaces (Wollenzien et al. 1995). In spite of our attempts to culture black colonies from the Persepolis samples only a few of them were successfully isolated onto media. Many of

black spots of MCF were observed on rocks but could not be taken into the culture. The low number of colonies generated indicated that the isolation procedures possibly could have been further improved and, mainly by varying the ranges of culture temperatures and osmotic concentrations of the isolation plates. Perhaps by mimicking the oligotrophic conditions that the fungi encounter in their natural environment, carrying out isolations at extreme temperatures and solute concentrations, and exposing isolation plates to ultraviolet radiation, the number and kind of isolates would have been further extended. Krumbein et al. (1996) explained that these microorganisms on one hand reduce the exchange capacity between the biological system of poikilotroph and bioinert natural bodies in its environment and the other hand make such organisms very resistant to cultivation attempts (http://www.biogema.de/ (Krumbein). By selection of this particular isolation method, our work concentrated on epilithic, non-lichenized fungi which grow extremely slowly on the surface and outer layers of stones exposed to extremely changing environmental conditions. Another reason could be explained that a response to a harsh environment conditions such as temperature and UV-radiation is rock penetration. Some species of fungi can be stimulated by higher light intensity to change from epilithic to endolithic growth and the pitting of rock or stone surfaces by microorganisms provide better microenvironmental habitats protected from extreme living condition as it was reported by Sterflinger and Krumbein (1997). Nature can nurture all extant microorganisms so far resisting to laboratory culture attempt, which is a considerable bias for the research on biodeterioration agents. Culture media with high content of organic material have to be used to isolate the organisms. These rarely if ever are encountered in nature, to which the MCF are adapted with maximum efficiency of utilization of rarely occurring nutritional and physical growth conditions. When dealing with the study of microbial communities under extremely changing poikilotroph conditions, present day culture media and cultivation times cannot reveal the real microorganism distributions. This explanation could be considered to fail the PF4 subculture. Airborne propagules and fast growing species are usually isolated instead of the really active microorganisms functioning in the ecosystem. Furthermore black spots can be caused by the secretion of metabolites that stain the stone or by the presence of pigmented fungal structures, espicially melanin left over after the death or elimination of a former microcolony. After fungal death the pigmented cell walls remain on the surface of the substratum, a phenomenon referred to as "melanin ghosts". Both of these structures might be taken into failing culture attempts. Cyanobacteria also produce black pigments, which could be erroneously taken as a MCF and invariably will fail to grow in the fungal

media. Finally, low diversity of fungi could be attributed to low relatively humidity and great seasonal contrast which is greater than the Mediteranean area described in many biodeterioration studies.

The rock inhabiting fungi isolates are difficult to describe in classical morphological terms because of the scarcity of diagnostic features and polymorphiosm characteristic. Therefore it was necessary to use molecular data in order to reveal information for their identification and classification. We investigated fungal ITS using ITS1F and ITS1_ for as the forward primer binding at the 3' end of the SSU rDNA and ITS4 as the reverse primer binding in the 5' region of the LSU rDNA. These primers (Fig.5) amplify the entire ITS region of both Ascomycetes and Basidiomycetes. Exception of PF8 less support was observed for the other strains. Relative short lengths of some sequences which yield relatively little signal for classification of the cell was problem. Perhaps an analysis of the entire 18S rRNA gene would have produced better results for the isolates. Ruibal et al. (2005) used ITS1/ITS2 rDNA sequences to characterize melanin fungi from limestone in Mallorca. Ruibal (2004) reported that although conserved rRNA gene could be used to identify the fungal strains they are not the best option in the analysis of sets of fungi that are phylogenically close.

Our results showed that ITS1F/ITS4 and ITS1_for/ITS4, when paired efficiently amplified DNA from all fungi in this work, although the problem with ITS1F/ITS4 was reported to be gel smearing (Martin and Rygiewicz 2005) and had less conserved sequences. Although by the CTAB method total DNA is extracted, the presence of DNA digestions, co-precipitation of inhibitory compounds might be hindered to get sufficient DNA. The thick cell wall of these rock-inhabiting fungi could be the other reason of unsuccessful extraction by using the CTAB method.

In this study sequencing was used to identify microcolonial black fungi. Landeweert et al. (2003) mentioned that sequence similarity of ≥99% in rDNA reveal identification to species; sequence similarity of 95 to 99%, identification to genus level; sequence similarity of ≤95%, identification to family or ordinal level although others considered 87% or 94% homology is sufficient for placing of unknown sequences in known fungal taxa (Chambers et al. 1999; McKendrick et al. 2000). Whereas ITS fragment has less conserved sequencing than whole rRNA gene 90% similarity of this fragment could have been explained as a species. Results of a previous study have demonstrated that rock surfaces were colonized by a very diverse and complex assemblage of ascomycetes, with many representatives among the classes Dothideomycetes and Chaetothyriomycetes (Ruibal et al. 2005). However it was

reported that many of the fungi belong to an undetermined number of orders and families, or did not seem to correspond with any known family of Ascomycetes. Of the symbiotic fungi, many are lichenized - an immense group of organisms, which have adapted to an extremely wide range of ecological niches and conditions. The phylum Ascomycota includes more than 98% of known lichenized fungal species. Their total number is ca. 13.500 and they are currently classified in over a dozen of orders. More than 10.000 of these species belong to the class of Lecanoromycetes. In recent years, rDNA sequence data has been used in reconstructing the phylogeny of them (http://www.fmnh.helsinki.fi/english/botany/mycology/ascomycete/research/lecphyl.htm). However, only a few taxonomic groups have been a target for most analyses, while other groups still remain poorly solved. Persoh et al. (2004) reported that the Lecanoromycetes were subdivided into eight monophyletic clades representing the following orders: Acarosporales, Agyriales, Baeomycetales, Gyalectales, Icmadophilales, Lecanorales, Ostropales, and Pertusariales. These clades are distributed in two monophyletic groups corresponding to the two different types of ascomatal ontogeny. It is necessary to do more efforts significantly to increase the amount of multi-gene sequence data for fungal systematics. These data could be combinable with other data for Lecanoromycetes. This needs coordination efforts with the major fungal project. Among these, it should be considered that some mycobionts belonging to lichens may also separately reside on rock surfaces as they were found in this study (Ruibal 2004; Kondratyeva et al. 2006).

The rate of deterioration of building stones and construction materials has increased remarkably since industrialization began (Krumbein 1988). Krumbein suggested that 10 mm erosion of a rock surface exposed in the desert takes 1000 years, but it takes only 250 years when the same rock type used as building material is exposed in any city. Danin and Caneva (1990) estimated rate of pitting 1 mm per 40 years. There is strong evidence that pollution-linked chemical weathering may increase the damaging effect of certain aggressive lichen species which survived in urban regions (Krumbein 1988; Seaward and Edwards 1995). Furthermore as Caneva et al. (1995) reported humidity as another most important limiting factor for the lithophytic microflora. This factor, however, in Persepolis monuments is less than Mediteranean area which was reported before.

Finally, in the present study we could demonstrate that biopits are often not, or no longer, colonized by microorganisms and the alterations and material losses are progressive and the lichens are the predominant microbiota. This is probably caused by changes in microclimatic and macroclimatic conditions through the influence of excavation and

perhaps air pollution. Gorbushina (personal communication) counted a percentage of 2-5 microcolonial fungi (MCF) among 100 empty biopits on biopitted chert in the Negev desert. From documentation of the Germany archaeologist who started the excavation of Persepolis monuments in 1931, it could be explained that most of the biodecay phenomena had developed during 75 years after excavation. Similar time frames were reported by Sterflinger and Krumbein (1997) at the French excavations at Delos. Sand and aerosols, air pollution and secondary invasions of bacteria, algae, fungi and endo- or epilithic lichens, found in the pits create favourable niches for developing in abandoned biopits. These could contribute to subsequent pit enlargement not really related to the original biopit producers.

4.2 Chemical Inhibitors of Fungal Biofilms

4.2.1 Fluorometry

Physical treatments

Microcolonies can consist of hundred of cells but will yield a count of only one when plated. When we talk about efficiency of chemicals, there are different susceptibilities between one cell and microcolonies in biological tests. To overcome this problem, a combination of chemical and physical procedures is usually necessary. There are many chemical agents that can be used to disrupt protein binding of cells and separate them. EDTA, Tween, Junlon and many other detergents were reported (Bowman et al. 2002; Amin et al. 2004). Because of the biocide toxicity assay, which was one of the aims in this study, it was important to us not to use other chemicals. For this reason the homogenising and sonicating method was used to break up cell clumps. No single technique is universally effective, and each technique has its drawbacks, but we have had good success using the physical disruption techniques. The effectiveness of the various methods differs for different microbial species. In general, mechanical methods are non-specific, but their efficiency is higher and application broader in comparison to any of the other methods.

There are few reports to study the milling effect on the viability of fungal cells. A reduction effect (20%) has been previously observed under milling condition described in other

studies with some soil-born fungi (Ingham and Klein 1982; Hassan et al. 2002). In this study milling caused different amounts of reduction in viability and culturability of A18 and PF6. A18 showed less reduction in fluorescence intensity probably due to the fungal cell wall as well as the hyphal septa that have a high resistance to this type of mechanical stress, protecting the cellular protoplasm. The septated nature of A18 and the possible balance between the damage and the recovery of cells could explain this finding but it was not enough to stimulate the culturability of cells. Therefore, esterase was able to remain high and active up to a time after a given milling treatment. Dissimilarity of cell wall components could be the reason of different fluorescence intensity of A18 and PF6.

Ultrasonic waves are another method to disintegrate clusters of cells which are used in industrial and dairy microbiology. This method varies according to the amplitude of the ultrasonic waves, the exposure/contact time with the microorganisms, the type of microorganism, age of culture, the volume of cell suspension, the temperature of treatment and other factors (Geciova et al. 2002). If organisms produce glycoproteinaceous sheath material such as rock fungi, harsh methods are necessary (Hirsch et al. 1995). Ultrasonic energy was used as pre-treatment to disintegrate sludge flocs and disrupt bacterial cells' walls, and improvement of hydrolysis in order to increase rate of sludge digestion and methane production (Wang et al. 2005). Ho (2006) reported that 90 minute sonication could disturb *Escherichia coli* in order to release the HBcAg. Floros and Liang (1994) noted the use of low intensity high-frequency ultrasound for improvement of food product/process monitoring due to the acceleration of diffusion. High frequencies in the range of 0.1 to 20 MHz, pulsed operation and low power levels (100 mW) are used for nondestructive testing to evaluate milk coagulation (Gunasekaran and Ay 1994). Floros and Liang (1994) also listed direct process improvements such as cleaning surfaces, enhancement of dewatering, drying and filtration, inactivation of microorganisms and enzymes, disruption of cells, degassing of liquids, acceleration of heat transfer and extraction processes and enhancement of any process dependent upon diffusion. Hughes and Nyborg (1962) attributed the bactericidal effect of sonication to intracellular cavitation. It is proposed that micro-mechanical shocks are created by making and breaking microscopic bubbles induced by fluctuating pressures under the ultrasonication process (Hughes and Nyborg 1962). These shocks probably disrupt cellular structural and functional components up to the point of cell lysis. Garcia et al. (1989) reported that stationary-phase cells were more resistant to sonication than those in exponential-phase growth stage. Moskowitz et al. (2004) used 5 minute sonication to remove mature biofilm of *Pseudomonas sp*. from peg lids. No

mathematical model has been formulated for the inactivation of microorganisms by ultrasonic methods. Review of the literature indicates that no published information on microbial inactivation rates is available. Such information is necessary to generate kinetic data. Two fungi were tested in this study showed different fluorescence intensity. It seems A18 is resistant to sonication and due to prepare separate cells amount of fluorescence was increased. The result for PF6 shows reduction in fluorescence. Different structure of cell wall can be involved in this result as it mentioned above. Also generation times of these black fungi, which grow slow, are different. It means PF6 grows slower than A18. It could be concluded that PF6 cells were in exponential-phase. These modifications of inoculums appear to affect the results of CFU and fluorometry and importantly, improve the methods which use in biocide activity in microbiological study. Further information of ultrastructure of fungal cell wall is necessary to evaluate alteration of component in fungal cell wall.

4.2.1.1 Fluorometric Methods

Fluorochromes are available that indicate cell viability by difference in cytoplasmic redox potential, electron transport chain activity, enzymatic activity, cell membrane potential and membrane integrity (Kepner and Pratt 1994; Breeuwer and Abee 2000). To assay viability of different kind of cells become common. There are some reports about using the fluoroscent dyes (May and Lewis 1988) or chemicals which detect enzymatic activity (Warscheid 1990; Taylor 1992). The fluorescence of FDA-stained cells was visible under the fluorescence microscope qualitatively. Ziegler et al. (1975) and Sörderström (1977) have described membrane properties in active, passive and dead microorganisms with respect to FDA fluorescence activity and were confident that this dye only stains metabolically active cells. Ingham and Klein (1982) showed that FDA-staining was related to hyphal growth and was 10% or greater during fungal growth and less than 10% during the late growth, stationary, and death phases. They supposed that growth stages can be assessed by the percentage of FDA-stained hyphae. Stewart et al. (1999) determined esterase activity in dead and live cells and stated that the measure of the esterase in the culture media could permit identification of potential cellular lysis during the growth and decline phases of culture in order to better identify the effect of culture parameters on cell death. Hassan et al. (2002) showed that FDA-stained *Trichoderma harzianum* made it easy to distinguish

metabolically active portions from metabolically inactive fragments of fungal hyphea. Viability of *Mycobacteria*, which grow very slow in culture, can be determined in samples by using the fluorescence stains (Jarnagin and Luchsinger 1980). Epifluorescence microscopy is reported as the method of choice for enumeration of microorganisms in environmental samples (Kepner and Pratt 1994). Gaspar et al. (2001) and Morris et al. (1997) used FDA to measure fungal biomass in soil. Tuszynska et al. (2006) by using the fluorescence stain studied soil fungal responses to zinc. A limitation of epifluorescence microscopy is that with environmental samples such as soil, rock and sediment, the fluorochrome may also bind to organic matter present, leading to overestimation of the number of microorganisms present. Some chemicals induce enzyme activation (unspecific esterase), and loss of cellular material during the washing procedure, and intensive melanization for rock fungi are the other disadvantages. To overcome these disadvantages it was considered some notation. The first points may be overcome by initial control experiments for any new cell. Washing procedures may delete the unspecific chemical effects and decrease the back ground hydrolysis of FDA as it was mentioned by Clarke et al. (2001). A third point, in the present study, especially for filamentous fungi has been considered. In this case longer time of centrifugation was suggested. Furthermore, using physical treatment to separate the cluster of cells mildly increases stainability of cells. In this work, a method to determine fluorometrically the amount of rock fungal cells after treatment by chemicals was elaborated and tested. The fluorometry technique offers several general advantages; (a) the method is rapid and permits a larger number of samples to be analyzed with a minimum of cost and labour; (b) this procedure is relatively simple and no hazardous material is needed (c) the microformat requires only small amounts of reagents and biological material which decreases the monetary costs and permits analyses of limited availability; (d) the dyes are non-destructive, allowing further working on the cells such as following alteration of enzyme activity of cells during the time which is very important viewpoint in susceptibility test and conservation and restoration aspect.

4.2.1.2 Stains

FDA derivates have been commonly used as a vital stain and applied to enumerate viable bacterial cells in fresh water, waste water and also in dairy and probiotic products (Porter et al. 1995; Bunthof et al. 1999; Bunthof and Abee 2002; Lahtinen et al. 2005; Lahtinen et al. 2006). It has also been reported that numbers of FDA-stained cells in herbal medicines were not similar to numbers of bacteria with growth potential determined by quantitative direct viable count method (Nakajima et al. 2005). Lass-Flrl et al. (2001) used viability staining in antifungal test and showed that to kill hyphae required higher concentration of antimicrobial agents than the conidia. Bowman et al. (2002) stated to assay antifungal activity of fungi static drugs which could not decrease CFU, showed changes in pattern of staining by fluorescence stains. Yang et al. (1995) showed that by using an ionic intensified FDA spore of several fungi could be distinguished. Chand et al. (1994) used this technique to evaluate antimicrobial activity of extracts and marine natural products. Fluorescent stain was also used to evaluate susceptibility of microorganisms in broth microdilution method (Liao et al. 2001).

CAL AM, another fluorochrome, which was reported could differentiate live and dead cells. It may be extruded by the transporter protein of multi-drug-resistant mammalian and eukaryotic cells, which resulted in low fluorescence of cells in comparison with control cells (Millard et al. 1997). Yang (2001) reported that the uncleaved CAL AM does not fluoresce and allows direct measurement of a cell suspension without removing the indicator outside the cells. They also showed that CAL AM could not discriminate the drug resistant *Candida albicans* cells from control cells of *Candida albicans.* In this work, under the fluorescence microscope and using fluorometry measurements, there were, however, low fluorescence intensity and weak fluorescence staining for CAL AM. This observation is consistent with the low stainability of CAL AM against *Saccharomyces cerevisiae* cells previously reported (Kaneshiro et al. 1993). The low fluorescent capacity probably came from the thick cell wall impermeable for CAL AM or differences in the physical location of the stains within the cells. Different amounts of melanin in fungal cell walls could be explained the different stainability of fungal cells to CAL AM. It seems melanin reduces the porosity of the fungal cell wall. Thus CAL AM was not reliable for fluorometric assessment of rock inhabiting fungal cells. Therefore, as the results showed, FDA can be considered as a vital stain to

assay chemical effect on fungal cells in fluorometry and fluorescence microscopy and CAL AM was not used for further experiments.

Jones and Senft (1985) showed that simultaneously double staining with FDA-PI was consistent over prolonged periods of exposure to the dye and it was a reliable method to determine cell viability. Lopes et al. (2003) used fluorescence stains, FDA and ethidium bromide, to demonstrate dermatophyte growth stages as well as the perfect differentiation between viable and non viable cells. Hickey et al. (2005) have found that combining of the stains FDA and PI works extremely well for confocal microscopy. Diaz et al. (2003) and Bunthof et al. (1999) suggested that FDA-PI two-colour fluorescence is a suitable method to study antibody-mediated cytotoxicity by endogenous or exogenous complements for various pig cells and to study impact of different stresses on *Lactococcus lactis* respectively. Sipkema et al. (2004) extended the combined use of FDA and PI to assay the effect of number relevant parameters on the viability of sponge cell cultures. PI is a membrane impermeant and generally excluded from viable cells. Permeabilization of cells is required for counterstaining with PI. Our results showed that using two-colour fluorescence and PI lonely was not suitable may be due to the thickness of cell walls and reduced porosity of membrane. The knowledge about ultrastructure and cytoplasmic components is not sufficient to explain these observations. Probably the presence of strongly melanized cells and other components, which was shown to absorb in the UV spectrum, could be considered. Furthermore underestimating of fluorescence intensity of FDA with two colours staining in two steps which were shown in result could be attributed to the number of washing steps.

Why have not been obsereved increasing in stainability of fungal cells by using H_2O_2? Some components such as manitol, melanin have been suggested to have anti-oxidant properties thus allowing the organisms to resistence against these kind of products (San-Blas and Calderone 2004). Jacobson et al. (1995) reported that to neutralize H_2O_2 by melanin under neutral condition higher concentration of anti-oxidant must be consumed. He explained reaction of melanin with H_2O_2 require alkaline condition. Amin et al. (2004) proved that toxic low level of hydrogen peroxide decreased cell viability was associated with apoptosis. It seems that concentration of hydrogen peroxide which used in neutral condition in this study was not lethal for these fungi and they showed fluorescence after treatment when it checked through the fluorescence microscope. Furthermore, hydrogen peroxide did not increase considerable stainability of cells. There is some explanation for this result: (a) melanin is an extra cellular component, whereas hydrogen peroxide is neutral

and diffuses readily into the cell; (b) hydrogen peroxide reacts with melanin only under alkaline conditions which there was not this condition in my work.

4.2.1.3 The Time Effect on Intensity of Fluorometry

Why fluorescence intensity of rock fungi was increased during time? Stewart and Deacon (1995), and Yang et al. (1998) showed FDA fluorescence to fade over short exposure times to UV light. Rotman and Papermas (1966) suggested that mammalian cells could be held for a short periods of time after staining and it depended on temperature of holding. In the same paper, it was reported that 7 h at 10 °C was the maximum time of holding. Similar results were mentioned for human tumour cell lines (Larsson and Nygren 1989). Hassan et al. (2002) reported that 50% of fluorescence intensity of *Trichoderma sp.* was decayed after 30 s of illumination in UV light. Another report mentioned that FDA might be extruded by a specific transporter of *Candida albicans* on the cell membranes before the molecule penetrates into cytoplasm (Yang et al., 2001). FDA has been previously reported to diffuse passively into the cytoplasm of *Saccharomyces cerevisiae* cells (Breeuwer et al. 1995). Yang et al. (2001) suggested that FDA was actively extruded from the drug resistant *Candida albicans* cells, and the penetration of the prefluorochrome into the cell was correlated inversely with over-expression of multi-drug resistance. Likewise, resembling results have been reported for *Lactococcus lactis* (Bunthof et al. 1999). In spite of reports based upon the stain effluxes in some cells, fluorescence intensity of cells in this work was found for a considerable period of time. This finding shows, that holding time of the rock inhabiting black fungi at 4 °C after 30 h of staining not only does not decrease the fluorescence but also the intensity of fluorescence increase throughout the experiments. The same results were seen at room temperature. It seems that all transported FDA was not immediately hydrolyzed after penetrating inside the cells and fluorescence formation is most likely limited by the hydrolysis rate. It was also reported that if the intracellular concentration of fluorescence and its derivation are too elevated, quenching occurs (Breeuwer et al. 1995) and in this study concentration of stain was not too elevated to quench. Furthermore when the rock inhabiting fungi are grown under less optimal condition such as are found in rock and soil or in our experiment; fungal esterase activity can be increased or remained elevated. Finally, this result might reflect the loss of the transport

system to extrude the stains from the rock inhabiting fungi which was studied under this condition. Another more important result was achieved in this work was increasing fluorescence intensity of chemical treated cells during the time. It probably reflects the capability of damaged cells to recover injury and increasing the fluorescence. This observation could be very important for conservators.

4.2.1.4 Chemical treatments

4.2.1.4.1 Biocides

There are numerous studies by which the action of biocides has been tested on stone against the activity and growth of microorganisms (John 1988; Richardson 1988). Toxic chemical washes have been used to eradicate or remove unsightly biological growths from stone. It is necessary to mention in all fields of conservations the last choice is chemical treatments and solution containing biocides should be applied if any other sterilization method cannot be used because it is either insufficient or not applicable for the material. Orial and Brunet (1991) present a satisfying account of the use of streptomycin and kanamycin to eliminate bacteria from stonework for a period of more than seven years, with a resulting cessation of decay. The potency of biocides may be influenced by the ability of microorganisms to oxidize or modify them, possibly in conjunction with UV light (John 1988). It is important to keep in mind that none of the biocides available in market has any long-term preventive effect against recolonization. Thus, controlling the humidity, temperature, light, nutrients and combinations of these environmental parameters is the only way to control microbiological contamination and to prevent reinfection. The knowledge about the prevalence of resistant microorganisms to the chemicals has increased significantly during the past decade. Although the mechanism(s) of resistance vary from agent to agent they typically involve one or more of: (a) alteration of the drug target in the bacterial cell; (b) enzymatic modification or destruction of the drug itself; (c) limitation of drug accumulation as a result of drug exclusion; (d) active drug efflux. For some classes of biocides to be less resistant this is common and likely reflects the multiplicity of targets within the cell as well as the general lack of known detoxifying enzymes. The multidrug efflux systems can be

found in some opportunistic pathogens. *Pseudomonas aeruginosa* is an example where they play an important role in the noted intrinsic and acquired resistance of this organism to antibiotics (Poole 2002). For this reason, the development of new chemicals or new application methods, preferably with novel mechanisms of action, is an urgent need.

White et al. (1938) used BAC as a germicide and reported that it was effective to remove contamination from skins and infected wounds. The products containing quaternary ammonium salts have abroad spectrum. Sung et al.(2003) reported that BAC component caused a change in components of the Vibrio community and heterotrophic bacteria in fresh water culture ponds of shrimp. He reported that this compound may affect phagocytosis and apoptosis in shrimp. Lunden et al. (2003) observed after exposure in sub-lethal quantities of two quaternary compounds and tertiary alkyl amines, that they increased the resistance of *Listeria monocytogenes* to these compounds. These findings suggest that sustaining high disinfectant effectiveness may be unsuccessful by rotation, even when using agents with different mechanisms of action. Tabata et al. (2003) reported adapted resistance to BAC in *Pseudomonas aeroginosa* due to expression of OprR gene in the resistance species. These results suggested that OprR significantly participate in the adaptation of *P. aeroginosa* to QACS, such as BAC and N-dodecylpyridinium iodide. It is obvious that characteristics accompanying resistance included alterations in outer membrane proteins, uptake of BAC, cell surface charge and hydrophobicity, and fatty acid content of the cytoplasmic membrane of *P. aeroginosa* (Loughlin et al. 2002). Richardson (1988) and May et al. (1993) reported a developed using the quaternary ammonium compounds which are surface active and be bactericidal or bacteristatic depending on concentration have wide spectrum activity against microorganisms which is concordance with our results. This chemical can alter membrane component and through these effects had inhibitory activity against fungal cells. The other biocide which was tested in this work was Preventol A8. Inhibition of fungal lanosterol-14 alpha-demethylase is the principle of the antifungal activity of this compound which have been used extensively in agricultur and medicine (Trosken et al. 2006). The intensive use of site-specific fungicides in agricultural production provides a potent selective mechanism for increasing the frequency of fungicide-resistant isolates in pathogen populations. This component has an impact to the complete disorganization of the prokaryotic cyanobacterial cell (Ascaso et al. 2002). Our results showed that this chemical is effective against the strains which used in this test and sensitisation of cells by the other chemical increased the effectiveness of drug. Alakomi et al. used incorporating chemical and physical agents to prevent biofilm growth *in vitro* and model biofilm growth on inorganic support and showed

that combination of physical and biocides PV A8 significantly reduced biofilms growth (Alakomi et al.). Inasmuch as this chemical shows cytotoxicity in warm-blood animals and mammalian, reducing the concentration of biocide due to accompanying with the other chemicals could be friendlier for environment.

4.2.1.4.2 EPS inhibitor (BisBAL)

Surfaces are often of considerable importance as microbial habitants because nutrients can adsorb to them, in the microenvironment a surface, nutrient levels maybe much higher than they are in the bulk solution or water (Brock and Madigan 1991). Hutchinson et al. (2006) studied the effect of substrate surface roughness on intertidal biofilms and reported that rock roughness affects both the biofilm and algal species that recruit and their abundance. Microorganisms in biofilms are recalcitrant to chemical biocides and antibiotics (Brown et al. 1988). Thus biofilms are a protected niche for microorganisms, where they safe from antibiotic treatment. Embedded cells encounter a different microenvironment with higher osmolarity conditions, nutrient limitation and higher cell density and behave differently with respect to growth rates and gene transcription than planktonic cells. The removal and killing of established biofilms requires harsh treatments, mostly using oxidising biocides. Depending on the nature of the biofilms, different chemicals may be useful and the best biocide for a specific biofilm still has to be determined under practical conditions. Why biofilms are more resistance than planktonic cells to biocides? Transport limitation due to neutralization of antimicrobial agent in the biofilm is more quickly than it can diffuse in it and absorption of the antimicrobial agent is mentioned to explain this enhanced resistance in biofilm microorganisms (Steward et al. 1999; Wu et al. 2002; Abdi-Ali et al. 2006). The second explanation for biofilm resistance to chemicals is based on physiological differences between biofilm and planktonic cells (Brown et al. 1988; Abdi-Ali et al. 2006). Nutrient depletion creates zones of altered activity. Outer layers of biofilm cells absorb damage and inner layers of biofilms have more time to initiate stress response. Microorganisms grown under stress factors such as phosphate or nitrogen starvation tend to produce more extra cellular polysaccharides, which occur in the interior of biofilms. These are also candidates for reduced susceptibility and persister cells may be present in higher numbers. The research showed that the most persister cells stay in stationary phase and virtually all antimicrobials are more effective at killing rapidly growing than stationary cells (Eng Rh et al. 1991).

Castanier et al. (2006) reported that biofilms developed in fractures of rock can persist for extended periods at reduced hydraulic conductivity when exposed to a long-term starvation. In this condition microenvironment alters and microniches introduce. It was reported that the action of simple non-antibiotic antibacterial agent, such as substituted phenols, biguanides, quaternary ammonium compounds can be altered up to 1000-fold by changes in growth rate and nutrient limitation (Brown et al. 1988). As previously reported for MIC (minimal inhibitory concentration) testing, the greatest numbers of serious errors for BIC (biofilm inhibitory concentration) testing were seen with ß-lactam antibiotics by deactivation of antibiotics in the surface layers, and the greatest numbers of minor errors with the positively charged aminoglycosides was retarted by binding to the negatively charged extracellular matrix (Moskowitz et al. 2004). These mechanisms suggest that the EPS play an important role in biofilm resistance. Based on microscopy, sequential steps of biofilm development have been characterized by microbial attachment; the formation of microcolonies, biofilm maturation and detachment. An alternative approach to control biofilm formation could be to eliminate of planktonic cells before they adhere to the surface and initiate biofilm formation. The other approach could be to inhibit production of the biofilm matrix material. Microbial capsule and slime can be inhibited by bismuth compounds, especially when complexed with lipophilic thiol chelators, Bismuth dimercaprol (BisBAL). Investigations show *Klebsiella pneumonia* capsule expression repressed which were exposed to sub MIC levels of BisBal (Domenico et al. 1999). Huang and Stewart (1999) showed that BisBAL did not initially kill attached *Pseudomonas aeruginosa* but was enough to reduce polysaccharide production. As treatment proceeded, the normalized polysaccharide content was reduced and those cells attached became susceptible to BisBAL (Huang and Stewart 1999). LPS as well as lipid A were affected by Bismuth-Thiols in *Pseudomonas aeruginosa.* The same result was reported against staphylococci and staphylococcal biofilms (Domenico et al. 2001). Wu et al. (2002) reported that Bismuth-Thiol was effective against *P.aeroginosa* and the combination of it may help to prevent or resolve respiratory tract infection. BisBAL shows a new approach to inhibit biofilms with less caustic activity. At sub-minimum inhibition concentration levels, BisBAL uniquely inhibits microbial exopolysaccharide, thereby retarding biofilm formation. Thus, the combination of biocide and EPS inhibitors may prevent slime build-up on the surfaces as it is reported on drinking water biofilms by Codony et al. (2003). Zhang et al. (2005) showed that BisBAL at low concentration did not inhibit the growth of *Escherichia coli, Klebsiella pneumonia, Enterobacter*, and *Enterococcus* which cultured on

the surface of stents**.** It did, however, significantly decrease the amount of bacteria adhering to the surface of stents for all bacterial strains except *Escherichia coli*. BisBAL in concentration 20 μM significantly inhibited the growth of *Escherichia coli*, *Klebsiella pneumoniae*, and *Enterobacter* and, thereby, significantly decreased the amount of these bacteria adhering to the surface of stents and therefore has unique bactericidal and antibiofilm activities.

Fungal biofilm are formed by the aggregation of cells into multicellular structures. It was observed that a large family of glycoprotein involved in adherence of fungal cells, reduces desiccation and provides the fungus with a favourable microenvironment (Moloshok et al. 1993; Nicholson 1996). In my work, BisBAL concentration was sufficient to reduce viability of cells, although this reduction was not alike for both fungi cultures (A18 and PF6). A18 showed more susceptibility to this chemical. Different pathways for this chemical action, different amount and distinctive component of EPS in these fungi may explain the differences. Why BisBAL had a biocidal effect? One explanation of this phenomenon is that BisBAL might be adsorbed by the EPS of the biofilm microorganisms. Consequently the local BisBAL concentration might be higher than the concentration of the chemical in the medium. Many bacteria communicate via the production and sensing of autoinducer ´pheromones` in order to control the expression of specific genes in response to population density which was named quorum-sensing signals (De Kievit and Iglewski 2000). It seems that reduction of extracellular matrix disturb quorum-sensing signals which induce protective stationary phase genes in the biofilms and alter biofilms susceptibility to the chemical. Combination of PV and BisBAL increased reduction of fluorometry. Reduction in viability of cells may be due to the reduction of the polysaccharide production and better influence of PV. On the other hand, they did work synergically with each other as reported by Wu et al. (2002). Combinations of PV with BisBAL and PEI, and PV, BisBAL, TC and PEI have shown significant differences. To distinguish specific or selective toxicity of chemicals on the cells need further and deeper biochemical analysis works.

4.2.1.4.3 Melanin Inhibitor (TCA)

There is a large class of potentially invasive fungi, which have in common the production of melanin. Melanized cell walls exhibit evidence of cross-linking that they are more resistant to enzymatic lyses (Jacobson 2000). Melanin functions to provide the fungal cell with resistance to a variety of environmental stresses. It was reported that melanin can reduce susceptibility to the anti-fungal chemicals in black fungi (San-Blas and Calderone 2004). These fungi have been termed dematiaceous or phaeohyphomycetous fungi. Melanin contains a widely conjugated aromatic structure, it was suggested that the energy of free radicals has a function as a sink for potentially harmful unpaired electrons. Wang and Casadevall (1994) studied protection against oxygen free radicals and nitrogen free radicals by melanin on *Cryptococcus neoformans*. They showed that melanized cells survived approximately 10-fold better than non-melanized cells and the system was proposed to model a role for melanin in virulence as protection for pathogens against generated free radicals. Melanized *C. neoformans* shows increases at the electron spin resonance signal when they were incubated in a solution containing free radicals. These results were thought to demonstrate the transfer of unpaired electrons to the melanin of melanized cells (Wang et al. 1995). A specialized cellular apparatus in a variety of dematiaceous fungi, the appressorium, produces osmotically generated pressure when exposed to hypotonic solutions. DHN-melanin is a necessary component to generate high pressure. Melanin reduces the porosity of the appressorial wall, enabling the fungal cell to retain glycerol which participates to generate pressure in this apparatus (Jacobson 2000). Inhibition of the melanin synthesis pathway by tricyclazole increases susceptibility of fungi against macrophages (Cunha et al. 2005). Various fungi have different sensitivities to TCA, indicating different metabolisms toward them. This commercial fungicide produces strong growth inhibitions only when it is used at high doses, indicating that it does not influence the growth of the fungi, but rather the pigmentation (Mares et al. 2004). It was proved that this substance interferes with the normal production of ergosterol. TCA retarded production of *B. cinerea* sclerotia and provoked a strong inhibition of the melanin layer of its wall, causing also deep alterations of the morphology of sclerotia seen at the TEM conferring resistance to heating in some instances (Rehnstrom and Free 1996). Therefore, it is able to alter wall permeability, causing an increase in water entry and, thus, an increase in pressure, as was demonstrated by other authors, and in particular by Howard et al. (1991) and Mares

et al. (2004). This structure was not reported in some other fungi having morphologically and physiologically abnormal structures to generate high pressure.

In our work TCA caused decrease in fluorescence intensity of A18. When combination of TCA and biocide was used intensity of fluorescence was reduced again. This can be due to wall alteration and higher penetration of biocide into the cell as it was reported by other authors. Significant differences were seen in combination of TC with other chemicals for PF6, indicating synergistic effects of chemicals as well as different metabolic pathways.

4.2.1.4.4 Permeabilizer (PEI)

Permeabilizers affect integrity of cells by different manner: they (a) chelate divalent cations such as EDTA; (b) release LPS like EDTA and HCL; (c) change the cells without releasing LPS such as polymixin B and PEI. The effect of PEI was comparable to that brought about by the well-known permeabilizer EDTA. PEI also increased the susceptibility of microorganisms to the hydrophobic antibiotics clindamycin, erythromycin, fucidin, novobiocin and rifampicin, without being directly bactericidal (Helander et al. 1997). Zlochevskaya et al. (1975) were the first group to study PEI effects on certain fungi. They reported that the PEI had no influences on fungal spore formation and altering the microscopic and macroscopic characterization of the fungi in solid medium, although it caused prolongation of the lag phase and a reduction of fungal biomass due to the antiseptic effect. No permeabilizer effect such as LPS releasing of PEI on the fungi which studied by them, was reported. Van Boxtel (1990) reported that using permeabilizer increased the antimicrobial susceptibility of *Mycobacterium paratuberculosis* and PEI could sensitize the bacterial cells to the action of detergent where the cells were pretreated with PEI. Helander et al. (1997) reported that where PEI and biocide were simultaneously present no sensitization was observed, indicating that PEI and biocide were inactivating each other. Alakomi et al. (2006) found that PEI supplementation enhanced the activity of benzalkonium chloride toward the gram negative bacteria which participate on biofilm stones as a biodeteriorant.

Our results show that PEI cause fluoresence intensity reduction for both fungi although this effect was stronger for PF6. Combination of PV and PEI decreased the fluorescence intensity of PF6 due to increasing permeability of cells to biocide, whereas a significant

difference was not observed for A18. This result for PF6 indicated that PEI and biocide had a synergistic effect on PF6. On the other hand PEI increase permeability of cells to hydrophobic and macromolecular substances, where as PV diffuses into the cells. In assays where PEI and biocide were simultaneously present, no sensitization was observed for A18, indicating that there were no intensified effects of each other due to alteration of cell wall components, which could have been changed by the PEI effect. The reduction of fluorometry for other combinations was significant, exception of combination of PV with TC and PEI for A18. The result indicated the neutralization of chemicals effect by PEI. This finding is in disagreement with the work of Helander et al. (1997).Using of this kind of chemicals in biocide formulations would enable the use of decreased concentrations of the active biocide ingredient, thereby providing environmentally friendlier products. These conclusions were supported by plate counting agar technique for A18.

4.2.2 Plate Counting Agar

CFU is the commonly used method to estimate viability in bacterial, algal and fungal cells. However, most researchers share the opinion that CFU is not a reliable method of measuring the number of viable cells. Because non-growing cells are usually found in cultures, it is important to confirm that they are still viable when experiments are performed. Furthermore, enumeration of viable cells for slow-growing cultures is time-consuming and laborious. Traditional techniques for quantifying growth provide no information about the quality, activity, or viability of microorganisms. Accurate viability assesment can help to improve the design for many different processes for example in fermentation process control or for physiological studies and those involving monitoring and control of these bioprocesses or bioassay of antibiotics. However, the major problem is to properly define ‘’Viability’’. Viable cells can be generally regarded as those cells, which are capable of performing all cell functions necessary for survival under given conditions. As stated by Barer and Harwood (1999), culturability is the only functional expression of viability. Furthermore, ’’biomass quality’’ is used to indicate the specific level of one cell component (such as ATP, RNA, DNA, or protein) of a group of cell components, while ‘’Biomass activity’’ is used to indicate the ability of biomass to metabolize a particular substrate, and so describes the biocatalytic potential of biomass (Gikas and Livingston 1998). The most

frequently used methods are those measuring the culturability as the colony forming unit, CFU and the growth performance test, i.e. capacity of reproduction in liquid culture. The use of dilution and incubation in the CFU method increases the probability of error, especially in the case of filamentous organisms where the particle or individual cell size is not well defined. Some fungal cells are arranged in filaments. Any attempt to separate individual viable cells invariably leads to uncontrolled losses of viable cells. Furthermore, high osmotic stress affects cellular viability in solid culture medium (Raynal et al. 1994). Quinn (1984) has shown that using cytochemical techniques reveals far higher numbers of viable and active bacteria than plate counts and suggests substrate-accelerated death may be partially responsible for the apparent non-culturability of high percentage of colony forming units found on artificial media. Schading et al. (1995) reported that damaged conidia maintained a measure of viability and fluoresced green but did not fully germinate. It was also found that direct cell counts, as determined by epifluorescence microscopy, measured ten times more microorganisms than viable counts on selective media, indicating that the majority of soil microorganisms are viable but nonculturable. Likewise, as Kell et al. (1998) said that validity of a cytological assay can be confirmed only by correlation with culture assays for a specific mechanisms of cell death. Therefore, it is necessary to evaluate the validity of the fluorescence assays as indicators for particular practical aspects of viability by comparing the fluorescence parameters with plate count and the other physiological aspect. Raynal et al. (1994) found to correlate closely with the colony-forming unit (CFU) method when used under the osmotic conditions with the epifluorescence microscopy but this method proved to be more versatile in that viability measurements (irrespective of their culturability) could actually be carried out at low water activities (a (w)), whereas the reference CFU method required an increase in a (w). Nishikaku and Burger (2003) compared fluorescent stain with plating count for some pathogenic fungi and could not detect any CFU counts from the blood samples whereas, fluorescence stain showed fungal cells in the sample. Statistic analysis of data in this work showed that there is no correlation between fluorometry and plate counting agar methods which means the conventional colony count method could not detect most of the physiologically active fungi in tests and that the fluorescent vital staining method with FDA made it possible to evaluate chemicals with antimicrobial activity against detectable and undetectable cells by the conventional culture method. These chemical stresses induced a non-culturable status, as shown by reduced ability to form colonies even though the cells remained intact and showed intracellular enzyme activity. To achieve CFU an intact cell wall is necessary. The differently treated

structure of fungal cell walls may explain different CFU results for A18 and PF6. This result could be useful for scientists who work in cultural heritage objects because they show the importance of analysing each individual participant in the total process of biodeterioration.

5 Conclusion

- The Persepolis monuments are situated in a harsh continental climate of the Fars Province. They have a long record of subaerial exposure. Excavations and restoration since 1931 have resulted in an advanced growth of microbial communities. The expressed presence of lichens evidences the next succession step after the dominance of free-living microorganisms (bacteria, fungi, algae). Though the lichens prevailed in our samples, free-living ascomycetes and other accompanying microorgansisms are present in the subaerial communities of these calcareous surfaces. It seems that the changing microclimatic and macroclimatic conditions through the influence of excavation and perhaps air pollution increase the influence of aggressive microbiota on the biodeterioration of Persepolis.
- Bioweathering patterns on Persepolis stones revealed biopitting, etching patterns or imprints of fruiting bodies, chipping, and crumbling. The microflora has an important role on biodeterioration, biopitting and biocorrosion in the samples analyzed. From SEM analysis it can be concluded that there are pits that reveal the size of lichen thallus, bacterial and fungal hyphae and in some instances fruiting bodies which were almost evenly distributed on Persepolis stones.
- Although macroscopic and microscopic photographs show the abundance and variety of different cells, the number of isolated rock-inhabiting fungi was less than expected. Therefore, it could be concluded that culturing techniques must be improved to optimize culture conditions for such poikilotrophic microorganisms which adapted to extremely changing environmental conditions. It is also anticipated that in such harsh environment with great seasonal temperature contrasts and low humidity a reduced diversity of rock fungi could be expected.
- To assay susceptibility of rock inhabiting fungi against chemicals it is necessary to improve methodological approaches. Conventional plating methods that were used to enumerate microorganisms on particular medium and temperature must been revised. Most of the fungi could not be detected by the conventional plate counting technique. Fluorometry measurement can overcome this problem. These data with a simple, rapid method to detect cytotoxicity of biocides against microbial growth would be useful to conservators and in the study of biodeterioration and bioweathering. This work compared the fungal culturability (as defined by ability to grow) with enzyme activity of fungi (as done by fluorescence

stains). The double staining method with FDA and PI could not be appreciated and developed into a rapid microbial monitoring test for chemical damages on rock inhabiting fungi. Furthermore, total count of cells is not a reliable method for quantitative analyses of rock inhabiting and biodeteriorating fungi although qualititively rocks decay by lichens and fungi could be established.

- Despite of many reports related to bleaching or quenching of fluorescence signals by compounds our work resulted that not only fluoroscence intensity of rock inhabiting fungi was not bleached but also fluorescence intensity of these cells was being increased after staining them by FDA. This result might also be interesting for mycologist to study the ultrastructure of fungal cells and also to follow the effect of chemicals after treatments. On the other hand some fungal cells could probably recover from injuries and get their ability to live or grow after chemical damages.
- This research points out that although rock inhabiting-fungi show different ways in fluorescence intensity, there appears to be a quantifiable relation between the percentages of fluorescence intensity and the chemical activity of fungal cells.
- Biocides, which were used in this study, were effective against rock inhabiting fungi. The combination of PV with BB, TC, and PEI had a stronger effectiveness. It would be beneficial to do further research to determine the minimum concentration required to inhibit the growth of fungal biofilms. The results of the present study demonstrated the susceptibility of rock inhabiting fungi in microscale or laboratory scale. It is necessary to assay efficiency of chemicals on the mesoscale in the future.

6 Summary

The problem of the deterioration of monuments made of rock is particularly relevant in countries like Iran being rich in such cultural heritage. The Persepolis monument is situated in a harsh continental climate of the Fars province and has a long record of subaerial exposure, which resulted in an advanced growth of microbial communities. By using the LM, SEM, Thin section, PAS staining, maceration, culturing and molecular techniques different biodeteriorative agents were to be identified on and in the stone materials of Persepolis monuments and the observations showed that the growth of the microbiota is not restricted to the surfaces and algal cells and fungal hyphae are found in the depth of the samples. Biopitting, crumbling, chipping and etching are the main destructive phenomena observed on persepolis samples. Biopitting is caused by the activity of epi- and endolithic lichens as well as fungal hyphae. Two shapes of pits were abundant in Persepolis samples. The diameter of the larger pit was about 800 µm (mesopit). The smallest pit is about 10 µm (micropit). Micropits inside of a larger pit could also be observed indicating the recolonization of these pits by algal cells or individual hyphae. Macropits were not seen in Persepolis stones. Crumbling is caused by penetration of hyphae inside the stone. The extent of hyphae penetration into the stone was considerable. The highest depth was up to 2 mm and sometimes even deeper penetration was seen. In some samples bundles of fungal hyphae growing in a certain depth are arranged parallel to the surface. They seem to arrange at a layer of optimum humidity. Chipping is often related to the endolithoic photobiont. Different size and different shape of bacteria and algal cells in some samples as well as fruiting bodies of lichens form considerable etching phenomena on the surface of stones and these patterns could only be seen in connection with these organisms. External polysaccharide material is covering the cortical and medular hyphae. Casts of lichens on Persepolis stone surfaces are readily visible which are correlated to the individual constituents of the endolithic lichens. Biomineralization of surface was shown in some samples and depth of these covers was on average 200 to 800 µm. Furthermore, the biomineralized cracks inside the stone produce exfoliation patterns on Persepolis stones. As a result many rock surfaces of the antique monuments at Persepolis are intensively inhabited by lichen colonies. The expressed presence of lichens evidences the next succession step after the dominance of free-living microorganisms (bacteria, fungi, algae). Though the lichens prevailed in our samples, rock inhabiting fungi, free-living ascomycetes and other

accompanying microorganisms are present in the subaerial communities of these calcareous surfaces. One can draw the conclusion that changing microclimatic and macroclimatic condition through the influence of excavation increases the impact of aggressive microbiota on the biodeterioration of Persepolis.

Our results show that the abundance and variety of isolated rock-inhabiting fungal species were less than expected. Our work concentrated on epilithic, non-lichenized fungi which grow extremely slowly on the surface and outer layers of stones exposed to extremely changing environmental conditions and a response to a harsh environment conditions such as temperature and UV-radiation is rock penetration. Some species of fungi can be stimulated by higher light intensity to change from epilithic to endolithic growth and the pitting of rock surfaces by microorganisms provides better microenvironmental habitats protected from extreme living conditions directly at the surface. Therefore culturing techniques must be improved to optimize culture condition for such poikilotrophic microorganisms, which adapted to extreme condition. Another reason for low diversity of fungi could be attributed to low relatively humidity and great seasonal contrast, which is greater than in the Mediterranean area described in many biodeterioration studies. Nature can nurture all extant microorganisms so far resisting to laboratory culture attempt, which is a considerable bias for the research on biodeterioration agents. Culture media with high content of organic material have to be used to isolate the organisms. When dealing with the study of microbial communities under extremely changing poikilotroph conditions, present day culture media and cultivation times cannot reveal the real microorganism distributions. Furthermore black spots can be caused by the secretion of metabolites that stain the stone or by the presence of pigmented fungal structures, especially melanin left over after the death or elimination of a former microcolony. Both of these structures might be sampled for invariably failing culture attempts. Finally, cyanobacteria also produce black pigments, which could be erroneously taken as a MCF and will fail to grow in the fungal media.

For the first time it was shown that the fluorometry technique developed during this work could be used to assay biocide activity against black rock inhabiting fungi which produce subaerial biofilms. To control and prevent the development of biodeteriogenic organisms growing on and in monuments made of rock, it is necessary to assay biocidal activity before applying this kind of products to artefacts. For this reason efficiency of some chemicals to inhibit biofilm growth was assayed in *in vitro* experiments. The point, which must be taken in consideration is, that modifications of any fungal biofilm inoculum is necessary to assay efficiency of chemicals in laboratory experiments. Homoginization and ultrasonicating

treatment, which is used to get a homogenised inoculum could achieve different degrees of microbial inactivation. Two methods have been used for evaluation; plate count agar and fluorescent activity stains. During this work the fluorometry techniques were improved and used to assay efficiency of chemicals on rock fungi. For this reason, work concentrated on the effectiveness of fluorometric techniques in rock treatment controls. In this study viability testing by the fluorescent stains FDA, CAL AM, and PI on rock inhabiting fungi was compared with viability of cells by cultivation method. The ressults show, that conventional plating method that was used to enumerate microorganisms on particular medium and temperature must be revised and after chemical treatment some of the fungi could not be detected by conventional plate counting techniques. Fluorometry measurement can overcome this problem and a technique based on esterase activity on cells and staining method with FDA could be applied to rapidly monitor chemical damages in microbial cells. These data on a simple, rapid method to detect cytotoxicity of biocides against microbial growth would be useful in biodeterioration and bioweathering studies. The double staining of cells by FDA and PI, however, could not be applied in a rapid monitoring test for chemical damages on rock inhabiting fungi. Furthermore, despite of many reports related to bleaching or quenching of fluorescence signals by mineral and organic compounds work presented here demonstrates that not only fluorescence intensity of rock inhabiting fungi was not bleached but also fluorescence intensity of these cells was being increased after staining the cells by FDA. This result might also be interesting for mycologists in the study of the ultrastructure of fungal cells, and to follow the effect of chemicals. On the other hand some fungal cells could probably recover from injuries after chemical damages and get back to integrity to live or grow.

The result of the chemical assay showed that although rock inhabiting fungi show different ways in fluorescence intensity to PV and BAC, there appears to be a quantifiable relation between the percentages of fluorescence intensity and the chemical activity on fungi cells. Furthermore, combination of PV with BB, TC, and PEI increased considerably efficiency of biocide against both fungal test organisms A18 and PF6. It would be beneficial to do further research to determine the minimum concentration required to inhibit the growth of fungal biofilms. The results of the present study demonstrated the susceptibility of rock inhabiting fungi in microscale or laboratory scale. It is neccessary to assay efficiency of chemicals on the mesoscale and field scale in the future.

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8 Acknowledgement

This work was carried out at the Geomicrobiology section of the Institute of Chemistry and Biology of the Marine Environment (ICBM), faculty of Mathematics and Science at Carl von Ossietzky University, Oldenburg.

The work was supported by Science, Research and Technology Ministry of Iran through a scholarship and by the EU project BIODAM.

I owe my gratitude to all following persons:

My supervisor Prof. Dr. Dr. h.c. mult. Wolfgang. E. Krumbein who introduced me into the world of biodeterioration of stonework and helped in the lingual improvement of the manuscript.

Dr. Anna. A. Gorbushina who has helped me throughout my technical and laboratory research and reviewed this thesis and gave me valuable comments.

Dr. Jürgen Marquardt who helped me in molecular work.

Renate Kort and Sophie Martyna gave me kindly help in Scanning Electron Microscopy.

Andrea Schlingloff organized fluorescence spectroscopy access for me, work which was carried out at ICBM, in the group of Prof. Dr. Heribert Cypionka.

Dr. Erhard Riel that gave me friendly valuable advice and was available when I had any questions.

In addition, all technicians especially Silke Ammermann and Annete Schulte were always ready to give skilful technical help.

Warm thanks go to friends specially Vasco and Silvia who made a very friendly atmosphere in the laboratory and were available when I needed them.

What I owe to my family mother, father, brothers, sister and husband cannot be expressed by words. Staying outside from work they have been my strength and had an important role in sustaining connection to real life.

My biggest thanks for my daughters Reyhaneh and Hanieh for their patience during these days, spending many hours without me while I was working in the laboratory and as well as during writing the manuscript and gave me strong feeling and chance to finalize this work.

9 Appendix

Tables

Table.1 Geographic data of Persepolis and Shiraz was compared.

Persepolis Geographic Data		Shiraz Geographic Data	
Height	1740 m	Height	1488 m
Geographic Position	29°, 55', 60" North 52°, 54', 00" East	Geographic Position	29°, 36' North 52°, 32' East
Weather	Semiarid - Semitropic	Weather	Semiarid - Semitropic

Table.2 Effect of physical treatment on viability A18 and PF6 by using the fluorometry technique, fluorescence of buffer and autofluorescence of cells were measured parallel with the treated cells.

Fungi	A18	SD A18	% Fluorometry	PF6	SD PF6	% Fluorometry
PBS	181	4	0	174	6	1
Autofluorescent	568	54	1	126	10	0
FDA	8689	931	15	8689	2	931
YNB	761	69	1	761	0	69
Control	51129	2383	86	43126	18157	171
Homoginated treatment	44834	873	75	25284	3794	100
Sonicated treatment	59726	1480	100	20902	6641	83

Table.3 Fluorescence intensity of A18 and J26 with different stains was measured. The fluorescence intensity of treated cells by BAC 1% was measured parallel the experiments.

Fungi	A18	SD A18	J26	SD J26
FDA Control	48073	8186	2495	19
FDA BAC 1%	7553	177	1587	10
BAC 0,1%	5807	882	1650	35
BAC 0,01%	27287	892	2106	67
CAL Control	1005	3	980	5
CAL BAC 1%	590	4	476	25

Table.4 Fluorescence intensity of different stains on A18, a part of cell was killed as described in Material and Method and a 50/50 mixture of live and dead cells used to evaluate the efficiency of staining by different stains and different staining method**.**

	A18	SD A18	% Fluorometry
Buffer	134,7	5,5	4,0
Autofluorescence	123,3	2,1	3,6
FDA Control	3389,0	870,5	100,0
PI Control	15,3	1,5	0,5
FDA Dead/Live cells	1862,3	66,0	55,0
PI Dead/Live cells	16,0	1,7	0,5
Mix staining Dead/Live cells	1075,3	140,2	31,7
Two step staining Dead/Live cells	288,7	26,6	8,5

Table.5 Time effect on fluorescence intensity of A18; fluorescence intensity of FDA control and buffer, as well as autofluorescence and chemical treating was measured.

Fungi	00	SD A18(00)	06h	SD A18(6h)	30h	SD A18(30h)
Buffer	243	9	223	12	204	19
Autofluorescence	246	12	218	14	195	19
FDA Control	10742	2814	16517	4494	34466	9717
BAC 1%	2828	883	5062	1592	13222	3313
BAC 0,1%	3716	723	6405	1401	16214	4582
BAC 0,01%	8636	2568	15156	5347	36462	13535

Table.6 Time effect on fluorescence intensity of PF6; fluorescence intensity of FDA control and buffer, as well as autofluorescence and chemical treating was measured.

Fungi	00	SD PF6(0h)	06h	SD PF6(6h)	30h	SD PF6(30h)
Buffer	239	5	212	7	200	12
Autofluorescence	259	18	217	35	175	35
FDA Control	6348	1496	11386	3299	28991	7623
BAC 1%	3561	330	6359	581	17131	1529
BAC 0,1%	5918	1027	10732	2189	26473	6397
BAC 0,01%	7258	2079	13382	3344	33066	8535

Table.7 Comparison of effect of three concentrations of two biocides PV and BAC and susceptibility of fungi A18, PF6 and J26 was shown. The fungal cells were stained by FDA. The experiments were performed as described in Material and Method and are presented as mean of quadruplicate of trials as one typical experiment of three.

Chemicals/Strains	A18	SD A18	PF6	SD PF6	J26	SD J26	% Fluorometry A18	% Fluorometry PF6	% Fluorometry J26
Buffer	224	6	219	4	251	6	2	2	2
Autofluorescence	246	12	259	18	251	8	2	2	2
FDA Control	10742	2814	6348	1496	2971	4701	100	59	28
PV 1%	1379	148	876	292	1627	172	13	8	15
PV 0.1%	6031	1185	1007	545	1083	32	56	9	10
PV 0.01%	6716	1972	1313	545	1713	536	63	12	16
BAC 1%	2828	883	3561	330	1641	51	26	33	15
BAC 0.1%	3716	723	5918	1027	1424	35	35	55	13
BAC 0.01%	8636	2568	7258	2079	2158	67	80	68	20

Table.8 Results of fluorometry A18 in different chemical treatments and combination of them was shown. The results are expressed in arbitrary units and presented as mean values of four individual trials of one typical experiment of three. The concentration of chemicals were 0,1% PV; 5mM BB; 30µg/ml TC and 30µg/ml PEI.

Fungi	AVG Fluorometry A18	% Fluorometry A18	SD A18	% SD
PBS	182	00	2	00
Autofluorescence	116	00	9	00
FDA control	57077	100	4893	9
PV	10218	18	399	1
BB	39640	69	3821	7
TC	29747	52	1385	2
PEI	28501	50	6165	11
PV+BB	9525	17	251	00
PV+TC	8084	14	333	1
PV+PEI	10797	19	1290	2
PV+BB+PEI	9303	16	730	1
PV+TC+PEI	10172	18	1499	3
PV+BB+TC+PEI	6848	12	387	1

Table.9 Results of fluorometry PF6 in different chemical treatments and combination of them was shown. The results are expressed in arbitrary units and presented as mean values of four individual trials of one typical experiment of three. The concentration of chemicals were 0,1% PV; 5mM BB; 30µg/ml TC and 30µg/ml PEI.

Fungi	AVG Fluorometry PF6	% Fluorometry PF6	SD PF6	% SD
PBS	174	1	6	00
Autofluorescence	126	1	10	00
FDA control	20902	100	6641	32
PV	17665	85	4018	19
BB	19211	92	6133	29
TC	12307	59	1451	7
PEI	7578	36	1094	5
PV+BB	12343	59	2738	13
PV+TC	8766	42	2062	10
PV+PEI	9496	45	2022	10
PV+BB+PEI	8989	43	1944	9
PV+TC+PEI	6629	32	1723	8
PV+BB+TC+PEI	4595	22	1147	6

Table.10 Results of different chemical and physical treatments on A18 & PF6 CFU. These results are presented as mean values of four individual trials and were done twice. The concentration of chemicals were 0,1% PV; 5mM BB; 30µg/ml TC and 30µg/ml PEI.

Fungi	CFU A18	SD	CFU PF6	SD
Control	44200	0	1005	114
Homoginated treatment	12523	9060	855	92
Sonicated treatment	10068	7778	913	163
PV	5500	1381	0	0
BB	1042	540	822	128
TC	5368	2469	807	189
PEI	1746	592	1298	235
PV+BB	419	0	0	0
PV+TC	0	0	12	6
PV+PEI	5	0	13	12
PV+BB+PEI	13	9	3	6
PV+TC+PEI	6	0	0	0
PV+BB+TC+PEI	0	0	0	0

Table.11 Correlation between Viability Measurements with FDA Fluorescence Intensity (FI) and Culturability Measurement (CFU); p-value≥0.05 so corrolation is not significant, N indicated the number of test. The analysis was done by SPSS software.

	AVGCFU A18	AVGCFU PF6
Pearson Correlation AVGFI	-0,193	-0,039
Significant p-value (2-tailed)	0,527	0,899
N	13	13

Table.12 The analysis is done after classifying data for A18 and the significance difference is done with pair comparison of t test. In the significant difference analysis, if p-value is less than .05 there is significant difference between two variables, AVG, N, SD,SEAVG, t, and Sig(2-tailed) indicated the average of fluorescence intensity measurments, the number of test, standard deviation, standard error average, t test and significant differences between two variables, respectively.

Paired Samples Statistics		AVG	N	SD	SEAVG
Pair 1	A18PV	10218,50	4,00	326,04	163,02
	PV (%0,1)+BB (5mM)	9525,25	4,00	204,56	102,28
Pair 2	A18PV	10218,50	4,00	326,04	163,02
	PV (%0,1)+TC (30µg/ml)a18	8084,00	4,00	271,78	135,89
Pair 3	A18PV	10218,50	4,00	326,04	163,02
	PV (%0,1)+PEI (30µg/ml)a18]	10796,75	4,00	1053,08	526,54
Pair 4	A18PV	10360,33	3,00	196,87	113,66
	PV (%0,1)+BB (5mM)+PEI (30µg/ml)a18	9302,67	3,00	516,50	298,20
Pair 5	A18PV	10218,50	4,00	326,04	163,02
	PV (%0,1)+TC (30µg/ml)+PEI (30µg/ml)a18	10172,00	4,00	1223,63	611,82
Pair 6	A18PV	10218,50	4,00	326,04	163,02
	PV (%0,1)+BB (5mM)+TC (30µg/ml)+PEI (30µg/ml)a18	6848,00	4,00	315,71	157,85

Paired Differences		AVG	SD	SE AVG	95% Confidence Interval of the Difference		t	Sig (2-tailed)
					Lower	Upper		
Pair1	A18PV - PV (%0,1)+BB (5mM)	693,25	444,49	222,25	-14,03	1400,53	3,12	0,05
Pair2	A18PV - PV (%0,1)+TC (30µg/ml)	2134,50	185,03	92,52	1840,07	2428,93	23,07	0,00
Pair3	A18PV - PV (%0,1)+PEI (30µg/ml)	-578,25	907,04	453,52	-2021,55	865,05	-1,28	0,29
Pair4	A18PV - PV (%0,1)+BB (5mM)+PEI (30µg/ml)	1057,67	383,45	221,39	105,12	2010,21	4,78	0,04
Pair5	A18PV - PV (%0,1)+TC (30µg/ml)+PEI (30µg/ml)	46,50	1298,39	649,20	-2019,53	2112,53	0,07	0,95
Pair6	A18PV - PV (%0,1)+BB (5mM)+TC (30µg/ml)+PEI (30µg/ml)	3370,50	320,72	160,36	2860,17	3880,83	21,02	0,00

Table.13 The analysis is done after classifying data for PF6 and the significance difference is done with pair comparison of t test. In the significant difference analysis, if p-value is less than .05 there is significant difference between two variables, AVG, N, SD,SEAVG, t, and Sig(2-tailed) indicated the average of fluorescence intensity measurment, the number of test, standard deviation, standard error average, t test and significant differences between two variables, respectively.

Paired Samples Statistics		AVG	N	SD	SEAVG
Pair 1	PVPF6	17665,00	4,00	3281,08	1640,54
	PV (%0,1)+BB (5mM)	12343,00	4,00	2235,62	1117,81
Pair 2	PVPF6	17665,00	4,00	3281,08	1640,54
	PV (%0,1)+TC (30µg/ml)	8766,25	4,00	1683,51	841,76
Pair 3	PVPF6	17665,00	4,00	3281,08	1640,54
	PV (%0,1)+PEI (30µg/ml)	9495,50	4,00	1650,89	825,44
Pair 4	PVPF6	17665,00	4,00	3281,08	1640,54
	PV (%0,1)+BB (5mM)+PEI (30µg/ml)	8988,75	4,00	1587,23	793,61
Pair 5	PVPF6	17665,00	4,00	3281,08	1640,54
	PV (%0,1)+TC (30µg/ml)+PEI (30µg/ml)	6629,25	4,00	1406,75	703,37
Pair 6	PVPF6	17665,00	4,00	3281,08	1640,54
	PV (%0,1)+BB (5mM)+TC (30µg/ml)+PEI (30µg/ml)	4595,25	4,00	936,17	468,08

	Paired Differences	AVG	SD	SE AVG	95% Confidence Interval of the Difference		t	Sig (2-tailed)
					Lower	Upper		
Pair 1	PF6PV - PV (%0,1)+BB (5mM)	5322,00	2384,21	1192,11	1528,19	9115,81	4,46	0,021
Pair 2	PF6PV - PV (%0,1)+TC (30µg/ml)	8898,75	2755,44	1377,72	4514,24	13283,26	6,46	0,005
Pair 3	PF6PV - PV (%0,1)+PEI (30µg/ml)	8169,50	1840,37	920,19	5241,05	11097,95	8,88	0,003
Pair 4	PF6PV - PV (%0,1)+BB (5mM)+PEI (30µg/ml)	8676,25	3060,12	1530,06	3806,91	13545,59	5,67	0,011
Pair 5	PF6PV - PV (%0,1)+TC (30µg/ml)+PEI (30µg/ml)	11035,75	3294,28	1647,14	5793,82	16277,68	6,70	0,007
Pair 6	PF6PV - PV(%0,1)+BB(5mM)+TC(30µg/ml)+PEI(30µg/ml)	13069,75	3884,27	1942,14	6889,01	19250,50	6,73	0,007

List of the used chemials:

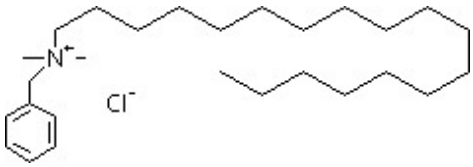
Agar	Roth
Agarose	Serva
BAC	Pharmacy
BisBAL	Sigma
CAL AM	Sigma
$CaOCl_2$	Merck
Chloramphenicol	Fluka
Dichloran	Sigma
dNTP	Promega
EDTA	Merck
Ethanol	Merck
FDA	Sigma
Glucose	Acros
Glutaraldehyde	Agar Scientific
Isopropanol	Merck
ITS1F, ITS1-for, and IST4 primers	Promega
K_2CO_3	Acros
K_2HPO_4	Merck
Malt Agar	Merck
$MgSO_4$* 7 H_20	Scharlu
PCR mixture	Promega
PEI	Sigma
Pepton	Roth
PI	Sigma
PV A8	LANXESS
QIA quick Spin kit	QIAGEN
Rose Bengal	Sigma
Silicon Carbide Powder	Buehler
Spurr Resin	Agar Sceintific
TC	Sigma
Tris-buffer	Scharlau
Ultra clean ^{TM} Kit	MO BIO
Yeast Extract Agar	ADSA Micro
YNB medium	Difco

List of the used instruments:

Autoclave	Varioklave
Centrifuge	Eppendorf 5415
Centrifuge	Heraeus
Digital Camera Olympus	3030 analySIS
Fluoroscan-spectrometer	FLUOstar OPTIMA
Homoginizer	Ultra-Turrax T25
Metal Block Thermostat	Biometra
Microtome saw	Leitz-1600
Optical microscopy	Axioscope II Zeiss
Power Supply	BIORAD
Saw	Beuhler
Scaning Electron Microscope	Hitachi S-3200
Sputter coater	Blazer SCD-005
Stereomicroscope	Zeiss
Thermocycler	Hybaid
Ultra-sonicator	Bandelin Sonorex RK100
Vaccum concentrator	Bachofer GmbH, Reutlingen

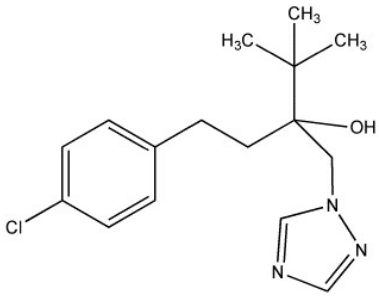
Chemical Structure of Chemicals

Benzalkoniumchloride or Alkylbenzyldimethylammoniumchloride (BAC) [C6H5CH2N(CH3)2R]Cl

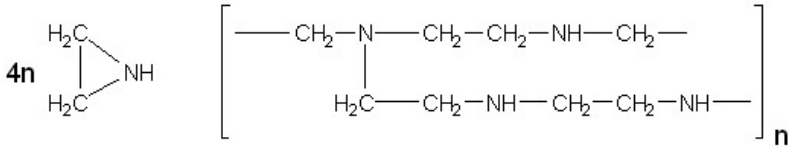


Preventol A8 or Tebuconazol

C16H22ClN3O

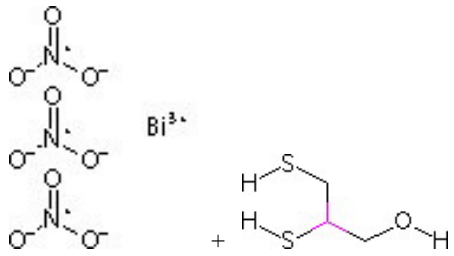


Polyethyleneimine (PEI) $(C_2H_5N)_n$



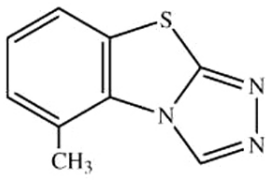
Ethyleneimine polyethyleneimine

Bismuth dimercaprol (BisBAL or BB)



bismuth nitrate dimercaprol

Tricyclazole (5-methyl-1,2,4-triazolo[3,4-*b*]benzothiazole)



List of Abbreviations

AVG	Average
BAC	Alkyl Benzyl Dimethyl Ammonium Chloride
BB	Bismuth-2,3-dimercaptopropanol (BisBAL)
CAL AM	Calcein AM
CFU	Colony Forming Unit
DNA	Deoxyribonuleic Acid
dNTPs	Deoxyribonucleic triphosphate (dATP, dCTP, dGTP, dTTP)
DRBC	Dichloran Rose Bengal Chloramphenicol
DW	Distilled Water
EDTA	Ethylene Diamine Tetraacetic Acid
EPS	Extracellular polysaccharides
FDA	Fluorescein diacetate
FI	Fluorescence Intensity
IGS1	Intergenic spacer1
IGS2	Intergenic spacer2
ITS1	Internal spacer1
ITS2	Internal spacer2
LM	Light Microscope
MEA	Malt Extract Agar
MCF	Microcolonial fungi
PAS	Periodic acid Schiff
PBS	Phosphate Buffer Saline
PCR	Polymerase Chain Reaction
PEI	Polyethyleneimine
PI	Propidium Iodide
PV	2 phenyl phenol or ortho-phenyl phenol, OPP (Preventol A8)
rDNA	ribosomal Deoxyribonuleic Acids
RNA	Ribonucleic Acids
rRNA	ribosomal Ribonucleic Acids
SD	Standard Deviation
SEA	Standard Error Average
SEM	Scaning Electron Microscope
SOD	Super Oxide Dismutases
TC	5-methyl-1,2,4-triazolo[3,4-*b*]benzothiazole (Tricyclazole)
UV	Ultra Violet
YNB medium	Yeast Nitrogen Base medium

Curriculum Vitae

Personal Detail

First Name:	Parisa
Surname:	Mohammadi
Date of Birth:	22.05.1966
Place of Birth:	Tehran/Iran
Marital Status:	Married, 2 Children

Education

10.1972-06.1977	Elementary School, Tehran, Iran
10.1977-06.1980	Ordinary School, Tehran, Iran
10.1980-06.1984	High School, Tehran, Iran
11.1984-02.1989	B.Sc. Microbiology, Faculty of Science, University of Tehran, Iran
02.1990-03.1994	M.Sc. Microbiology, Faculty of Science, University of Tehran, Iran (Vacation due to the birth of child)
2004	Ph-D Student at the University of Oldenburg/ICBM

Experience

1987-1988	Collaboratore of Dr. Ziaee in "Mutagenicity of Food" project, Institute of Biochemistry & Biophysics (IBB), Tehran, Iran
1996-2003	Lecturer at Azzahra University, Tehran, Iran

Workshops

1997	PCR, The Iranian Research & Developing Center, Tehran, Iran
1998	Electophoresis, The Jahad Academic Center,Tehran, Iran
2000	MIC, Minstry of Health & Medical Education, Tehran, Iran
2002	Stem Cells Bone marrow Culture, Tarbiat Modarres University, Tehran, Iran

List of Submitted Publications and Presentations

Biodeterioration of cultural Heritage in Iran; Persepolis (ISSM 2005)
P. Mohammadi, W. E. Krumbein, A. A. Gorbushina

Biodeteriorating microbial communities on Rock materials of the cultural heritage site at Persepolis (Iran); VAAM 2005
P. Mohammadi, A. A. Gorbushina, W. E. Krumbein

Morphological and molecular biological characterization of rock-inhabiting fungi from Persepolis (Iran); VAAM 2006
P. Mohammadi, J. Marquardt, W. E. Krumbein, A. A. Gorbushina

A study of biopitting on stones from the Persepolis monuments (Iran); VAAM 2006
P. Mohammadi, W. E. Krumbein

Rapid Screening of antimicrobial agents against Rock Biofilms; (ISME-11 2006)
P. Mohammadi, A. A. Gorbushina, W. E. Krumbein.

Assay of Antimicrobial Agents against Rock Biofilms: VAAM 2007
Comparison of FDA Fluorometry and CFU Determinations
P. Mohammadi, A. A. Gorbushina, W. E. Krumbein

Biopitting of ancient stone materials from the Persepolis monuments (Iran)
P. Mohammadi, W. E. Krumbein, Geomicrobiology, ICBM, Carl von Ossietzky University Oldenburg, D-26111 Oldenburg Germany.
Submitted to Aerobiology

Declaration

I, Parisa Mohammadi carried out all the experimental works including isolation and characterization of rock inhabiting fungi (exception of rDNA sequencing), preparation of stone samples for ligth microscopy, thin section, PAS reactions, maceration technique, and SEM under supervision of Geomicrobiological specialists. I designed and carried out the tests for the fluorometry method for rock inhabiting fungi which were measured at ICBM. I have personally completed this thesis using only the materials and aids indicated in the text.

Oldenburg, Germany

Parisa Mohammadi