BIOCURĂȚAREA PICTURII MURALE CU ESTERAZE MICROBIENE IMOBILIZATE ÎN AGARART
BIOCLEANING OF WALL PAINTING WITH MICROBIAL ESTERASES IMMOBILISED IN AGARART

I. GOMOIU[1]* , M. ENACHE[1], S. NEAGU[1] , R. RUGINESCU[1], M. DUMBRĂVICIAN[2], R. RADVAN[3], L. GHERVASE[3], I. MOHANU[4], R. COJOC[1]

[1] *Institutul de Biologie București, str. Splaiul independenței, nr. 296, cod 060031, București, România*
[2] *Universitatea Națională de Arte, str. General Budișteanu, nr. 19, cod 010773, București, România*
[3] *Institutul Național de Cercetare-Dezvoltare pentru Optoelectronică INOE 2000, str. Atomiștilor, nr. 409, cod 077125, Măgurele, România*
[4] *CEPROCIM S.A., str. Preciziei, nr. 6, cod 062203, București, România*

Biological cleaning of the restored of artworks is a green methodology which avoids the toxicity of solvents used in dry cleaning and protects restorers and environment. Biocleaning with viable bacteria cultures has been used successfully to remove black crust, mineral salts deposits, and organic matter residues, due to selective and specific metabolites proving to be more effective than chemical methods. Biocleaning treatments were applied for removing of the organic materials used in previous restoration or accidentally filed onto an artwork. Gels captured the attention of restorers and their applicability quickly extended to murals, stone, paper, even metals. Agarart hydrogel is recommended both for cleaning of artworks and as a delivery system. This study aimed to remove consolidants (acrylic resin Paraloid B72, Transparent Dispersion of Casein) and accidental organic deposits (beewax, sunflower oil and soot) from the surface of the murals with esterolytic enzymes produced by halotolerant bacteria Bacillus sp. BA N P3.3, applied directly or integrated in Agarart. The experiments were carried out on laboratory models aged by exposure to variations of temperature and humidity respectively to UV-A/UV-B radiation. Although biocleaning was achieved by applying of the esterolytic enzymes entrapped in Agarart gel (E-Agarart) or directly on the surface of organic deposit and then covered with Agarart gel (E+Agarart), to avoid the development of efflorescences, treatment with esterolytic enzymes integrated in Agarart gel is recommended.

Biocurățarea operelor de artă restaurate este o metodologie ecologică care evită folosirea solvenților toxici caracteristici pentru curățarea uscată, protejând astfel atât restauratorii cât și mediul înconjurător. Biocurățarea bazată pe culturi bacteriene viabile s-a dovedit a fi mai eficientă decât cea chimică deoarece a contribuit prin metaboliți specifici la îndepărtarea crustelor negre, depunerilor de săruri și a materialelor organice. Biocurățarea se aplică cu succes atât pentru îndepărtarea unor materiale folosite la restaurarea anterioară cât și a unor depuneri organice accidentale. Gelurile au atras interesul restauratorilor iar aplicarea acestora s-a extins la pictura murală, obiecte litice, documente și chiar la obiecte metalice. Hidrogelul Agarart este recomandat atât pentru curățarea operelor de artă cât și ca suport de imobilizare. Prezenta lucrare are ca scop atât îndepărtarea consolidanților (rășina acrilică Paraloid B72 și Dispersia Transparentă de Cazeină) cât și a depunerilor organice accidentale (ceara de albine, uleiul de floarea soarelui, gudroanele de cărbune) de pe suprafața picturii murale folosind enzime esterolitice produse de bacteria halotolerantă Bacillus sp. BA N P3.3, aplicate direct sau incluzionate în gelul Agarart. Experimentele s-au efectuat pe modele de laborator executate în tehnica al fresco, îmbătrânite prin expunere la variații de temperatură și umiditate, respectiv la radiații UV-A/UV-B. Deși biocurățarea s-a obținut atât prin aplicarea soluției enzimatice integrate (E-Agarart) cât și prin aplicarea preparatului enzimatic direct pe suprafața modelului experimental urmată de acoperirea cu gelul Agarart (E+Agarart), pentru evitarea formării eflorescențelor, recomandăm tratamentul cu enzime esterolitice integrate în gelul Agarart.

Keywords: *halotolerant and halophilic microorganisms, biotechnologies for conservation and restoration, hydrolytic enzymes, integrated esterase in hidrogels, biocleaning with fungal cultures*

1.Introduction

Studies about biological cleaning of the restoration of artworks stared in the early 1990s, and developed limiting the use of conventional chemical products [1]. Biocleaning with viable bacteria cultures has been used successfully to remove black crust, mineral salts deposits, and organic matter residues, due to selective and specific metabolites proving to be more effective than chemical methods. Gauri et al. [2] proved for the first time that *Desulfovibrio desulfuricans*, can be used as a cleaning agent for the reduction of gypsum and black crust in marble. The samples had to be immersed in a growth medium for 84 h. Later on Cappitelli et al. [3] proposed a method based on the application of cell *D. desulfuricans* entrapped in a Carbogel carrier. The biological procedure proved homogeneous results in the removal of the surface deposits, a good preservation of historical layers below the crust (patina noble) and did not produced undesirable secondary products [4, 5].

The biocleaning procedure applied on samples of weathered stone in Failaka Island,

* Autor corespondent/*Corresponding author*,
E-mail: ioana.gomoiu@ibiol.ro

Kuwait with black gypsum [6]. The hydrobiogel-97 (a polymer created by an acrylic resin hydrogel) mixed with *D. desulfuricans* and covered by polyethylene was used on the Japanese Paper for 24h, three times. *D. desulfuricans* converted gypsum (calcium sulphate) into H_2S then, the free calcium Ca^{+2} released reacted with Carbon dioxide to form $CaCO_3$. In that way, cleaning and consolidation took place in the same time.

The salt efflorescence can cause the deterioration of the wall paintings. They are the consequence of high relative humidity and capillarity moisture or the result of biological processes. The natural aging of painting substances, residuals of restoration products favour microbial colonization, forming of efflorescences and microcracks. Bosh Roig et al. [7] applied a uniform layer of *Pseudomonas stutzeri* cells and then the following carriers: hydrophilic cotton, sepiolita, carbogel, European bacteriological agar and agarose. Biocleaning system based on *P. stutzeri* DSMZ 5190 and agar was the best to remove efflorescence from the wall paintings of the Santos Juanes church of Valencia. It was demonstrated that the method was non-invasive, specific, and non-toxic, acting in an eco-friendly manner. One of the most important advantages of the method is the use of safe substances for the environment, the artwork, and the people due to the fact that bacteria are not pathogenic and non-toxic materials are used.

Rampazzi et al. [8] performed biocleaning tests with laponite as delivery system instead of agar gel which needs a higher temperature before solidification. Laponite gel (control) and laponite gel with *P.stutzeri* A29 cells entrapped were applied onto the *ex situ* noble tufa rock surface using a sterile spatula, for 12 to 24 h, at room temperature of 25 °C ± 2 °C.

Romano et al. [9] suggested the possibility of using extremophilic bacteria, such as *Halomonas campaniensis* spp., for biocleaning of nitrate crusts on stone surfaces, demonstrating also the safety of use of this kind of biological approach.

Due to the fact that crusts have different composition (sulphate deposits, nitrate salts, carbonates, apatite, and proteins traces) they were cleaned by combined methods: *Desulfovibrio desulfuricans*-Carbogel application and mechanical pre-treatment [10] or chemical pre-treatment with a non-ionic detergent [11]. Alfano et al. [12] proposed the mixture of *D. vulgaris* ATCC 29579 and *P. pseudoalcaligenes* KF707 in a multilayer biosystem. Mazzoni et al. [13] recommended compresses containing microorganisms as micro-packs as fellows: *Cellulosimicrobium cellulans (*to solubilise calcium sulphate and carbonate*), Stenotrophomonas maltophilia (*to degrade protein) *and Pseudomonas koreensis* (to solubilise inorganic compounds and to degrade protein material).

Biocleaning treatments were also applied for removing residues of organic substances, such as casein, egg yolk, oil, and animal fat by the direct application onto an artwork. Ranalli et al. [14] used bacterial cells of the *P. stutzeri* A29 strain as the first step of the treatment followed by adding of a purified protease enzyme.

Lustrato et al. [15] removed in one step the casein and animal with viable bacterial cells of *P. stutzeri*, A29 strain (applied for 2 h) on the 14th-century fresco Stories of the Holy at Camposanto Monumentale in Pisa, Italy. The results confirmed the success of this advanced biological approach for recovering those valuable frescoes.

Bosch-Roig [16] proposed the biocleaning of insoluble animal glue on the frescoes of the central vault of the Santos Juanes (situated in the main old area of Valencia, Spain) with *P. stutzeri* 5190 due to its high protease activity.

Ranalli et al. [17] reported the *ex situ* large-scale biotreatment of Triumph of Death fresco at the Camposanto Monumental Cemetery (Pisa, Italy) to set up of a 'hi-tech restoration process' based on a two-steps biocleaning of the proteinaceous residues (mainly animal glue and casein) present both on the front and back of the fresco paint work as well as the removal of the old asbestos-cement support.

Ranalli et al. [18] proposed the short bioapplication contact time (between 3 and 12 h) of advanced agar-gauze gel activated with *P. stutzeri* cells as an alternative method to the traditional one.

Introduced in the early 1990s to clean paintings on canvas [19], gels captured the attention of restorers and their applicability quickly extended to murals and stone [20, 21, 13]. The gels used in cleaning are biphasic systems, made up of two or more components, mainly a polymer and a fluid phase. Agarart hydrogel is recommended both for cleaning of artworks and as a delivery system.

Cold active molecules extracted from marine invertebrates (sponges, jellyfish, sea anemones and crustaceans) were used to remove protein layers from the surface of the painting. Barresi et al. [22] removed casein layers with bioactive molecules containing protease. Comparative treatment with commercial enzymes demonstrated that biological enzymes do not need surface heating to 37 ^{o}C, which is an advantage for *in situ* cleaning. Bioactive molecules with esterase and proteolytic activity [23, 24] were applied to an experimental model obtained in the laboratory mimicking the "strappo" of mosaic tiles, made by gluing the tiles to a sheet of cloth. The results were promising for biocleaning.

This study aimed to remove consolidants (acrylic resin Paraloid® B72, Transparent Dispersion of Casein) and accidental organic deposits (beeswax, sunflower oil and soot) from the surface of the murals with esterolytic enzymes produced by halotolerant bacteria *Bacillus* sp. BA N P3.3 applied directly and integrated in Agarart.

2. Materials and Methods

2.1. Painted laboratory models were obtained by using bricks (22.5x11 cm), 2-3% of 0.5 mm sand, lime, tow, and pigments. Each laboratory model was divided in four sections, onto which red, ochre, blue and green pigments have been applied. Pigments (Venetian red 0315, Yellow ochre 0324, Pure Ultramarine blue 0516, and Green earth 0264) were acquired from CTS Company. According to the manufacturer, the chemical composition of the selected pigments is as follows: the red pigment contains Fe_2O_3, the yellow one contains α-FeO(OH), $CaCO_3$,$CaSO_4$, the blue pigment contains, approximately, $(Na,Ca)_4(Al,SiO_4)_3(SO_4,S,Cl)$, and the green one contains ferrous and ferric silicates of potassium, manganese and aluminium [25]. Then, three layers of acrylic resin Paraloid® B72 (B72), Transparent Dispersion of Casein (TDC), beeswax (BW), sunflower oil (SO) and soot (S) were applied [26]. The surface of each pigment covered by each deposit was divided in areas of $2cm^2$ for application of hydrogel Agarart.

2.2. Preparation of esterase

Bacillus sp. BA N P3.3 was grown on liquid medium HM [27] supplemented with 1% (v/v) Tween 80 for 4 days at 30 °C. Subsequently, the extracellular esterases present in the culture fluid were concentrated by acetone. The enzyme solution used in the biocleaning experiments was obtained by precipitation with acetone of the culture liquid of the *Bacillus* sp strain. In general, the volume of enzyme solution used was 1 mL/10 mL of buffer solution, corresponding to a final protein concentration of 0.48 mg/mL, with esterase activity of 0.124 U/mg.

2.3. Preparation of Agarart hydrogel

Different concentrations of Agarart (1.5%, 2% and 2.5%) were suspended in demineralized water, followed by heating and maintaining at 98 ^oC for 6 minutes. After the temperature drops to 45 ^oC, mixed gently with the wand and apply on a flat surface. After complete cooling to room temperature (24-26 ^oC), the gels were cut with a scalpel and applied to the surface to be cleaned. The pH of the gels was 5.7.

2.4. Aging of the painted laboratory models

In order to reduce, as much as possible, the research time, accelerated aging techniques were used. The artificial aging process can influence, in a relatively short time, the chemical and physical parameters (composition, internal and surface degradation, turbidity, viscosity, humidity, pH, mechanical stress, colour and brightness), thereby partially simulating the natural aging process. There are different methods of artificial ageing: by using UV and/or visible radiation, by varying the temperature and humidity in specially designed enclosures, by using chemicals, especially acids, or combinations.

Two aging methods were chosen for this experiment. For the first one (I1), a Memmert CTC256 climatic chamber was used, which allowed the variation of temperature, between -10 and 55 ^oC, and of the relative humidity, between 20 and 90 %. The maximum temperature level was chosen so as to be lower than the melting point of natural wax, which is approximately 61-65 °C. For each temperature/relative humidity extreme, a dwell time of 3 hours was set. In total, the samples were exposed to the modified atmosphere for ~190 h

For the second one (I2), exposure to UV radiation was selected and performed using two 36 W Philips UV lamp systems, emitting in the UV-B (280-315 nm) and UV-A (315-400 nm) ranges. Each colour zone was divided into four parts, one as a reference, one for UV-A, one for UV-B and one kept as a reserve. Custom-made masks were created so as to expose only one area at a time to specific radiation.

2.5. Microbiological analysis

The evaluation of total viable microorganisms was performed by taken samples on the sterile swabs which were analysed using the plate counting technique. The incubation took place at 28 ^oC for 48 hours in case of bacteria and 10 days in case of fungi. Then, the colony forming units (CFU) were expressed.

2.6. Biocleanig tests

In order to establish the optimal method of application of the esterolytic enzymes, it was applied to the surface of the aged experimental models, on which squares were delimited, as follows:

- directly on the surface of the organic deposit (E - box 1)
- directly on the surface of organic deposit and then covered with polyethylene film (E+PF - box 2)
- enzyme entrapped in Agarart gel (E-Agarart - box 3)
- directly on the surface of organic deposit and then covered with Agarart gel (E + Agarart - box 6)
- buffer solution applied on the surface of the organic deposit (box 7)

Each square was divided into two parts to evaluate the biocleaning efficiency after 5 and 10 hours. Incubation took place at 24^0C and was followed by mechanical cleaning.

2.7. Evaluation of the biocleaning by microscopy

Biocleaning is considered as an effect of hydrolytic decomposition of the deposits. Samples were analysed under optical microscope (Nikon AZ100) and Scanning Electron Microscope (SEM). The samples were gold sputtered and then

observed under a variable pressure scanning electron microscope JEOL JSM6610LV operated at high vacuum (accelerated voltage 10–20 kV) [28].

2.8. Evaluation of biocleaning through colorimetry

The evaluation of the hydrolytic decomposition of the deposits was also assessed based on the color variation of the samples, through colorimetry [29, 30]. An X-rite Ci64UV colorimeter was used, with 4 mm, standard illuminant D65/10°, CIELAB colour space, averaging three measurements in different points for each treated area. Principal component analysis was performed using the Unscrambler 11 software in order to better understand the data. The input dataset was created using the luminosity (L*), red-green (a*), and yellow-blue (b*) values recorded with the colorimeter.

3. Results and discussion

3.1. Analysis of aged painted laboratory models

Although low, the microbial load of the aged painted laboratory was different, depending on the type of deposition and sometimes the aging method. No fungal colony-forming units (CFU) were identified on the I2-aged models. The wax-coated painted laboratory aged by both methods did not contain bacterial CFU. Models coated with TDC, Paraloid B72 and sunflower oil had a low bacterial load (3.8 – 122 CFU bacteria/ml/cm^2).

Microscopy analyses did not reveal major changes after the aging of the experimental models by method I1. The Paraloid B72 was not changed after the aging treatment. It developed a fine film and, in some images, can be seen bubbles with or without a ceiling. The formation of bubbles was characteristic for this consolidant (Table 1). The wax, being a product of biological origin, deposited by hand, was very heterogeneous in terms of thickness but also from the morphological point of view. TDC formed a morphologically heterogeneous film. No microscopic changes were observed after aging. The oil formed a thin film on the surface of the colour layer on the yellow, blue and green pigments. Its fragmentation was not the result of aging but of application conditions. Soot was made up of particles of different sizes. The small ones can be detached by simply manipulating the experimental models and the large ones adhere firmly to the surface of the pictorial layer and have a network structure. Soot was not uniformly distributed. Colorimetric analyses revealed little changes in the case of consolidants (B72, DTC) and oil deposits and significant changes in the case of wax and soot deposits.

Through microscopic methods, it was found that the Paraloid B72 was not morphologically affected by UV-A radiation, but UV-B radiation produced point-like changes such as dislocations and the collapse of the bubble ceiling (Table 2). The wax was not morphologically affected by exposure to UV-A radiation, but showed point-like changes such as dislocations as a result of exposure to UV-B radiation. TDC did not change morphologically under the action of UV-A radiation. Conversely, UV-B radiation caused the fragmentation of TDC film and, in some areas, even destroyed it. The sunflower oil was affected by both UV-A and UV-B radiation, showing fragmentation and detachment. UV-A radiation had no effect on soot unlike UV-B radiation which caused significant areas to detach (but not the entire thickness of the deposit).

Colorimetric analysis revealed little changes in the case of consolidants (Paraloid B72, TDC) and sunflower oil deposits and significant changes in the case of wax and soot ones. Differences between the pigments on which the depositions were applied may be the result of uneven deposition. The morphological changes identified as well as the results of the colorimetry and spectroscopy analysis will represent the witnesses in the evaluation of biocleaning.

3.2. Assessment of biocleaning of painted laboratory models

3.2.1. Assessment of biocleaning of painted laboratory models aged by exposure to variations in temperature and humidity (I1)

Macroscopic analysis in direct and radiant light performed after removal of gels and mechanical cleaning did not reveal morphological/aesthetic changes on the deposits of Paraloid B72, TDC, wax and sunflower oil. Biocleaning was evident only in the case of soot deposit, mainly on the blue paint layer.

Table 1

Microscopic analysis (optical and electronic) of painted laboratory models aged by exposure to temperature and humidity variations; line 1- painted laboratory models covered by Paraloid B72 analyzed by optic microscopy (OM); line 2- painted laboratory models covered by Paraloid B72 analyzed by electronic microscopy (SEM); line 3- painted laboratory models covered by BW analyzed by OM; line 4- painted laboratory models covered by BW analyzed by SEM; line 5- painted laboratory models covered by TDC analyzed by OM; line 6- painted laboratory models covered by TDC analyzed by SEM; line 7- painted laboratory models covered by SO analyzed by OM; line 8- painted laboratory models covered by SO analyzed by SEM; line 9- painted laboratory models covered by S analyzed by OM; line 10- painted laboratory models covered by S analyzed by SEM. / *Analiza modelelor de laborator îmbătrânite prin expunere la variații de temperature și umiditate prin microscopia optică și electronică*; *rândul 1- modelul de laborator acoperit cu Paraloid B72 analizat la microscopul optic (OM); rândul 2- modelul de laborator acoperit cu Paraloid B72 analizat la microscopul electronic (SEM); rândul 3- modelul de laborator acoperit cu ceară de albine analizat la microscopul optic (OM); rândul 4- modelul de laborator acoperit cu ceară de albine analizat la SEM; rândul 5- modelul de laborator acoperit cu Dispersie Transparentă de Cazeină analizat la OM; rândul 6- modelul de laborator acoperit cu Dispersie Transparentă de Cazeină analizat la SEM; rândul 7- modelul de laborator acoperit cu ulei de floarea soarelui analizat la OM; rândul 8- modelul de laborator acoperit cu ulei de floarea soarelui analizat la SEM; ; rândul 9- modelul de laborator acoperit cu gudroane de cărbune analizat la OM; rândul 8- modelul de laborator acoperit cu gudroane de cărbune analizat la SEM* .

See table on next page

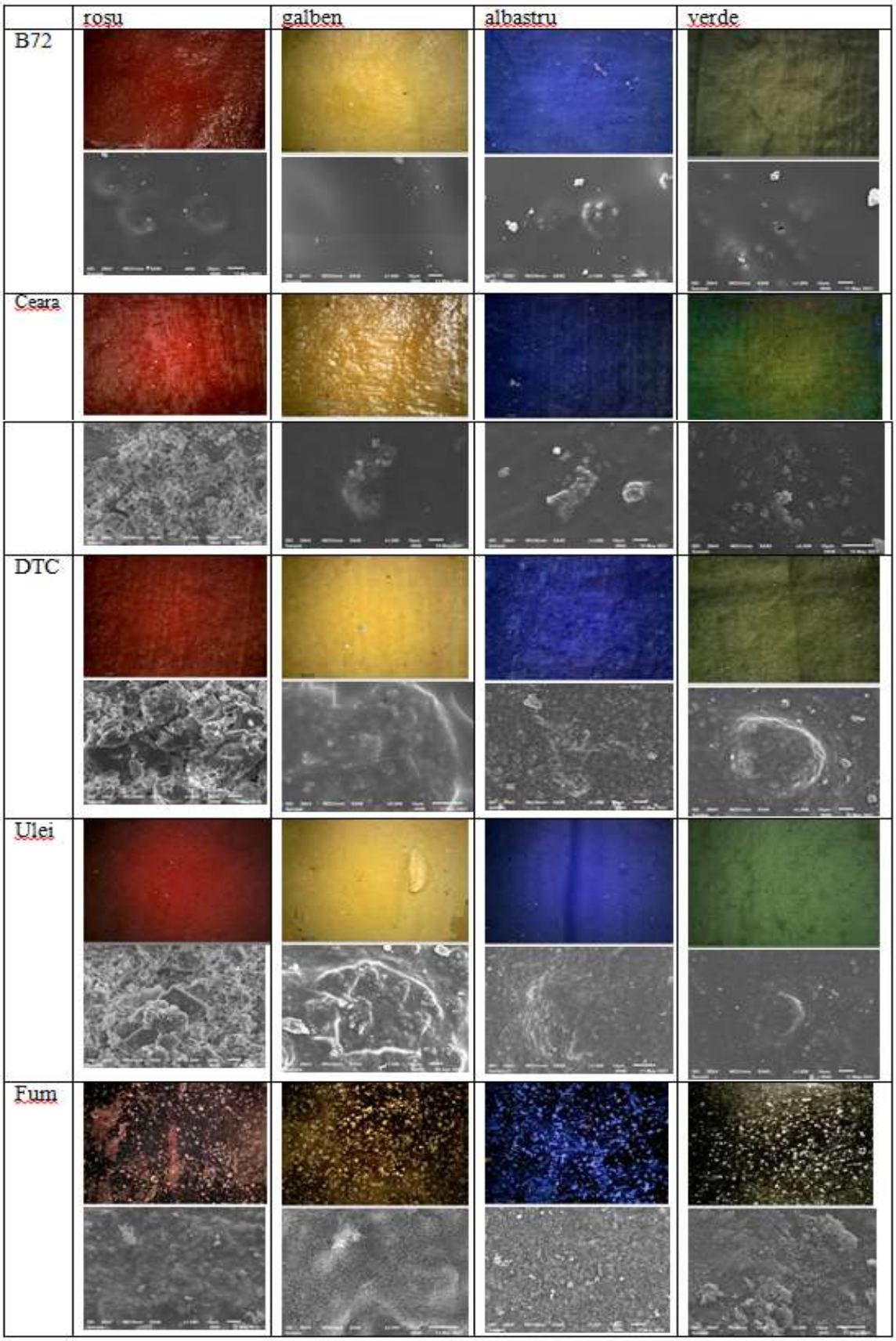


Table 2

Electron Microscope Analysis of UV-A and UV-B of painted laboratory models with red, yellow, blue and green pigment layers covered with Paraloid B72, BW, TDC, SO and S; line 1- painted laboratory models covered by Paraloid B72 aged by UV-A; line 2- painted laboratory models covered by Paraloid B72 aged by UV-B; line 3- painted laboratory models covered by BW aged by UV-A; line 4- painted laboratory models covered by BW aged by UV-B; line 5- painted laboratory models covered by TDC aged by UV-A; line 6- painted laboratory models covered by TDC aged by UV-B; line 7- painted laboratory models covered by SO aged by UV-A; line 8- painted laboratory models covered

by SO aged by UV-B; line 9- painted laboratory models covered by S aged by UV-A; line 10- painted laboratory models covered by S aged by UV-B. / *Analiza modelelor de laborator îmbătrânite prin expunere la radiații UV-A și UB-B prin microscopie electronică*; *rândul 1- modelul de laborator acoperit cu Paraloid B72 îmbătrânit prin expunere la UV-A; rândul 2- modelul de laborator acoperit cu Paraloid B72 îmbătrânit prin expunere la UV-B; rândul 3- modelul de laborator acoperit cu ceară de albine îmbătrânit prin expunere la UV-A; rândul 4- modelul de laborator acoperit cu ceară de albine îmbătrânit prin expunere la UV-B; rândul 5- modelul de laborator acoperit cu Dispersie Transparentă de Cazeină îmbătrânit prin expunere la UV-A; rândul 6- modelul de laborator acoperit cu Dispersie Transparentă de Cazeină îmbătrânit prin expunere la UV-B; rândul 7- modelul de laborator acoperit cu ulei de floarea soarelui îmbătrânit prin expunere la UV-A; rândul 8- modelul de laborator acoperit cu ulei de floarea soarelui îmbătrânit prin expunere la UV-B; ; rândul 9- modelul de laborator acoperit cu gudroane de carbune îmbătrânit prin expunere la UV-A; rândul 8- modelul de laborator acoperit cu gudroane de carbune îmbătrânit prin expunere la UV-B .*

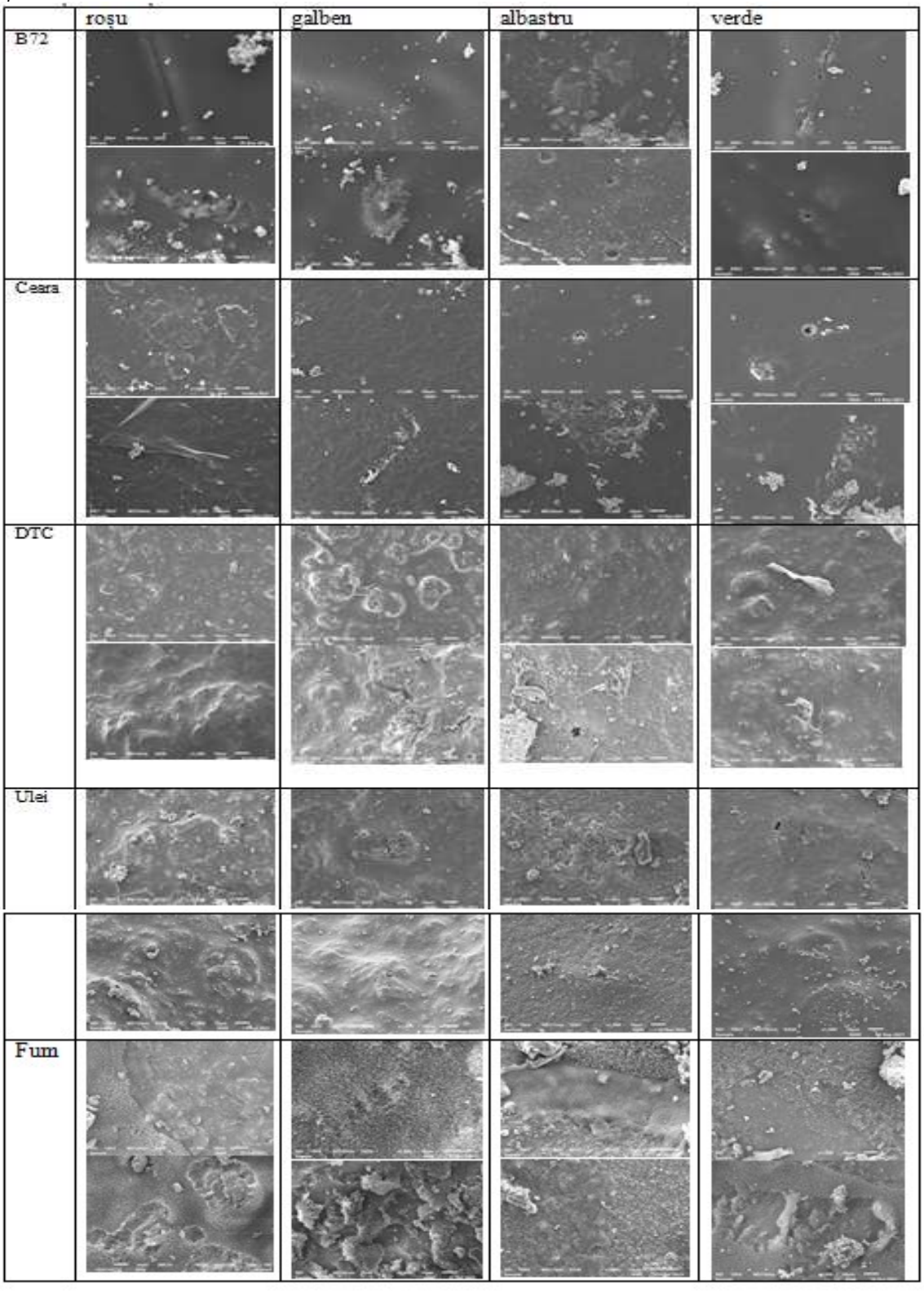
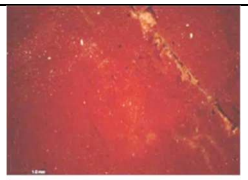
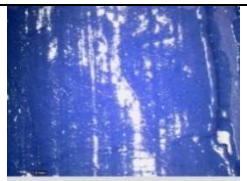
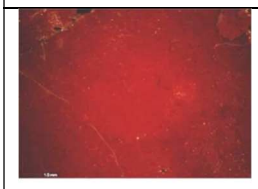
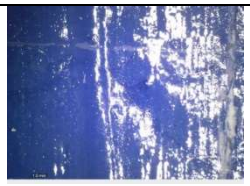
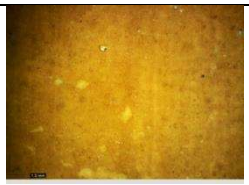
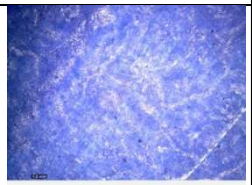
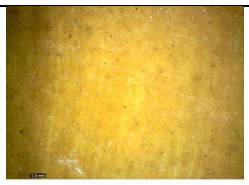
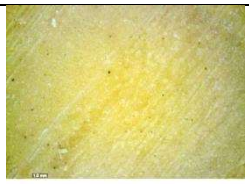
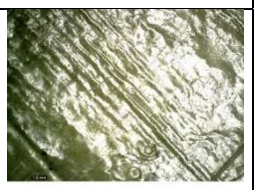
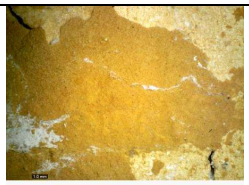
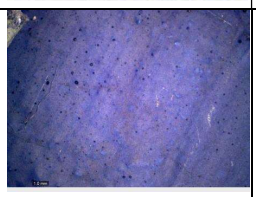


Table 3.

Evaluation of biocleaning with E-Agarart and E+Agarart (I1), by optical microscope analysis; line 1- painted laboratory models covered by Paraloid B72 cleaned with E-Agarart ; line 2- painted laboratory models covered by Paraloid B72 cleaned with E+Agarart; line 3- painted laboratory models covered by TDC cleaned with E-Agarart; line 4- painted laboratory models covered by TDC cleaned with E+Agarart; line 5- painted laboratory models covered by BW cleaned with E-Agarart; line 6- painted laboratory models covered by BW cleaned with E+Agarart; line 7- painted laboratory models covered by SO cleaned with E-Agarart; line 8- painted laboratory models covered by SO cleaned E+Agarart; line 9- painted laboratory model covered by S cleaned with E-Agarart; line 10- painted laboratory model covered by S cleaned with E+Agarart./ *Evaluarea biocurățării cu E-Agarart și E+Agarart (I1) prin analiza la microscopul optic; rândul 1- modelul de laborator acoperit cu Paraloid B72 curățat cu E-Agarart; rândul 2- modelul de laborator acoperit cu Paraloid B72 curățat cu E+Agarart; rândul 3- modelul de laborator acoperit cu Dispersie Transparentă de Cazeină curățat cu E-Agarart; rândul 4- modelul de laborator acoperit cu Dispersie Transparentă de Cazeină curățat cu E+Agarart; rândul 5- modelul de laborator acoperit cu ceară de albine curățat cu E-Agarart; rândul 6- modelul de laborator acoperit cu ceară de albine curățat cu E+Agarart; rândul 7- modelul de laborator acoperit cu ulei de floarea soarelui curățat cu E-Agarart; rândul 8- modelul de laborator acoperit cu ulei de floarea soarelui curățat cu E+Agarart; rândul 9- modelul de laborator acoperit cu gudroane de carbune curățat cu E-Agarart; rândul 10- modelul de laborator acoperit cu gudroane de carbune curățat cu E+Agarart.*

Deposit/Depunere	Red/Roșu	Yellow/Galben	Blue/Albastru	Green/Verde
Paraloid B72/ Paraloid B72/				
				
TDC/ Dispersie Transparentă de Cazeină				
				
Beeswax/ceară de albine				
				
Sunflower oil/Ulei de floarea soarelui				

See next page

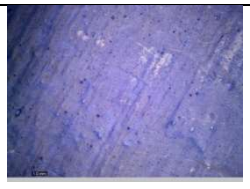
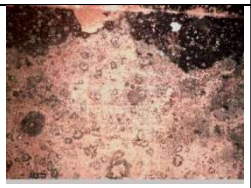
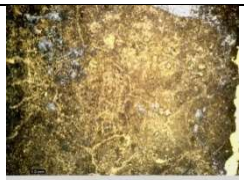
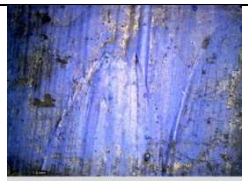
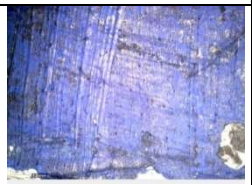
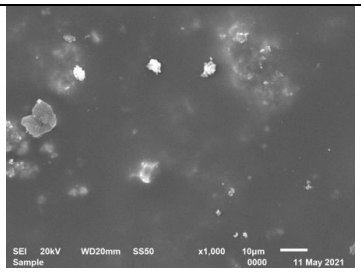
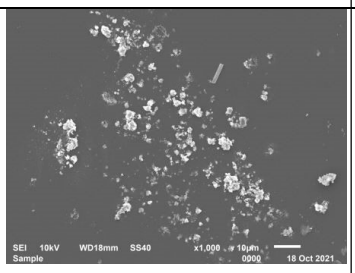
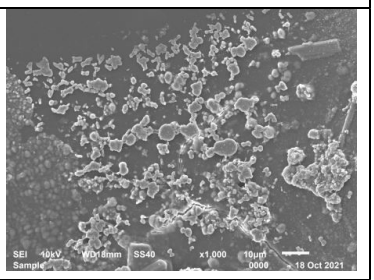
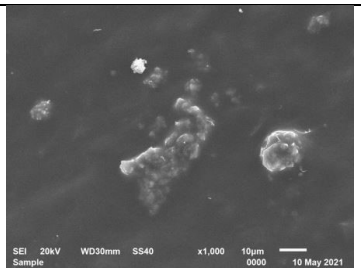
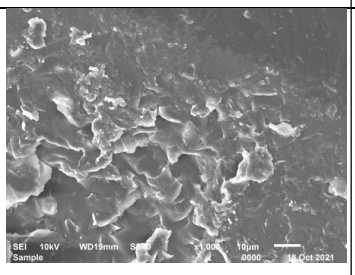
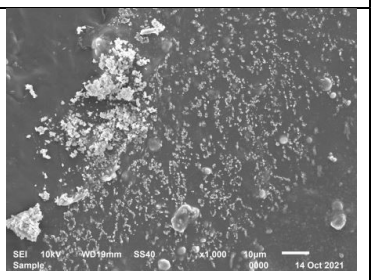
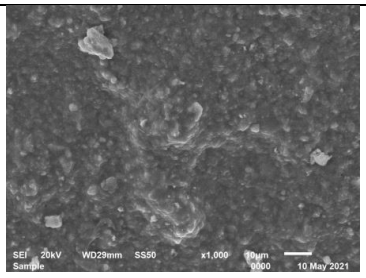
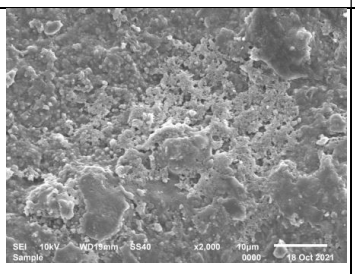
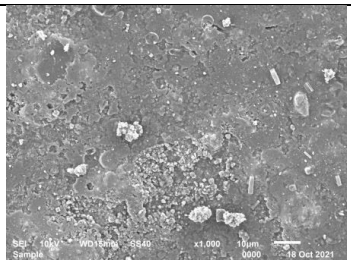
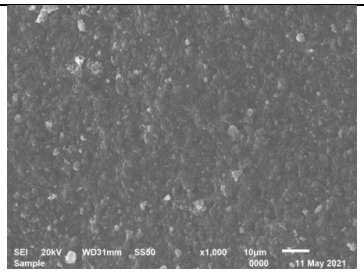
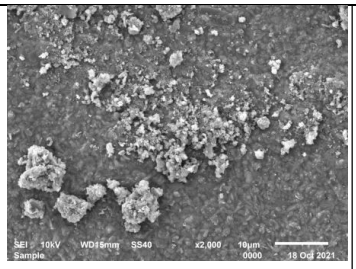
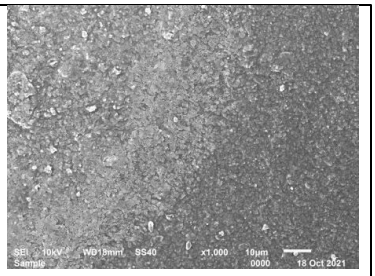
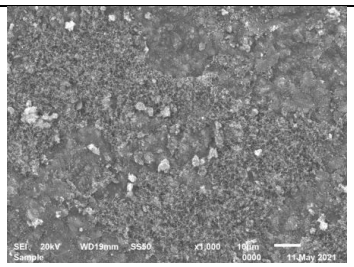
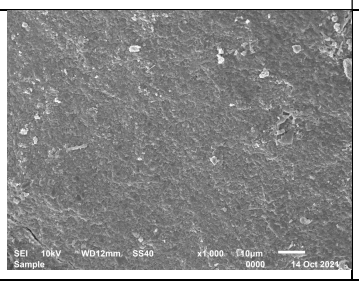
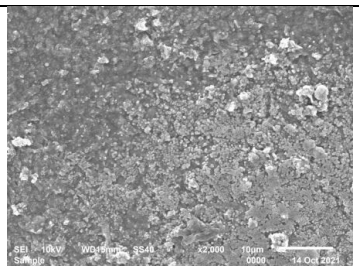
				
Soot/ Gudroane de cărbune				
				

Table 4

Evaluation of biocleaning of experimental models (I1) by SEM examination; line 1- painted laboratory models covered by Paraloid B72: control, cleaned with E-Agarart and E+Agarart; line 2- painted laboratory models covered by BW: control, cleaned with E-Agarart and E+Agarart; line 3- painted laboratory models covered by TDC: control, cleaned with E-Agarart and E+Agarart; line 4- painted laboratory models covered by SO: control, cleaned with E-Agarart and E+Agarart; line 5- painted laboratory models covered by S: control, cleaned with E-Agarart and E+Agarart; / *Evaluarea biocurățării modelelor experimentale (I1) prin examinarea la SEM*; *rândul 1- modelul de laborator acoperit cu Paraloid B72: control, curățat cu E-Agarart și E+Agarart; rândul 2- modelul de laborator acoperit cu ceară: control, curățat cu E-Agarart și E+Agarart; rândul 3- modelul de laborator acoperit cu Dispersie transparentă de cazeină : control, curățat cu E-Agarart și E+Agarart; rândul 4- modelul de laborator acoperit cu ulei de floarea soarelui: control, curățat cu E-Agarart și E+Agarart; rândul 5- modelul de laborator acoperit cu gudroane de cărbune: control, curățat cu E-Agarart și E+Agarart.*

Deposit/ Depunere	Control/Control	E-Agarart/ E-Agarart	E+Agarart/ E+Agarart
Paraloid B72/ Paraloid B72			
Beeswax/ Ceară de albine			
TDC/ Dispersie Transparentă de Cazeină			
See next page			

Sunflower oil/ ulei de floarea soarelui			
Soot/gudroane de cărbune			

Optical microscope analysis. The direct application of the esterases solution unprotected or protected by the polyethylene film was not effective because, during the treatment, the liquid phase evaporated and the enzyme no longer exerted its hydrolytic activity. Consequently, on boxes 1, 2 and 7, efflorescences (from phosphate buffer and precipitated enzyme) were highlighted. Images were representative of E-Agarart (for 5 hours and 10 hours-box 3) and E + Agarart (for 5 hours and 10 hours-box 6) only. The Paraloid B72 consolidant applied to the 4 types of pigments gives them a good fixation. Table 3 shows the areas with different degrees of discoloration assimilated to hydrolysis. Both application/treatment methods seem to be effective.

The TDC gave also a good fixation of pictorial layer containing 4 pigments. Table 3 shows that after mechanical cleaning, except for the green pigment, in some areas were noticed cracks. The optimal biocleaning time was 10 hours.

The sunflower oil deposit gave the surfaces a shiny aspect. As Table 3 shows after mechanical cleaning, changes occurred as follows: on the red colour layer, in the case of E+Agarart application, efflorescences appeared, most likely as a result of the more intense reaction between esterases and broken down oil; on the yellow pigment layer there was a massive loss of pigment, most likely it reacted chemically with the oil and its decomposition products; on the blue pigment layer there was a partial loss of pigment, most likely it was more resistant to the action of sunflower oil; on the green pigment layer the loss of pigment after mechanical cleaning was reduced (compared to the blue pigment layer). By application of E-Agarart development of the efflorescences was avoided.

The soot deposited on the 4 layers of pigments profoundly changed the aesthetic appearance of the experimental model. Only the black colour caused by soot is observed. The analysis of Table 3 showed the following:

the efficiency of biocleaning on the red pigment layer was highlighted by embedding the enzyme in the Agarart gel (5 hours); in the case of the yellow and green pigment layer, the efficiency of biocleaning was highlighted by both methods of enzyme application (10 hours); on the blue pigment layer, the efficiency of biocleaning was clearer than in the case of the yellow one and was highlighted by both methods of enzyme application (10 hours) - we consider that the soot adheres less to the blue pigment layer.

Although biocleaning is achieved through both methods, to avoid the development of efflorescents, treatment with esterolytic enzymes integrated in Agarart is recommended. For increased efficiency, the optimal treatment time is 24 hours.

Electron microscope analysis. Samples were collected from the models aged by exposure to variations in temperature and humidity, before and after the enzymatic treatment, with the aim of scanning electron microscope (SEM) analysis. The results obtained after the application of the esterases treatment by different methods allowed to select for this stage, the enzyme solution immobilized in Agarart and the enzyme solution applied directly on the deposit, followed by the placement of the Agarart gel. The treatment time was 10 hours. Thus, the chemical reaction determined by the immobilized esterases was confirmed, highlighting areas of different sizes with intermediate decomposition products of the Paraloid B72, DTC, wax, sunflower oil and soot deposits (Table 4). The SEM observations made it possible to recommend the use for future experiments of E-Agarart for the hydrolysis of Paraloid B72 deposits, TDC and soot, and for the decomposition of wax and oil, E+Agarart.

Colorimetry confirmed very good hydrolysis in the case of soot deposits (I1) for both E-Agarart and E+Agarart (Fig.1-Fig.4). Biocleaning by both methods gave good results for the wax deposited

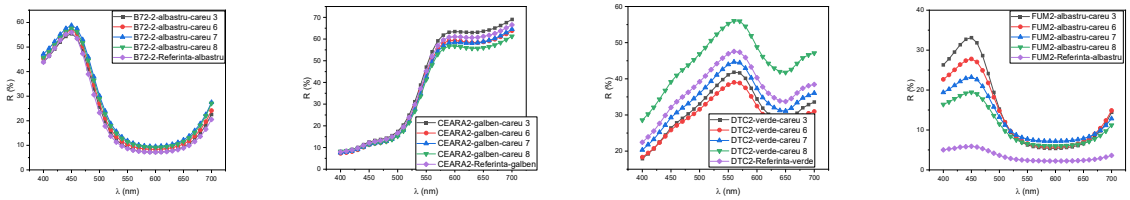


Fig.1 – Fig.4. Evaluation of biocleaning efficiency of experimental models with E-Agarart and E+Agarart by colorimetry (I1)/ *Evaluarea eficienței biocurățării modelelor experimentale cu E-Agarart and E+Agarart prin colorimetrie (I1).*

on the blue pigment and the oil deposited on the yellow and green pigments. Both methods gave minimal results for B72 (blue pigment), DTC (red pigment), wax (red, yellow, green pigments), oil (red and blue pigments). FTIR results (not included here) for the oil coating on the red pigment demonstrated a shift of the broad band attributed to silicate from about 1100 cm^{-1} to lower wavenumbers for the E-Agarart cleanup. The region between 1610 – 1680 cm^{-1} shows that certain characteristic bands of the pigment are broadened in the cleaning area with E-Agarart.

3.2.2. Assessment of biocleaning of painted laboratory models aged by exposure to UV-A and UV-B (I2)

The experimental models aged with the I2 method were biocleaned with E-Agarart because this gel does not cause the colour layer to tear. The treatment had the same positive effects as in the case of experimental models aged by I2 methods (Table 5). An effect unwanted by the restorers occurs, namely the cracking, which is more extensive.

Evaluation by electron microscope examination showed the effectiveness of biocleaning with E-Agarart of the experimental models aged by method I2 only in the case of consolidants (B72, DTC) and wax and soot deposits (Table 6). The best results were obtained in the case of soot deposits (UV-A and UV-B). Good results were obtained in the case of UV-B exposure, for the green pigment of the B72-coated model, and the blue pigment of the DTC-coated model.

Colorimetry (Fig.5-Fig.8) confirmed very good hydrolysis in the case of soot deposits (I2) for both UV-A and UV-B exposure. Biocleaning gave good results on models aged by exposure to UV-A only in the case of Paraloid B72 (green pigment layer). In the case of those exposed to UV-B radiation the same results were obtained for Paraloid B72 (green pigment) and TDC (blue pigment). Negative results were obtained for sunflower oil (all pigments), beeswax (red, blue, green pigments), TDC (red and green pigments) and Paraloid B72 (red pigment). Through colorimetry and spectrophotometry, some positive results were confirmed, but not the intensity of the biocleaning process.

Table 5

Evaluation of biocleaning with E-Agarart (I2), by optical microscope analysis; line 1 - painted laboratory models with red, yellow, blue and green pigment covered by Paraloid B72 aged by UV-A; line 2- painted laboratory models with red, yellow, blue and green pigment covered by Paraloid B72 aged by UV-B; line 3- painted laboratory models with red, yellow, blue and green pigment covered by TDC aged by UV-A; line 4- painted laboratory models with red, yellow, blue and green pigment covered by TDC aged by UV-B; line 5- painted laboratory models with red, yellow, blue and green pigment covered by BW aged by UV-A; line 6- painted laboratory models with red, yellow, blue and green pigment covered by BW aged by UV-B; line 7- painted laboratory models with red, yellow, blue and green pigment covered by SO aged by UV-A; line 8- painted laboratory models with red, yellow, blue and green pigment covered by SO aged by UV-B; line 9- painted laboratory models with red, yellow, blue and green pigment covered by S aged by UV-A; line 10- painted laboratory models with red, yellow, blue and green pigment covered by S aged by UV-B; */ Evaluarea biocurățării cu E-Agarart (I2) prin analizarea la microscopul optic*; *rândul 1 - model experimental cu strat de culoare roșu, galben, albastru, roșu acoperit cu Paraloid B72 și îmbătrânit cu UV-A; rândul 2 - model experimental cu strat de culoare roșu, galben, albastru, roșu acoperit cu Paraloid B72 și îmbătrânit cu UV-B; rândul 3 - model experimental cu strat de culoare roșu, galben, albastru, roșu acoperit cu Dispersie transparentă de cazeină și îmbătrânit cu UV-A; rândul 4 - model experimental cu strat de culoare roșu, galben, albastru, roșu acoperit cu Dispersie transparentă de cazeină și îmbătrânit cu UV-B; rândul 5 - model experimental cu strat de culoare roșu, galben, albastru, roșu acoperit cu ceară de albine și îmbătrânit cu UV-A; rândul 6 - model experimental cu strat de culoare roșu, galben, albastru, roșu acoperit cu ceară de albine și îmbătrânit cu UV-B; rândul 7 - model experimental cu strat de culoare roșu, galben, albastru, roșu acoperit cu ulei de floarea soarelui și îmbătrânit cu UV-A; rândul 8 - model experimental cu strat de culoare roșu, galben, albastru, roșu acoperit cu ulei de floarea soarelui și îmbătrânit cu UV-B; rândul 9 - model experimental cu strat de culoare roșu, galben, albastru, roșu acoperit cu gudroane de cărbune și îmbătrânit cu UV-A; rândul 10 - model experimental cu strat de culoare roșu, galben, albastru, roșu acoperit cu gudroane de cărbune și îmbătrânit cu UV-B.*

Deposit /Depunere	Red/Roșu	Yellow/Galben	Blue/Albastru	Green/Verde
Paraloid B72 (UV-A and UV-B)/ Paraloid B72 (UV-A și UV-B)/				

See next page

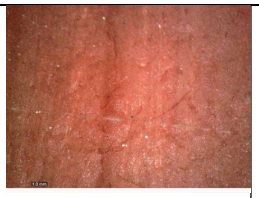
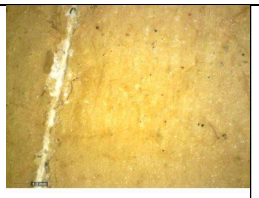
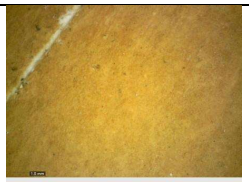
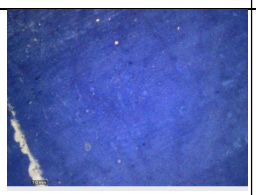
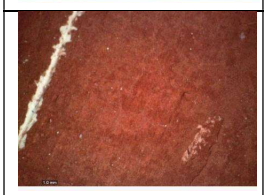
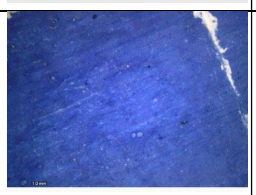
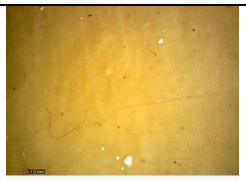
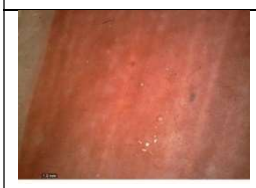
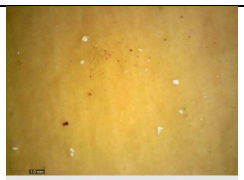
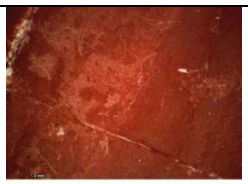
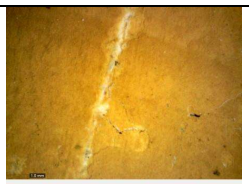
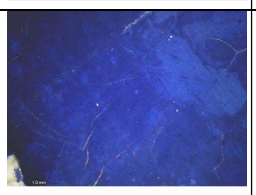
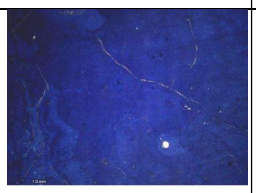
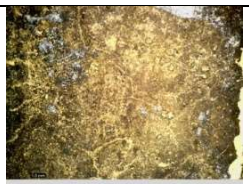
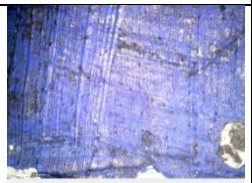
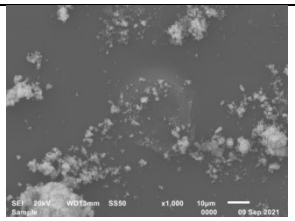
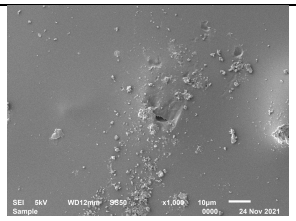
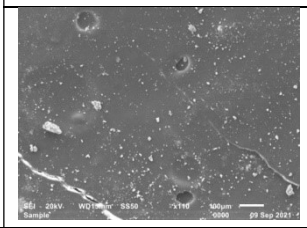
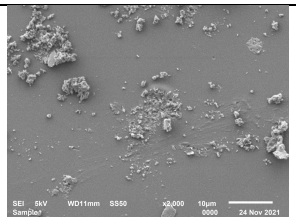
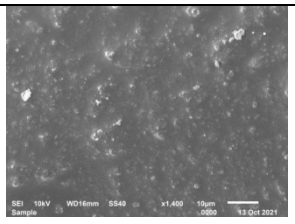
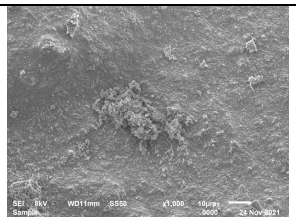
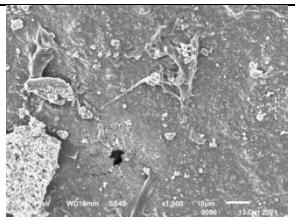
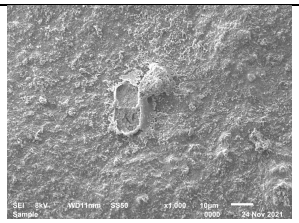
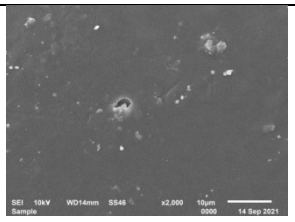
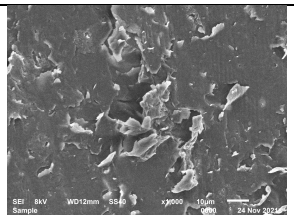
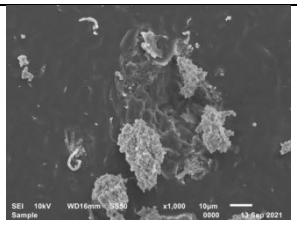
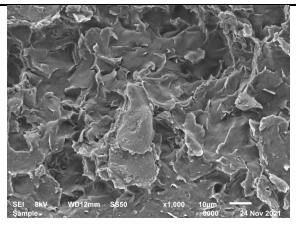
				
TDC (UV A and UV B)/ Dispersie Transparentă de Cazeină(UV A și UV B)				
				
Beeswax (UV-A and UV- B)/ Ceară de albine (UV-A și UV- B)				
				
Sunflower oil (UV-A and UV- B)/ ulei de floarea soarelui(UV-A și UV- B)/				
				
Soot (UV-A and UV- B)/ gudroane de cărbune (UV-A și UV- B)				
				

Table 6

Appearance of aged experimental models (I2) biocleaned with E-Agarart evaluated by SEM; line 1 – painted laboratory models covered by Paraloid B72 aged by UV-A, Control and biocleaned; line 2 – painted laboratory models covered by Paraloid B72 aged by UV-B, Control and biocleaned; line 3 – painted laboratory models covered by TDC aged by UV-A, Control and biocleaned; line 4 – painted laboratory models covered by TDC aged by UV-B, Control and biocleaned; line 5 – painted laboratory models covered by BW aged by UV-A, Control

and biocleaned; line 6 – painted laboratory models covered by BW aged by UV-B, Control and biocleaned; line 7 – painted laboratory models covered by SO aged by UV-A, Control and biocleaned; line 8 – painted laboratory models covered by SO aged by UV-B, Control and biocleaned; line 9 – painted laboratory models covered by S aged by UV-A, Control and biocleaned; line 10 – painted laboratory models covered by S aged by UV-B, Control and biocleaned;/ *Aspectul modelelor experimentale (I2) biocurățate cu E-Agarart evaluat prin examinare la SEM; rândul 1 - model experimental acoperit cu Paraloid B72 ,îmbătrânit cu UV-A, Control și biocurățat; rândul 2 - model experimental acoperit cu Paraloid B72 ,îmbătrânit cu UV-B, Control și biocurățat; rândul 3 - model experimental acoperit cu Dispersie Transparentă de Cazeină, îmbătrânit cu UV-A, Control și biocurățat; rândul 4- model experimental acoperit cu Dispersie Transparentă de Cazeină, îmbătrânit cu UV-B, Control și biocurățat; rândul 5 - model experimental acoperit cu ceară de albine, îmbătrânit cu UV-A, Control și biocurățat; rândul 6 - model experimental acoperit cu ceară de albine, îmbătrânit cu UV-B, Control și biocurățat; rândul 7- model experimental acoperit cu ulei de floarea soarelui, îmbătrânit cu UV-A, Control și biocurățat; rândul 8- model experimental acoperit cu ulei de floarea soarelui, îmbătrânit cu UV-B, Control și biocurățat; rândul 9- model experimental acoperit cu gudroane de cărbune, îmbătrânit cu UV-A, Control și biocurățat; rândul 9- model experimental acoperit cu gudroane de cărbune, îmbătrânit cu UV-B, Control și biocurățat.*

Deposit / Depunere	Control UV A/UV B / Control UV A/UV B	Biocleaned UV A/UVB / Biocurățat UV A/UVB
Paraloid B72 / Paraloid B72		
		
TDC / Dispersie Transparentă de Cazeină		
		
Beeswax / ceară de albine		
See next page		

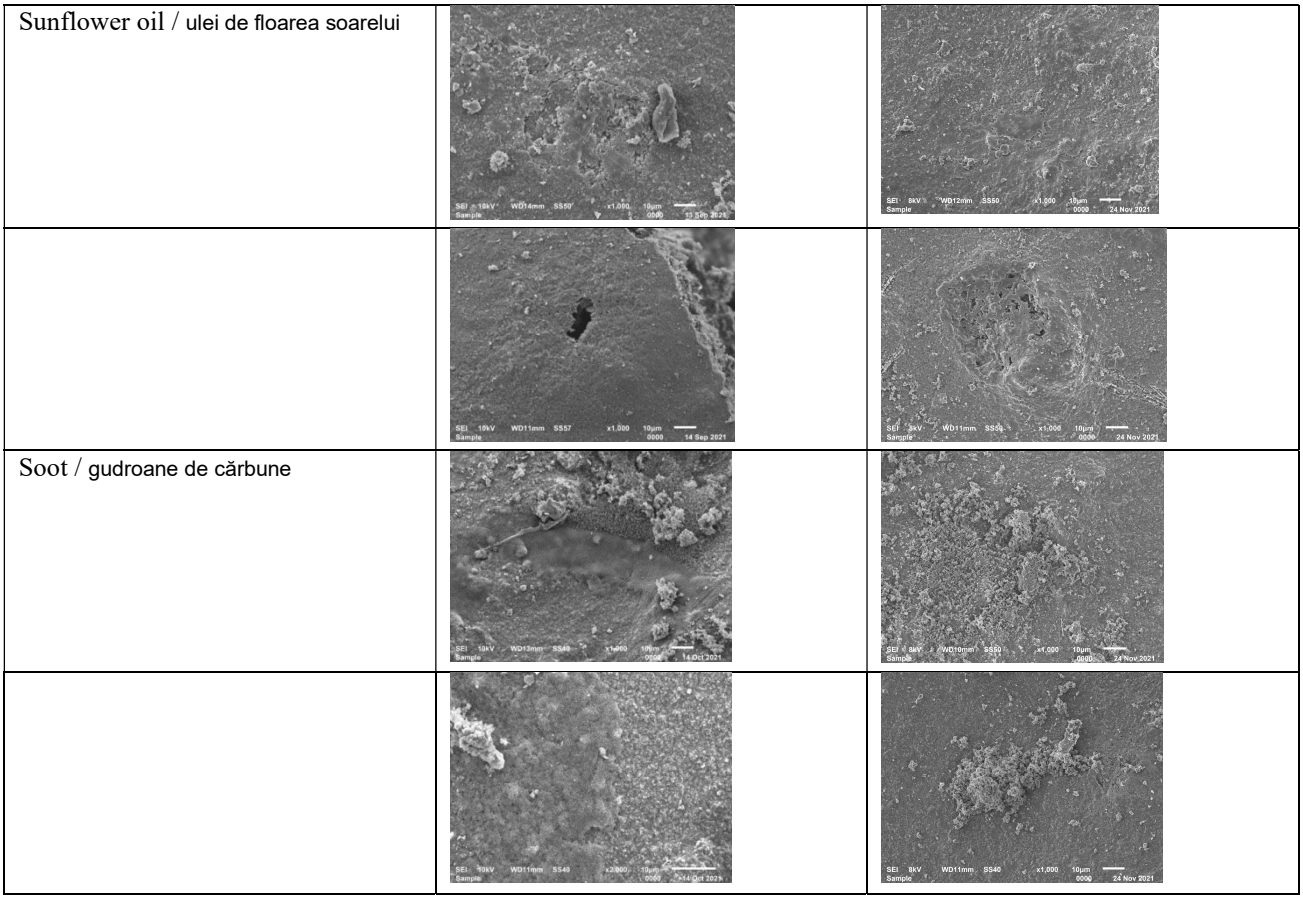


Fig.5-Fig.8. Evaluation of biocleaning efficiency of experimental models cleaned with E-Agarart and E+Agarart by colorimetry (I2). / *Evaluarea eficienței biocurățării modelelor experimentale cu E-Agarart și E+Agarart, prin colorimetrie (I2).*

4. Conclusion

Painted laboratory models in al fresco technique, esterolytic enzymes produced by *Bacillus* sp. BA NP3.3 and Agarart hydrogel have been obtained to establish a green method to remove consolidants and other organic deposits. In order to reduce, as much as possible, the research time, two aging methods were chosen: variation of the temperature and relative humidity (I1) and exposure to UV-A/UV-B radiation (I2). Microscopically, some morphological changes were highlighted. These were not confirmed by the colorimetric method due to their small size. Although biocleaning is achieved by applying of the esterolitic enzymes entrapped in Agarart gel (E-Agarart) or directly on the surface of organic deposit and then covered with Agarart gel (E+Agarart), to avoid the development of efflorescences, treatment with esterolytic enzymes integrated in Agarart is recommended. For increased efficiency, the optimal treatment time is 24 hours.

Acknowledgements

This research was funded by The Executive Agency for Higher Education, Research, Development and Innovation Funding (UEFISCDI), grant number 570PED/2020 and by The Romanian Academy, grant number RO1567-IBB05/2021.

REFERENCES

[I] Soffritti, M. D'Accolti, L. Lanzoni, A. Volta, M. Bisi, S. Mazzacane, E. Caselli, E. The potential use of microorganisms as restorative agents: an update. Sustainability, 2019, **11**(14), 3853.

[2] K. L. Gauri, L. Parks, J. Jaynes, R. Atlas, Removal of sulphated-crust from marble using sulphate-reducing bacteria. In Stone cleaning and the nature, soiling and decay mechanisms of stone: proceedings of the international conference held in Edinburgh, UK, 14-16 April 1992 (pp. 160-165).

[3] F. Cappitelli, E. Zanardini, G. Ranalli, E. Mello, D. Daffonchio, C. Sorlini, Improved methodology for bioremoval of black crusts on historical stone artworks by use of sulfate-reducing bacteria, Applied and Environmental Microbiology, 2006, **72**(5), 3733.

[4] F. Cappitelli, L. Toniolo, A. Sansonetti, D. Gulotta, G. Ranalli, E. Zanardini, C. Sorlini, Advantages of using microbial technology over traditional chemical technology in removal of black crusts from stone surfaces of historical monuments, Applied and environmental microbiology, 2007, **73**(17), 5671.

[5] G. Ranalli, M. Chiavarini, V.,Guidetti, F. Marsala, M. Matteini, E. Zanardini, C. Sorlini, The use of microorganisms for the removal of sulphates on artistic stoneworks. International biodeterioration & biodegradation, 1997, **40**(2-4), 255.

[6] A. F., Elhagrassy, A. Hakeem, A. F. Alhagrassy, Comparative study of biological cleaning and laser techniques for conservation of weathered stone in Failaka island, Kuwait. Sci. Cult, 2018, **4**, 43.

[7] P. B. Bosch-Roig, J. L. R. Ros, R. M. Estellés, Biocleaning of nitrate alterations on wall paintings by *Pseudomonas stutzeri*, International Biodeterioration & Biodegradation, 2013, **84**, 266.

[8] L. Rampazzi, A. Andreotti, M. Bressan, M. P. Colombini, C. Corti, O. Cuzman, G. Ranalli, An interdisciplinary approach to a knowledge-based restoration: The dark alteration on Matera Cathedral (Italy), Applied Surface Science, 2018, **458**, 529-539.

[9] I. Romano, M. Abbate, A. Poli, L. D'Orazio, Bio-cleaning of nitrate salt efflorescence on stone samples using extremophilic bacteria, Scientific reports, 2019, **9**(1), 1.

[10] E. Gioventù, P. F. Lorenzi, Valutazione dell'efficacia nell'utilizzo di microrganismi per la biorimozione delle croste nere dai materiali lapidei ed approfondimento delle metodologie applicative, OPD restauro, 2010, **22**, 127.

[11] F. Troiano, D. Gulotta, A. Balloi, A. Polo, L. Toniolo, E. Lombardi, F. Cappitelli, Successful combination of chemical and biological treatments for the cleaning of stone artworks, International Biodeterioration & Biodegradation, 2013 **85**, 294.

[12] G. Alfano, G. Lustrato, C. Belli, E. Zanardini, F. Cappitelli, E. Mello, G. Ranalli, G. The bioremoval of nitrate and sulfate alterations on artistic stonework: the case-study of Matera Cathedral after six years from the treatment. International Biodeterioration & Biodegradation, 2011, **65**(7), 1004.

[13] M. Mazzoni, C. Alisi, F. Tasso, A. Cecchini, P., Marconi, A. R., Sprocati, Laponite micro-packs for the selective cleaning of multiple coherent deposits on wall paintings: The case study of Casina Farnese on the Palatine Hill (Rome-Italy). International Biodeterioration & Biodegradation, 2014, **94**, 1-11.

[14] G. Ranalli, G. Alfano, C. Belli, G. Lustrato, M. P. Colombini, I. Bonaduce, C. Sorlini, Biotechnology applied to cultural heritage: biorestoration of frescoes using viable bacterial cells and enzymes. Journal of applied microbiology, 2005, **98**(1), 73.

[15] G. Lustrato, G. Alfano, A. Andreotti, M. P. Colombini, M. P, G. Ranalli, Fast biocleaning of mediaeval frescoes using viable bacterial cells. International Biodeterioration & Biodegradation, 2012, **69**, 51.

[16] M. D. P. Bosch Roig, J. L. Regidor Ros, M.P. Soriano Sancho, R. M. Montes Estellés, Biocleaning of animal glue on wall paintings by Pseudomonas stutzeri. Chimica Oggi–Chemistry Today, 2013, **31**(1), 50.

[17] G. Ranalli, E. Zanardini, A. Andreotti, M. P. Colombini, C. Corti, C., M. D. P. Bosch-Roig, D. Zari, Hi-tech restoration by two-steps biocleaning process of Triumph of Death fresco at the Camposanto Monumental Cemetery (Pisa, Italy). Journal of applied microbiology, 2018, **125**(3), 800.

[18] G. Ranalli, E. Zanardini, L. Rampazzi, C. Corti, A. Andreotti, M. P. Colombini, P. Virilli, Onsite advanced biocleaning system for historical wall paintings using new agar-gauze bacteria gel. Journal of applied microbiology, 2019, **126**(6), 1785.

[19] A. Byrne, Wolbers Cleaning Methods: Introduction, AICCM Bulletin, 1991, **17**(3-4), 3.

[20] P. Bosch-Roig, G. Lustrato, E. Zanardini, G. Ranalli, Biocleaning of Cultural Heritage stone surfaces and frescoes: which delivery system can be the most appropriate?, Annals of Microbiology, 2015, **65**(3), 1227.

[21] T. Giraud, A. Gomez, S. Lemoine, C. Pelé-Meziani, A. Raimon, E. Guilminot, Use of gels for the cleaning of archaeological metals. Case study of silver-plated copper alloy coins, Journal of Cultural Heritage, 2021, **52**, 73.

[22] G. Barresi, E. Di Carlo, M. R. Trapani, M. G. Parisi, C. Chille, C., M. F. Mule, F. Palla, Marine organisms as source of bioactive molecules applied in restoration projects, Heritage Science, 2015, **3**(1), 1.

[23] F. Palla, Bioactive molecules: innovative contributions of biotechnology to the restoration of cultural heritage. Conservation Science in Cultural Heritage, 2013, **13**, 369.

[24] F. Palla, V. Rotolo, A. Giordano, A., Biotechnology a source of knowledge in agreement with green strategies for the conservation of cultural assets, Conservation Science in Cultural Heritage, 2019, **19**, 69-80.

[25] N. Bevilacqua, CTS Pure Artis's Pigments; 2010;

[26] R. Ruginescu, M. Enache, O. Popescu, I. Gomoiu, R. Cojoc, C. Batrinescu-Moteau, C.; M. Dumbravician, S. Neagu, Characterization of Some Salt-Tolerant Bacterial Hydrolases with Potential Utility in Cultural Heritage Bio-Cleaning. Microorganisms, 2022, **10**, doi:10.3390/microorganisms10030644.

[27] R. Ruginescu, I. Gomoiu, O. Popescu, R. Cojoc, S. Neagu, I. Lucaci, I. Batrinescu-Moteau, C., M. Enache, Bioprospecting for Novel Halophilic and Halotolerant Sources of Hydrolytic Enzymes in Brackish, Saline and Hypersaline Lakes of Romania. Microorganisms, 2020, 8, 1903, doi:10.3390/microorganisms8121903.

[28] R. Cojoc, M. Enache, S. Neagu, M. Lungulescu, R. Setnescu, R. Ruginescu, I. Gomoiu, Carotenoids Produced by Halophilic Bacterial Strains on Mural Paintings and Laboratory Conditions. FEMS Microbiology Letters, 2019, **366**, fnz243, doi:10.1093/femsle/fnz243.

[29] H.E. Ahmed, F. N. Kolisis, An Investigation into the Removal of Starch Paste Adhesives from Historical Textiles by Using the Enzyme α-Amylase, Journal of Cultural Heritage, 2011, **12**, 169.

[30] E. Gioventù, P. Lorenzi, Bio-Removal of Black Crust from Marble Surface: Comparison with Traditional Methodologies and Application on a Sculpture from the Florence's English Cemetery. Procedia Chemistry, 2013, **8**, 123.

****