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DISCOLORATION OF ANCIENT EGYPTIAN MURAL PAINTINGS BY STREPTOMYCES STRAINS AND METHODS OF ITS REMOVAL

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Abstract

Streptomyces isolated from mural paintings at Tell Basta and Tanis tombs were identified using 16S rDNA sequencing method. These Streptomyces strains caused discoloration of mural paintings with irreversible red stains of carotenoid pigment. A mixture of n-hexan and acetone (92:8 v/v) was the best solvent for extracting and purification of red pigment from biomass of Streptomyces. Dimethyl sulfoxide (DMSO) and N,N-dimethylformamide (DMF) were the most effective in treatment of these red stains without changing the paintings or stone surfaces.

Keywords: *Carotenoid pigment; Dimethyl sulfoxide; N,N-dimethylformamide; Melanin pigment; Streptomyces; Tanis; Tell Basta*

Introduction

Streptomyces were the most dominant in colonizing deteriorated mural paintings and stone surfaces in hypogean environments, such as caves, grottos and tombs and the presence of *Streptomyces* is an indicator of advanced phase of deterioration [1].

The DNA-based methods such as PCR and 16S rDNA sequencing are promising methods in identification of *Streptomyces* colonizing mural paintings due to the problems caused by the morphological identification methods [2].

Understanding the accurate nature of biopigments produced by *Streptomyces* colonizing paintings and stone surfaces is important for the development of correct conservation and restoration strategies and for choosing the most appropriate solvent for the treatment of these biogenic pigments [3].

Discoloration of paintings and stone surfaces is one of the most complex problems encountered by the conservators and the microbiologists in the field of archaeology, because the stains discoloring paintings and stone surfaces are irreversible stains. Gurtner et al. [4] reported that wall paintings in Eilsum, Germany, were stained with rosy stains of carotenoid pigment whereas *Arthrobacter agilis* and *Nocardia corynebacteroides* were involved in causing the irreversible damage and hiding of these paintings.

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Discoloration of mural paintings with red pigment produced by *Streptomyces* was put into the evidence due to the ability of *Streptomyces* to produce two types of biopigments of melanin (brown-black group) and carotenoid (red-violet-pink and yellow pigment group) as metabolites which cause irreversible disfiguration of paintings and plaster layers, especially if these pigments are exolithic in nature [4-6, 8, 9]. In general, *Streptomyces* strains produce three types of carotenoid are *β*-carotenoid ($C_{4O}H_{54}O_2$), γ-carotenoids ($C_{40}H_{54}O_2$) and rhodoxanthin ($C_{4O}H_{54}O_2$) [10].

Groth and Saiz–Jimenez, [11] delineated that *Streptomyces* sp. were involved in the discoloration of the walls of the Circular Mausoleum, Carmona Necropolis, Spain, by producing violet pigments. Moreover, Urzi, [12] reported that *Streptomyces*, *Micromonospora* and *Geodermatophilus* colonizing marble slabs of the palace of Noto, Silicie, caused red stains of carotenoid pigment and found out that under laboratory conditions the production of this pigment should depend on the age of the strains, where pigment production was increased with the increasing age of the *Streptomyces* strains, media components and climate conditions.

Discoloration of paintings with rosy stains of carotenoids pigment was well documented in several instances such as Etruscan tombs, Italy [11], where paintings were scalled in form of red powder [13], also red stains in Luca Signorelli fresco paintings in St. Brizio Chapel (Orvieto Cathedral, Italy) were attributed to biological alteration [14]. The *Streptomyces, Micromonospora* and *Saccharothrix* were the most frequently isolated from paintings in Dona Trinidad cave (Spain), where litho paintings were stained with red pigment. Moreover, *Streptomycetes* involved in chromatic alteration of Necrópolis paintings of Carmona (Sevilla, Spain), where *Streptomyces* sp. produced violet pigment in cultures. On the other hand, paintings in Castle of Herberstein (Austeria) were disfigured by the growth and colonization of *Microbacterium aerolatum* sp. which formed yellow-pigmented colonies [4], whereas formation of rosy powders on fresco paintings is a well-known form of alteration [14]. Furthermore, *Streptomyces* strains caused disfiguration of paintings in Mayan mural paintings, Mexico, by forming a reddish biofilm containing carotenoid pigment [5].

Moreover, paintings in Orvieto Cathedral (Italy) showed different forms of deterioration such as chromatic alterations on both the stonework and the fresco paintings, also red stains found on the marble statues of the fountain of Villa Litta and on the marble façade of the Certosa of Pavia composed mainly of carotenoid pigment[14].

Streptomyces strains isolated from sandstone and mural paintings in Arbroath Abbey, Scottalnd, were attributed to *S. cirratus lavidinii*, *S. candidus, S. microflavus* and *S. vulgaris* and caused staining of these paintings and stone surfaces with red-brown stains [15].

However, *Streptomyces* strains may not produce carotenoid pigment under normal growth conditions, but could produce this pigment as a defense mechanism against adverse environmental conditions of hypersalinity, heavy metals, biocides, UV light and starvation, so these pigments are called protective pigments [16].

The role of red pigment produced by *Streptomyces* in disfiguration of Ancient Egyptian mural paintings is underestimated, although it was well documented that *Streptomyces* sp., *Micrococcus* and *Arthrobacter* disfigured mural paintings in the Abydos Temple, the tomb of the King Tutankhamen and the tomb of of Queen Nefertari, Upper Egypt, with red and brown stains and the colonized paintings were discolored and flaked off [17].

Several attempts had been done to remove biogenic stains produced by *Streptomyces* strains chemically using organic solvents. Szezepanowska and Lovett, [18] used Dimethyl sulfoxide (DMSO), 1,4-dioxan, N, N-dimethylformamide (DMF) and pyridine to remove the yellow stains of anthraquinones pigments produced by different fungi, they found that 1,4-

dioxan gave good results, but produces harmful vapors and flammable liquid and cause irritations of the skin and eye.

Isolation and identification of *Streptomyces* strains causing discoloration of stone surfaces with red pigment and removal of this pigmented stains will be described here.

Materials and Methods

Sampling

Three out of forty six isolates producing red pigment under normal and abnormal conditions of hypersalinity were taken from tombs of Ankh h3f, Ankh m b3st, (Tell Basta, 2420 B.C.) and tomb of Oserkon II, Tanis (Fig. 1), using sterile cotton swab method. Samples were cultivated and plated onto starch-nitrate-agar (SNA) plates (agar 20, starch 20, KH_2PO_4 1, $MgSO_4$ 0.5, NaCl 0.5, KNO_3 2 & $CaCO_3$ 3 g/L distilled water) supplemented with antifungal (Dermatine 10-50 μg / l) to inhibit the growth of competitive fungi. Plates were incubated for 45 days at 30°C. Bacterial isolates were identified morphologically and biochemically using identification keys of Kämpfer [19] and confirmed by 16S rDNA sequencing method. These isolates produced red pigment on the synthetic media.

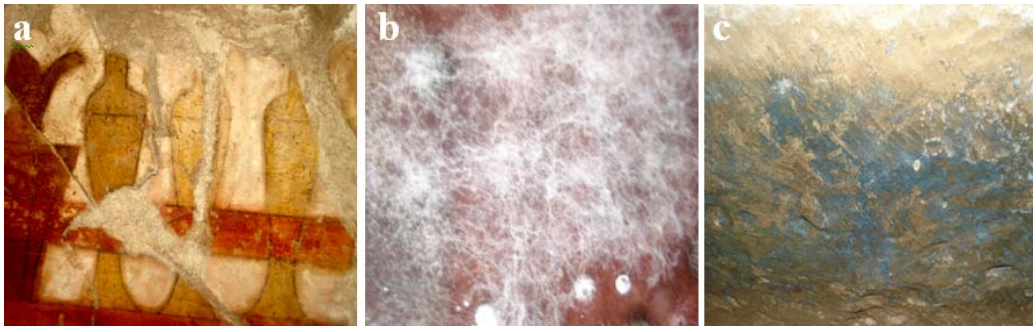

Fig. 1. Location of sampling: a - Red and yellow paintings, tomb Ankh h3 f, Tell Basta;
b - Red ochre, tomb Ankh m b3st; c - Azurite blue in tomb of Oserkon II with white crust, Tanis.

16S rDNA sequencing

The sequences of 16S rDNA s were determined by Polymerase Chain Reaction (PCR) as following: The DNA template for PCR was extracted from eight isolated colonies of actinobacteria [20]. The amplified 16S rRNA gene was obtained from each isolate by PCR with universal primers (forward primer [F27] 5'-AGAGTTTGATCCTGGCTCAG-3' [21] and reverse primer [R1492] 5'-GGTTACCTTGTTACGACTT-3') [21] which are targeted to universally conserved regions and permit the amplification of an approximately 1500 bp fragment. The PCR amplification was carried out in a Gene-Amp PCR system 9600 thermocycler (Perkin Elmer). The presence and yield of specific PCR product (16S rRNA gene) was monitored by 1% agarose. Then PCR product was cleaned up by using GeneJET™ PCR Purification Kit (Fermentas).

PCR product obtained from three *Streptomyces* strains produced red pigment with F27 and R1492 primers, and then partially sequenced using GATC German Company by using ABI 3730xl DNA sequencer using forward primer (F27). The 16S rDNA sequences which have been determined in the present study were deposited at NCBI web server (www.ncbi.nlm.nih.gov;

Table 1); through Basic Local Alignment Search Tool (BLAST) program (http://www.ncbi.nlm.nih.gov/blast) [22].

Table 1. Sampling location and *Streptomyces* identification

Streptomyces isolates	Location	Author' accession number	G+C content (%)	Observation
S. canaries	Blue color, ceiling burial tomb of Oserkon II, Tanis.	BankIt1507650 JQ625337	58.9	White efflorescence over the blue pigment, ceiling of burial chamber of the king Oserkon II; Tanis.
S. coelicolor	Red color, tomb Ankh m b3st, Tell Basta.	BankIt 1507648 JQ 625335	56.7	The red color is hematite pigment, spotting with dark spots
S. parvullus	Yellow color of southern wall of Ankh h3 f tomb	BankIt 1507645 JQ625333	59.2	The yellow color is limonite pigment

Discoloration of stone support and plaster layers

To investigate the discoloration of the stone support and plaster layers in mural paintings, the following procedures were carried out: Gypsum discs (d. 7 cm) were put into beakers 500 ml, each contains 50 ml of distilled water left in the bottom of the flask, then immersed for 24 h to saturate them with humidity before inoculation. Water agar (WA) medium was prepared from 25g agar in 1 L distilled water. A thin layer of WA was poured on plaster discs, then inoculated with *Streptomyces* strains isolated from deteriorated mural paintings. These discs were incubated at 30 °C for five months.

Effect of soduim chlorid on the production of red pigment

To investigate the effect of NaCl on the production of carotenoid pigment, *S. canaries* was cultured on SNA medium supplemented with different concentrations of NaCl (2.5, 5, 10 %). This strain was isolated from white effloresce of NaCl, tomb of Oserkon II, Tanis.

Extraction of red pigment

S. parvullus and *S. coelicolor* strains were cultured on broth starch-nitrate-agar meduim, incubated at 30 °C for 14 days. Red pigment was extracted according to Sterflinger, [10] using a mixture of *n*-hexane and acetone (92/8 v/v). 1 ml of filtrate was analyzed by JASCo. FT / IR 61000, (National Research Centre, Dokky, Giza).

Removal of red staining

Spots of red pigment on the stone surfaces were removed by using different solvents such as 1,4-dioxan, dimethyl sulfoxide (DMSO), ethanol, diethyl ether, pyridine, N,N-dimethylformamide (DMF), xylen, aceton and *n*-hexan in form of pultice of cotton on limestone blocks for different periods.

Results

The current results of 16S rDNA sequencing of the three bacterial isolates gave higher similarity to *S. parvullus, S. coelicolor* and *S. canarius* whereas accession numbers of the authors in the International Gen Banck shown in Table 1.

The current results indicated that *Streptomyces* strains produced red pigment and this pigment was exolithic in nature and caused discolorization of gypsum discs with irreversible stains (Fig. 2).

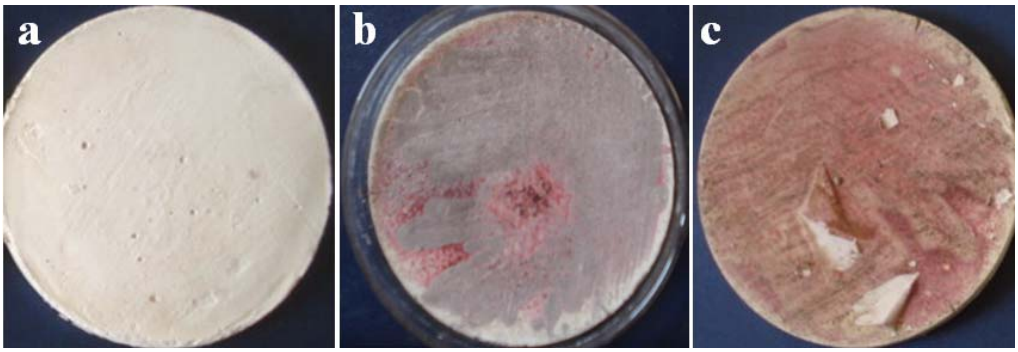

Fig. 2. Discoloration of plaster layer by the produced red pigment:
a – control, b - *S. parvullus*, c - *S. coelicolor* on stone discs.

Our finding indicated that *S. canaries* produced red pigment of carotenoid under hypersalinity of NaCl up to 10 % in comparison with normal growth conditions (Fig. 3), as this salt is the most common occurring in the Egyptian monuments.

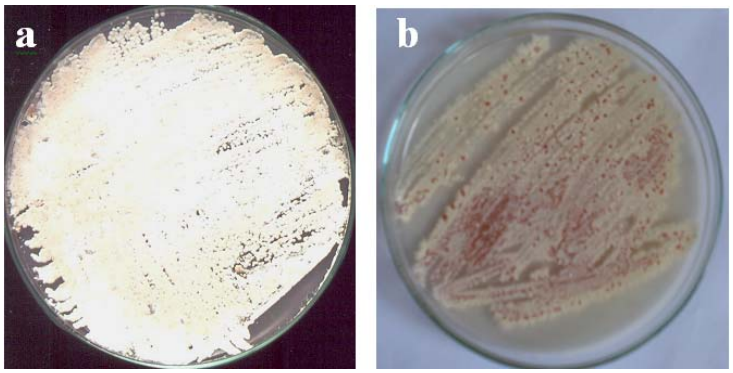

Fig. 3. Production of carotenoid pigment by *S. canrius*:
a - normal conditions, b - under hypersalinity of NaCl.

The current results indicated that a mixture of *n*-hexane and acetone (92/8 v/v) was the most suitable solvent for extracting the red pigment produced by *Streptomyces* strains and for purification of this red pigment on silica gel plates. The extracted pigment was non pH sensitive. The IR pattern of extracted red pigment gave an intense band at 3457 cm^{-1} (Fig. 4), indicated to the presence of quinonoxime (ON-O-R) group characterizing for carotenoid, so we can determine that this red pigment is carotenoid pigment.

With regard to removing the red pigment stains, the obtained results indicated that dimethyl sulfoxide (DMSO) removed the red stains produced by *S. coelicor* sucessfully except the patina layer on stone surface but N,N-dimethylformamide (DMF), 1,4-dioxan, n-hexan and ethanol had a moderate effect, whereas *n*-Hexan, aceton and xylen gave unsatisfactory results (Fig. 5 and 6). DMSO and DMF did not affect on paintings, and the effects of the used organic solvents are presented in Table 2.

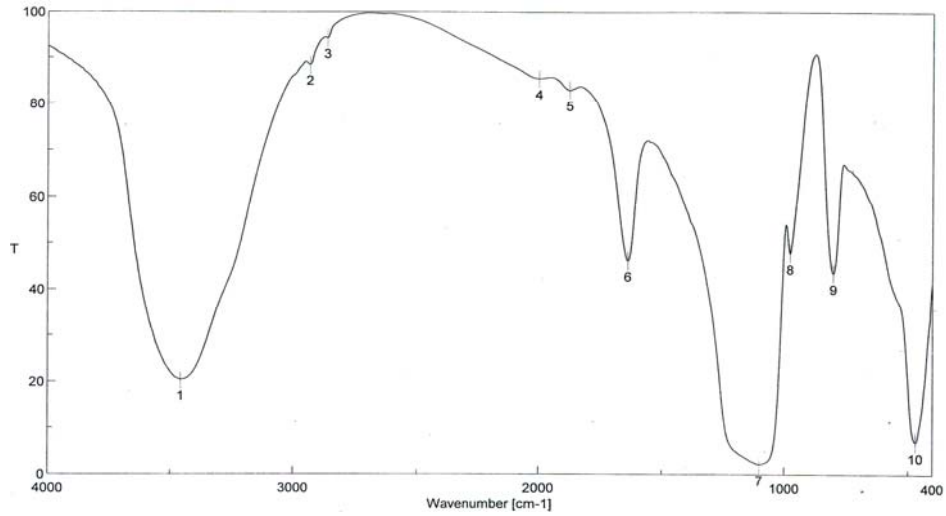

Fig. 4. IR pattern for carotenoid pigment produced by *S. parvullus*

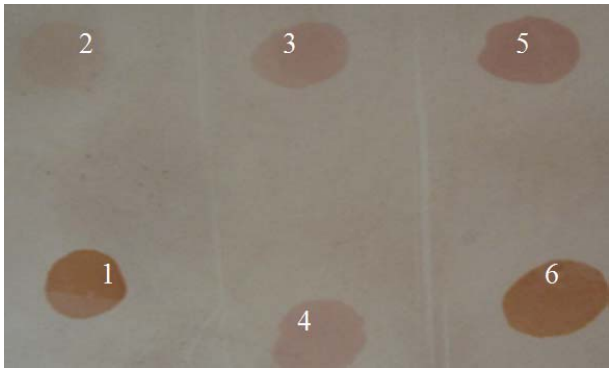

Fig. **5**. Removal of red stains produced by *S. parvullus* using different solvents:
1 - control, 2 - dimethyl formamide, 3 - Ethanol, 4 - doxian, 5 - Diethyl ether, 6 - *n*-Hexan.

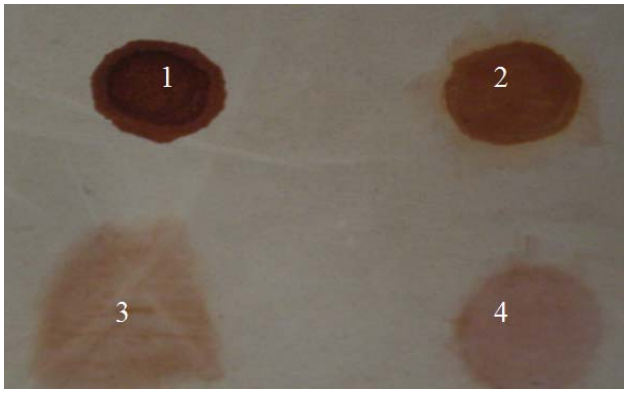

Fig. 6. Removal of red stains produced by *S. parvullus* using different solvents:
1 - Control, 2 - Xylen, 3 - Dimethylformamide, 4 - Pyridine.

Table 2. Removal of carnotenoid pigment by different organic solvents

Organic solvent	Removability
Dimethyl sulfoxide DMSO	++++
N, N-dimethylformamide DMF	+++
Pyridine	++
1,4-Dioxan	++
n-Hexan	++
Xylen	+
Ethanol	++
Diethylether	++

Discussions

The 16S rDNA data gave higher similarity to *Streptomyces* strains, whereas *Streptomyces* were the most dominant among associated microorganisms in deteriorated mural paintings and stone surface and considered an indicator for advanced phase of deterioration. Also *Streptomyces* considered the first and the typical colonizer for deteriorated cultural heritage objects [24]. Sequence analysis of the 16S rDNA proved to be a powerful tool for further characterization of isolates culturable or non-culturable bacteria of defined taxonomic groups, because using 16S rDNA sequence gave a surprising number of bacteria obtained and identified from microbial communities colonizing wall paintings [2].

The colonizing of stone surface and mural paintings caused irreversible pigmented stains, whereas *Streptomyces* strains produced two different groups of pigments were brown of melanin pigment and red of carotenoid pigment. The current results indicated that red pigment was carotenoid pigment, since carotenoids ($C_{40}H_{50}$) are a group of pigments naturally occuring widely abundant in plant kingdom and include three types: *ß*-carotene, γ-carotene, and rhodoxanthin.

The obtained results indicated that biopigments production should depend on several factors such as age of colonies where the production was increased with the age of *Streptomyces* strains, climate conditions, composition of media, presence of heavy metals and hypersalnity whereas presence of heavy metals and hypersalnity induced production of pigments as a defence mechanism against these adverse environmental conditions [23, 24].

Red pigment of carotenoid produced by *Streptpmyces* strains isolated from paintings in some Ancient Egyptian tombs caused irreversible stains of paintings and chromatic alteration to stone surface and plaster layers, whereas *Streptomyces* strains were involved in chromatic alteration of stone surfaces and fresco paintings of Orvieto Cathedral (Italy) [14] and stone surfaces at Noto, Sicily were discolorized with pale orange of carotenoid pigment produced by actinobacteria [25, 26].

Due to undesirable effects, the stains of *Streptpmyces* colonizing paintings and stone surfaces must be removed. Our results indicated that dimethyl sulfoxide (DMSO) and N,N-dimethylformamide (DMF) removed the red stains produced by *S. coelicor* and *S. parvullus* sucessfully, whereas Szezepanowska and Lovett, [18] used dimethyl sulfoxide (DMSO), 1,4-

dioxan, N, N-dimethylformamide (DMF), and pyridine for removal of yellow stains of anthraquinones pigments produced by different fungi and found that 1,4-dioxan gave good results.

Dimethyl sulfoxide (DMSO) is an organosulfur compound having two C-S bonds in its molecular structure and characterized with high polarity and the ability of DMSO and DMF to remove red stains produced by *Streptomyces* from stone and plaster layers was attributed to this high polarity [27], but 1,4-dioxan and N, N-dimethylformamide (DMF) produced harmful vapours and irritated the skin, lung, eyes and mucous membranes. Moreover, 1,4-dioxan is a flammable solvent.

Conclusions

The three *Streptomyces* strains isolated from some Ancient Egyptian tombs were identified using 16S rDNA sequencing method and these strains produced red pigment of carotenoid under normal and hypersalinity conditions. The obtained results indicated that dimethyl sulfoxide (DMSO) and N,N-dimethylformamide (DMF) could remove the red stains from stone surfaces and plaster layers produced by *Streptomyces* strains.

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