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APPLIED MICROBIOLOGY AND BIOTECHNOLOGY, 101 (18), 2017,pp: 7041–7052
DOI: 10.1007/s00253-017-8437-8

Enzymatic laundry for old clothes: immobilized alpha-amylase from *Bacillus sp.* for the biocleaning of an ancient Coptic tunic

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Abstract

The classification and conservation of ancient art-works (belonging to collections) is of important cultural, historical and economic concern. However, ancient textiles often display structural damage that renders them fragile and unsuitable for exhibition. One of the most common types of damage is linked to erroneous restoration treatments, among which the application of glues to consolidate cuts. Harsh strategies, such as mechanical or chemical treatments, are not suitable since they can cause further impairment of the fabric, whereas mild approaches, like wet cleaning, are often ineffective, as also demonstrated by the present study. Here, we have explored the possibility of using gellan-immobilized enzymes of bacterial origin (*Bacillus* alpha-amylase) to obtain a satisfactory starch removal from a damaged archaeological tunic-shroud from the Turin Egyptian Museum (Italy), without altering the original yarns or textile fibers. This method, already applied to clean casein-damaged wall paintings, as well as cotton, silk and linen fabrics, has proved to be optimal for the treatment of a wool burial shroud and to be able to definitively solve fragile textile restoration problems. Moreover, efforts have been made to obtain insights into the artwork: a multidisciplinary approach has allowed to obtain a correct chronological attribution (radiocarbon dating) and fabric fiber characterization (SEM-EDX) as well as shed light on the colored parts and dark stains (FORS+ IR-FC and XRF). Finally, the evaluation of the type of glue, by Fourier transform infrared spectroscopy, has suggested the best enzyme for glue removal. These results have demonstrated that a mild bio-based approach is a successful tool for the treatment of archaeological textiles in critical conditions.

Key words**: alpha-amylases, gellan-immobilized bacterial enzymes, biorestoration, archaeological textile, SEM-EDX, FT-IR.**

Introduction

Cultural heritage conservation is a crucial concern, especially in Italy where it constitutes an important historical and economic value. Several environmental factors (*e.g.*, weathering, microorganisms) as well as erroneous preservation attempts can contribute to the deterioration of artworks (Beutel et al. 2002; Sterflinger and Pinzari 2012). Unfortunately, recovering damaged artworks through traditional approaches (washing, wet cleaning, sewing, mechanical removal) has some critical aspects, since they risk further impairing the structure of the item, and, in the case of fabric, disrupting the fibers (Ahmed and Kolisis 2011).

Bio-restoration is a relatively recent mild strategy that enables the original status of an artwork to be recovered or partially restored. The use of *in toto* bacteria for both bio-mineralization (Dhami et al. 2014) and bio-cleaning (Ranalli et al. 2005) has been experimented on outdoor monuments and frescoes, respectively. On the other hand, the employment of purified enzymes appears very interesting. Actually, some erroneous treatments, performed with the aim of protecting or consolidating art-works, often use natural products, such as proteins or polysaccharides, both of which are sensitive to the action of hydrolytic enzymes (Gupta et al., 2002). Casein is employed on frescoes (Beutel et al. 2002), while collagen and starch are used to paste and consolidate degraded cellulose materials (books, ancient documents and textiles) (De La Chapelle et al. 1994).

The enzymatic approach (Cremonesi 2009) was suggested for paper conservation purposes (DeSantis 1983, Sandrine, 2002), and successfully employed to clean outdoor stone monuments (Christopher 1999), to treat casein-damaged medieval paintings (Beutel et al. 2002), glue-damaged paper (Banik et al. 1999; Corbi et al. 2005; Cremonesi et al. 2003) and textiles, mainly linen, silk and cotton fabrics (Ahmed and Kolisis 2011; Conti et al. 2010). The glues used for textile restoration are generally obtained using starch (Ahmed and Kolisis 2011; Whaap 2007). In this context, water-dissolved alpha amylase preparations have been applied locally either in solution (Ahmed and Kolisis 2011) or as poultice (Bott 1990; Chapman 1986; Shibayama and Eastop, 1996). However, the application of enzymes in solution is not always suitable for art-works, since it can cause further damage by flooding the fabric with excess water. This creates favorable conditions for mold and fungi colonization and growth. In addition, water treatments are necessary to remove the excess enzyme from textile samples, as referred by Ahmed and Kolisis (2011). For

all these reasons, the use of immobilized enzymes is preferable. Theoretically, all enzymes can be immobilized, but both the immobilization yield and the enzyme efficiency should be determined beforehand, to limit the loss of enzyme and catalytic activity. Moreover, the choice of the most suitable enzyme requires time-consuming tests, due to the rigorous parameters needed for catalysis (*e.g.*, optimum ionic force, temperature and pH). These features partly depend on the organism that produces the enzyme. The main sources of alpha amylases are pancreatic extracts of animals, cereal seeds and microorganisms.

In the present work, a damaged archaeological tunic-shroud, belonging to the Egyptian Museum of Torino (Italy, see supplementary material) (INV.17490, see supplementary material) (Fig. 1), that had been treated with glue in a previous restoration intervention during the 1950s in order to consolidate it, has been investigated. This adhesive material decreased the natural flexibility of the wool fibers and promoted tension inside the texture, especially in the case of bad handling (see supplementary material). The poor textile conditions rendered it fragile and unsuitable for exhibition.

The tunic that has been the subject of the present investigation dates back to the so-called Coptic period (or Byzantine period) of the Egyptian Christian culture. This refers the time interval between the division of the Roman Empire (A.D. 395) and the defeat of the Byzantine Empire during the Muslim invasion (A.D. 641). The term "*copt*" (derived from the Arab word "Qibt"=Egyptian) refers to native Egyptians, as opposed to Greek or Arab invaders. Although the Muslim defeat of Byzantium introduced Islam as well as the Arabic culture as the dominant influence, the Egyptian Coptic culture has been preserved until the present day. Traditionally, the chronological attribution of Coptic tissues was based on historical/stylistic features. For instance, the Egyptologist Du Bourguet (1910-1988), editor of the Catalogue of Coptic textiles hosted in the Louvre Museum (Du Bourguet 1964), allocated fragments of fabrics similar to the here studied tunic to the 10-12th centuries, that is, at the height of the Arab period. However, this ascribed chronology, based on the type of decoration, form, color scheme and technique of execution, has proved to be erroneous (Strydonck et al. 2004, Strydonck and Benazeth 2014). After the study of Du Bourguet, other authors have dated textiles without bothering to check the sources in a sort of "*domino effect*" that has sometimes led to patently erroneous results (Baginski and Tidhar 1980; Donadoni Roveri 1983; Peter 1976; Rutschowscaya 2000; Stauffer and Schmidt-Colinet 1991; Trilling 1982). For these reasons, a reconsideration of the dating of these artifacts, through

scientific approaches, is necessary. On the basis of the decoration motifs (*i.e.*, the valuable technique of the tapestry, the flying shuttle and the great attention to detail), the tunic has since been classified as an everyday "dress of life". However, the bad state of conservation also suggested that the tunic could be "dress of death" (*i.e.*, burial clothes). The origin of the tunic has been found to be related to Christian Egyptian burials. Following the spread of the Christian religion, Egyptians changed their funerary practices (see supplementary materials). The ancient mummification ritual was gradually abandoned, and the dead were buried in their own clothes (*i.e.*, tunic-shrouds) (Fig. S1).

Therefore, the present investigation was aimed at characterizing (*e.g.*, dating and better defining the use of the textile in the Coptic period) and restoring this art-work through a multidisciplinary experimental strategy.

Materials and Methods

Analytical procedures

Textile dating by means of* ^{14}C *analysis. Artwork dating based on artistic features has proved to be unreliable, as previously reported. ^{14}C dating is commonly used by art historians to confirm the ascribed chronology of art-works. One of the most important aspects of ^{14}C dating is the sampling strategy. In the present case, two samples of about 15 mg of wool fibers were collected. The sampling areas were selected according to three requirements: i) they had to be in good state of conservation ; ii) they had to be coeval, non-woven and far from the materials of the old restorations; iii) there had to be an absence of glue residues, which would have required a more aggressive strategy for the chemical pre-treatment of the sample, with a consequent greater loss in mass. Sampling was done in agreement with and under the guidance of the conservator of the tunic, as well as scholars involved in the historical and/or archaeological problem. In addition, several threads were collected in order to reduce ambiguity. The samples were pre-treated with an Acid-base-Acid procedure (incubation in 1M HCl at a temperature of about 80°C for 1 hr, incubation in 0.1M NaOH at room temperature for 10 min, incubation in 1M HCl at a temperature of about 80°C for 1 hr and drying in an oven at 100°C for about 12 hr). The samples were then analyzed accurately using the line of Accelerator Mass Spectrometry installed at the LABEC-

INFN Tandem accelerator in Florence (Italy) by means of the method reported by Fedi et al. (2007).

Thread analysis. Scanning electron microscopy-EDX (SEM-EDX) was employed to investigate the morphology, composition and state of conservation/deterioration of the textile fibers. Microsamples were harvested in correspondence to organic stains and were then observed, without any further treatment, using a scanning electron microscope (ZeissEVO60, variable pressure mode) for the morphological investigations of the yarns. The microprobe (Oxford EDX elemental analysis Penta FET) was used to obtain semi-quantitative analysis, which provided compositional information on the chemical elements present in the shroud.

Dye analysis. The shroud is decorated with red, green, yellow and purple squares and bands (see supplementary material). The dyes used for these parts of the tunic were analyzed by both false color infrared reflectography (IRFC) and fiber optic reflectance spectroscopy (FORS). These techniques, although not suitable for very pale colors, enabled an unambiguous identification of the wool fiber dyes to be obtained. By combining a visible picture with an infrared reflectography image (750-950 nm), an IRFC image can be achieved: an IRFC image provides an overview of chemical differences in materials and allows the different spectral behaviors of the pictorial and dyeing materials to be enhanced with respect to the near infrared region. Infrared reflectography images were acquired by a Fuji IS PRO IrUv camera, equipped with two filters (infrared Peca 914 and visible Peca 916), two Ianiro 800w diffused-light spotlights and a Macbeth color checker. FORS is a molecular, punctual and non-invasive procedure that is used for the characterization of pigments and dyes. Through the use of optical fibers and a halogen lamp (HL-2000-FHSA, Ocean Optics), the apparatus (spectrometer Ocean Optics HR2000+ES), used with optical geometry measurement (CIE) 2x45°/ 0°, provides the reflectance spectrum of the area under investigation (about 3 mm diameter), in the range between 350 nm and 900 nm (extensible to 1000 nm) with a 0.5 nm step resolution. The conducted FORS analyses were supported by investigations in multispectral false color infrared at 950 nm (950 nm IR-FC).

Stain analysis. Dark stains were present on the shroud. Their chemical composition was analyzed by means of X-ray induced fluorescence (XRF)**.** XRF is an accurate, non-invasive technique that allows the chemical elements present in the measurement area (0.65 mm -1.5 mm Ø) to be identified. Both the surface and the underlying layers can be analyzed to a depth of about 250 pm (depending on the atomic weight of the chemical elements themselves). Following atomic

excitation with suitable energy, the surveyed area emits the so-called X-ray fluorescence characteristic, which is revealed, as a function of its energy (energy dispersive XRF, EDXRF), by means of a solid state detector, which allows all the elements detectable in a single measure to be identified. By flushing the measurement area with Helium, the apparatus can detect even very light chemical elements (with an atomic number higher than 11, *i.e.* Sodium). The analyses were carried out by purging Helium, in order to improve the detecting threshold. The used system is a portable spectrometer (Bruker ARTAX 200 μ-EDXRF) with the following characteristics: X-ray generator 50 kV; fine focus X-ray source with a Molibdenum anode; ADC with 4096 channels; anode voltage adjustable from 0 to 50 kV; anode current adjustable from 0 to 1500 A (maximum 50 W).

Glue analyses. Two methods were used to determine the chemical nature of the glue used to consolidate the tunic in the 1950s and, in particular, to discriminate between vegetal (starch) and animal (collagen) types of glue. The analyses were performed on a textile sample of about 2 x 5 cm^2, which had been excised from the Coptic tunic. Since starch is the most common glue employed for textile restoration (De La Chapelle et al. 1994), we started our analyses with a Lugol test. This test is based on the reaction between the test sample and a solution of Lugol (Sigma-Aldrich) (0.1 g of potassium iodide and 1 g iodine dissolved in 100 ml of water), which turns dark blue in the presence of starch.

The Fourier Transform Infrared Spectroscopy (FT-IR) technique was employed to confirm the result. FT-IR allows the organic and inorganic components present in the sample to be identified. The used equipment was an FT-IR Bruker Vertex 70 spectrophotometer, coupled with an infrared microscope (Bruker Hyperion 3000). The analyses were carried out with a resolution of 4 cm^{-1} and 64 scans. The vibrational bands that appear in the infrared spectra provide information about the functional groups of a sample, which leads to a general characterization of the material, or even the identification of specific compounds. The technique was applied to confirm the results of the spot test on the starch glue.

Restoration procedures

Color stability and wet cleaning. Before proceeding with the wet cleaning, it was necessary to be sure that this procedure would not result in a loss of color. Therefore, a color stability assessment was performed on the wool wefts. Five samples, including colored (purple - red - green - orange) and undyed wool yarns, were tested. The yarns were incubated in glass tubes filled with

deionized water for 60 min at room temperature and subsequently at 45° C for 30 min, with the aim of revealing any loss of color. The samples were then placed between two sheets of blotter paper and were kept under a weight for 3 h, to verify whether any color migration had taken place. The result of the test was negative (no color loss according to the visual assessment) and, hence, it was possible to proceed with wet cleaning tests which consisted of vaporization-atomization (6 minutes at 30°C) and application of Agar-Agar and Gellan gum rigid gels (10 minutes), prior to removing the glue applied to the back of the fabric using a spatula.

Biocleaning: enzymes and supports employed. Since the glue used for the previous restoration of the tunic was starch, several commercially available amylases were considered for its removal. Starch is composed of two types of alpha-glucans: amylose (linear polysaccharide) and amylopectin (branched-chain polysaccharide), the latter being the main cause of the paste-effect. Both alpha and beta amylases can hydrolyze amylose and amylopectin through different catalytic mechanisms. Alpha amylases cleave internal α-1-4 glycosidic bonds of starch at random generating dextrins, while beta amylases hydrolyze amylose and amylopectine, starting from their non-reducing ends, and release maltose (Ray and Nanda 1996). For these reasons, beta-amylases are better suited when a complete saccharification of starch is needed, while alpha amylases are generally preferred for the fast removal of starch (Schwarz et al, 1999). Microbial amylolytic enzymes are excellent for applicative purposes, because they are generally stable over a wide range of temperatures and pH. Lyophilized alpha-amylase from *Bacillus sp*. Type IIA (Sigma-Aldrich) was finally selected, because of its high specific activity (> 1,500 units/mg protein). The alpha-amylase solution was prepared at a very low concentration, *i.e.*, 0.1% w/v in a 20 mM sodium-phosphate buffer with pH 6.9, according to the manufacturer's instructions and data reported in literature (Cremonesi 2009; De La Chapelle et al. 1994).

The application of enzymes in solution is inappropriate for the bio-cleaning of ancient textiles, since water flooding can damage the artifact. Therefore, in this study, the alpha-amylase from *Bacillus sp*. was immobilized as follows: 2 % w/v Agar-agar (polygalactose and sulphurpolygalactose, Bresciani SRL) and 2 % w/v Gellan gum (Phytagel, heteropolysaccharide, Bresciani SRL) in 20 mM sodium-phosphate buffer pH 6.9 were used as immobilization matrices. It is worth noting that such buffered systems were chosen considering the optimal pH (pH=6.9) for alpha-amylase activity. One cm thick low-viscosity "soft" gels were used for the alpha-amylase immobilization. The enzyme solution was brushed onto the solid gel surfaces. This immobilization

strategy was developed to facilitate the localization of the amylases at the interface between the textile and the support, and hence to maximize the glue removal efficiency.

Biocleaning: simulated textile sample preparation and enzyme application to the textiles. Before using the immobilized-enzyme system on the real art-work, a test was performed on a simulated sample. For this purpose, a textile sample with similar chemical-physical properties to those of the original Coptic shroud was woven and submitted to artificial aging by means of dry-incubation. Reproducing the original aging of artifacts is a challenging procedure. Coherently with the burial ground where the tunic had originally been found, a dry incubation at 90°C for 60 days was applied (instead of a wet-chamber) to induce accelerated aging in the simulated sample. A starch-glue was then applied to simulate the restoration treatment performed in the 1950s. Given the impossibility of performing diagnostic investigations to establish the type of starch used in the fifties to restore the original tunic, some hypotheses were made. Barley, corn, oat and rye starches were excluded, because these cereals are not commonly used in Italy, where the restoration was made. The choice was then restricted to potato, rice and wheat starches. Glues were prepared by dissolving 3 parts of each starch in 1 part of hot water, so as to obtain a thick paste. The three glues were then applied to the simulated sample and subjected to a three day-"aging" at 50°C to obtain a further thickening of the glue.

Gellan - and agar - (6X6 cm cold sheets) immobilized alpha-amylase was applied, first to the simulated samples and, once it had been established that the procedure did not cause damage to the textile, then to the original Coptic tunic. A control was also set up using agar and gellan alone.

Results

Characterization of the Coptic tunic

^{14}C datings

Traditionally, the chronological assignement of Coptic textiles was based on historical/stylistic features. The goal of the present radiocarbon measurement was to demonstrate how scientific analysis can complement humanistic studies. The analyses were performed on two samples of the tunic selected according to the following requirements: a good conservation state; coeval, non-

woven and far from old restoration areas; the absence of starch glue residues. The conventional radiocarbon ages obtained for the two samples were 1645 ± 50 and 1515 ± 40 years Before Present, respectively. Since the two samples are parts of the same specimen, we were able to combine them to evaluate the calibrated age (by using the IntCal13 calibration curve) (Reimer et al. 2013). These analyses indicated that the textile (Fig. 1) can be ascribed to 415-560 A.D. (95% probability).

Thread analysis by means of SEM-EDX

The studied tunic appeared visibly damaged because of its ancient origin and showed several stains which deserved investigation. As regards the chemical composition, SEM-EDX analysis on two samples of wool fibers (taken in correspondence to the organic stains) revealed the presence of silicon, aluminum and phosphorus. As far as the morphological analysis is concerned, the electron microscope observation allowed us to observe a variable diameter of the fibers, ranging between 10 and about 65 μm (Fig. S2). The larger diameter fibers did not exhibit the overlapping cuticle and displayed a smoother and more degraded surface, compared to the fibers with a smaller diameter. Other fibers were also found to have longitudinal and transverse lesions and crease marks around the central part of the cloth (Fig. S2).

Analyses of the dyes and stains

It was important to establish the origin (animal or vegetal) of the pigments used to decorate the colored parts of the tunic. Reflectance spectroscopy with optical fibers (FORS), supported by investigations in multispectral false color infrared (IR-FC), allowed two of the natural dyes used for the decorated parts of the shroud to be identified (see supplementary material) (Figs. 1, 2). A comparison between the absorption spectra measured in the artwork and those collected in the database of known samples of the Restoration and Conservation Centre “La Venaria Reale” (University of Turin) was a valuable aid for the characterization of the considered sample. Both the red and blue pigments were of plant origin: the red dyes had been obtained from *rubiaceae* and the blue dye had been obtained from indigotin. Moreover, our analyses demonstrated that dye mixtures (comprising an unidentified yellow pigment) had been employed to obtain the various colors (e.g., purple, orange and green).

Several dark stains were also present on the tunic as localized spots. These stains were investigated by means of X-ray fluorescence (XRF), which detected a chalky- and earth-based component (iron oxides). The presence of metals, such as Zinc, Copper and Silver, was evident in some areas.

Glue analyses by means of Spot Test and FT-IR

As shown in Fig. 3 (a, b) (before cleaning), the entire back of the art-work showed white areas of dull precipitated material, ascribable to glue. Taking into consideration the likely presence of starch, the commonly used Spot Test, with a Lugol iodine solution, was performed. The dark blue color the glue acquired on the wool textile fiber confirmed the presence of starch (Fig. 3c, d, Fig. S4). In addition, a Fourier Transform Infrared Spectroscopy Analysis (FT-IR) was performed on a micro-sample of the glue-damaged fabric. From a comparison with the FT-IR spectra of pure compound references present in the database of the Restoration and Conservation Centre "La Venaria Reale" (University of Turin), it was possible to ascertain the starch nature of the glue (Fig. 4).

Restoration of the tunic

Glue removal by means of wet-cleaning

Prior to wet cleaning, experiments had been carried out to test the color solidity of the wool wefts. Color solidity tests have the purpose of "stressing" the yarns to verify the stability of the dyes after a treatment with the solvent expected to be used for the wet cleaning (*i.e.*, deionized water). Relatively harsh washing parameters (*i.e.*, increased temperature and immersion time) were used to check the sealing of the fibers. Samples of colored (purple - red - green - orange) and undyed wool yarns were tested. No significant losses of color were observed after this treatment (visual assessment, data not shown), hence we were able to proceed with wet-cleaning to remove the glue.

Tests were performed to remove the starch paste with "traditional" wet cleaning system (vaporization-atomization and the application of rigid agar gel and gellan gum), but they proved to have little effect. In fact, video-microscope images (data not shown) revealed that the starch had swelled, but the mechanical removal with a spatula caused the superficial fibrils to break, due to the penetration of the softened glue inside the interlaced textile. This result prompted us to use a novel method.

Glue removal by means of immobilized amylase

Bacillus sp. alpha-amylase was selected from among the commercial amylolytic enzymes because it was the one that displayed the highest specific activity. Both gellan (heteropolysaccharide) and agar-agar (polygalactose and sulphurpolygalactose) were selected as supports for the enzyme immobilization. The gellan matrix appears to be more transparent than agar, and this constitutes an advantage for controlling the time-course removal of the glue (Fig. S3). However, both matrices were tested for enzyme immobilization and glue removal.

The alpha amylase solution was brushed onto the gel matrix surfaces (Fig. 5a), to locate the enzymes at the interface between the textile and the support, and hence to maximize the glue removal efficiency. The temperature on the matrix surface was 40°C, which is significantly lower than the optimal temperature for amylase activity (60°C). However, higher temperatures would have caused gel melting. After enzyme adsorption on the gel matrix, we applied the immobilized amylase to a simulated sample to test the hydrolytic action against the glue. The entire procedure is illustrated in Fig. 5.

A textile sample was woven with similar chemical-physical properties to the original Coptic tunic. Since it was not possible to ascribe the original wool fibers to sheep, goat or camel (the cuticle is similar in all these cases), a goat wool was chosen. Artificial aging was obtained by means of dry incubation (instead of a wet-chamber), considering the dry ground in which the artwork had been buried for more than 1000 years in Egypt. The textile sample was then treated with a starch glue to simulate the previous inappropriate restoration. A wheat starch glue was used for this purpose, since it was the one that had been found to cause the greatest alteration and stiffening of the textile fiber (data not shown). After a further aging to increase the glue thickening, gellan- and agar-immobilized alpha-amylase was applied to the simulated samples (data not shown). A control was also set up using agar and gellan alone.

The cleaning procedure adopted for the wheat starch glue is reported in Fig. 6a: it is evident that the aspecific action of the support was negligible, whereas the gellan-immobilized alpha-amylase was the best fitting. The Lugol test was repeated on the simulated textile sample before and after the alpha-amylase treatment (Fig. S4), and the efficient removal of the starch was confirmed. Therefore, we decided to apply the combination of the alpha-amylase plus gellan to the original Coptic linen (Fig 6b).

The application steps for the enzymatic treatment were the same as those described in the preliminary testing intervention. Alpha-amylase was applied to gellan sheets (Fig. 5a), and these "tablets" were placed on the fabric, interposing a thin sheet of medium weight Japanese paper to prevent the gel from seeping into the textile (Fig. 5b). A sheet of Melinex and a glass weight were then superimposed to create a protected "wet room" (Fig. 5c) with the aim of inhibiting water evaporation.

The poultice was applied for about 15 minutes, as described in the preliminary tests. The sticky residue that visually appeared swollen and liquefied was then removed with a moistened cotton swab (Fig. 5e). Finally, the superficial glue residues were excised with a spatula. After drying the cloth on a suction table with absorbent paper and glass weights (Fig. 5f), the textile, now free from the glue film, showed enhanced flexibility and elasticity (Fig. 6b). Only after the bio-cleaning was the needle consolidation carried out on the overall linen support (without the danger of breaking the textile due to the passage of the needle). The cleaning of the back of both fragments (about 4 m^2 of textile) was completed in 160 h of work.

Discussion

The results obtained in the present study by ^{14}C dating have demonstrated that the shroud belongs to a period between the 5^{th} and 6^{th} Century A.D., thus proving that the tunic is much older (about 500 years) than expected based solely on historical and artistic criteria. Radiocarbon investigations on other archaeological textiles, stylistically and technically similar to the tunic that was the subject of the present investigation can be found in the literature (De Moor and Fluck 2007; Schrenk 2004; Strydonck et al. 2004; Strydonck and Benazeth 2014). The data, in agreement with our results, showed that the samples were much older than the age inferred through art-historical criteria, and should in fact be traced back to the period between the 5^{th} and 7^{th} Century A.D., in the same way as for the tunic under investigation. These considerations underline that the dating of "Coptic" textiles is a critical issue and emphasize the need for scientific investigations to support art-historical studies.

Owing to the ancient origin of the investigated sample, a relatively high degree of degradation, which was visibly evident but also confirmed by the present analyses, was observed (Fig. S2). Silicon and aluminum were found, by means of SEM-EDX analyses, in correspondence

to organic stains that were probably derived from the burial ground terrain, these elements being the most abundant on the Earth. Phosphorus, presumably originating from decomposition products of living organisms, was also detected. It was not possible to ascribe the original wool fibers to sheep, goat or camel, since their cuticles are similar. Morphological analysis of the fibers showed lesions due to aging, mechanical load and traction, and crease marks around the central part of the textile, thus indicating that the tunic was probably worn belted (Fig. S2). All these results confirmed that the tunic was actually used a "dress of death/burial outfit" (*i.e.*, shroud)

The ancient textile (Fig. 1) is decorated with fine tapestry squares and bands characterized by several parts colored in red, green, yellow and purple (see the supplementary material). The results obtained by combining FORS and IR-FC revealed that red dyes from *rubiaceae* and blue dye obtained from indigotin had been used as the main pigments for these parts of the textile. These dyes had been mixed together to obtain purple. Furthermore, an unknown yellow dye had been mixed with the red *rubiaceae* to obtain different degrees of orange, and with indigo to obtain green, respectively, as also mentioned by Cardon et al. (2007). Zinc, Copper and Silver were detected in the stains that were found on the front and back of the tunic. It is tempting to hypothesize that the presence of these metals, especially copper and silver, may be due to contact with precious heirlooms placed next to the body of the deceased or in the folds of the tunic.

The back of the tunic appeared to be significantly damaged (Figs. 3, 6) as a result of a previous restoration intervention (during the 1950s) aimed at consolidating it with a glue of an unknown nature (see the supplementary material). This treatment caused an overall enhanced rigidity and structural fragility of the fabric. The wet cleaning procedure (based upon vaporization-atomization and application of rigid agar gel and gellan gum) performed here was unsuccessful in removing this paste (Fig. 6a). Therefore, considering the possibility of using an enzymatic cleaning approach (Beutel et al, 2002; Schwarz et al, 1999), we established the nature of the glue used to consolidate the tunic, which proved to be starch (Lugol test and FT-IR analysis) (Figs. 3, S4). Starch is generally preferred to protein glues since it is an easy-to-prepare paste that is commonly used to strengthen vegetal- and animal-derived textiles (De La Chapelle et al. 1994).

Hence, alpha amylase was chosen. Alpha amylases are endo-enzymes that generate dextrins, whereas beta amylases are exo-enzymes that release maltose units from the non-reducing ends of amylose and amylopectine (Ray and Nanda 1996). Therefore, beta-amylases have slower

catalytic mechanisms, but are more suitable for the complete saccharification of starch. However, alpha amylases are generally able to reduce viscosity and the adhesive properties of starch more quickly, and for this reason were preferred in this study to remove the glue (Schwarz et al, 1999).

Amylases are biosynthesized by bacteria, archaea and eukaryotic organisms. Microbial enzymes are preferable for applicative purposes, both because of their stability over a larger temperature and pH range, and because of their lower production costs (i.e., the use of growth media containing cheap substrates, recombinant microorganisms) (Mehta and Satyanarayana 2016). *Bacillus sp.* alpha amylase was chosen for the present application because of its high specific activity (Chand et al. 2014). However, using enzymes in solution is not suitable for this kind of application, due to the high water load that can damage the textile. Hence, an enzyme immobilization strategy was set up. The amount of water, which is essential for any catalytic reaction, can be contained to a minimum in immobilized enzymes. Furthermore, no additional by-products (except starch-derived small sugars) are formed (Schwarz et al, 1999).

Two polysaccharidic matrices, *i.e.* gellan and agar-agar, were selected. Both of these polymers are employed extensively in biotechnological applications: they possess good stability in the 6.5-7 pH range, are un-reactive, un-adhesive and suitable for generating films (Iannuccelli and Sotgiu 2007). Furthermore, the highly hygroscopic nature of these polymers limits water transfer to the fibers, and allows a gradual and controlled release of the enzyme into the textile without flooding it with liquids (Iannuccelli and Sotgiu 2007).

The application of an alpha amylase solution by simply brushing it onto the surface of the gel matrix facilitated the localization of the enzymes at the interface (between the textile and support), thus leading to a maximal efficiency of glue removal. Gellan, being less rigid than agar, proved to be better suited to adhere to the discontinuity of the textiles.

In a first experiment, a simulated wool sample, similar to the original Coptic textile, was used to test the cleaning effect of immobilized enzymes versus the matrices alone. First, it was necessary to submit this textile sample to artificial aging. It is worth noting that it is very difficult to reproduce the original aging process, since several variables, such as contamination from the decomposition liquids of the dead body and from the burial ground, could not be included in the artificial aging procedures. According to Ahmed and Kolisis (2011), 3 days of thermal aging of silk at 120°C correspond to about 25 years of natural aging. However, wool tends to become rough

and turn yellowish when incubated at 100°C, and to shrink at 130°C (Quaglierini 1985). Therefore, an incubation for 60 days at 90°C was used here to induce accelerated aging in the simulated sample and, after this time, starch glue was applied and left to further thicken, age and stiffen. Finally, agar and gellan were tested alone on the simulated samples, and then together with gellan- and agar-immobilized alpha-amylase to compare their cleaning action.

The enzymatic removal of the starch glue was clearly detectable at both a macroscopic and a microscopic level after only 15 minutes of treatment, whereas the effect of the matrices alone was negligible (Figs. 6A, S4). The liquefied glue residue was then removed and the textile appeared to have gained flexibility and softness. Hence, this method was applied to the original shroud (Fig. 5).

This strategy, based on enzyme adsorption, differs from the technique that has been used by other Authors (Beutel et al. 2002), which employed covalent-bound proteases to bioclean casein-damaged mural paintings. However, in spite of the weak interaction between the matrices and enzymes, and of the fact that the temperature used to prevent the gel from melting (40°C) was lower than the optimum temperature for the amylase enzyme (Schwarz et al, 1999), this biocleaning approach has proved to be excellent, as can be seen in Fig. 6B, C. It is worth noting that alpha amylases are commonly employed in the textile industry to desize fabrics (Chand et al. 2012). Compared to traditional cleaning methods, the application of immobilized amylases also led to a shorter contact time and milder mechanical action during the glue removal phase. The overall procedure demonstrates the suitability and effectiveness of enzyme-based bio-cleaning procedures for the restoration of archeological wool and linen textiles. Only a few reports on the enzyme-biocleaning of artworks are available in the literature, and they pertain to the use of: i) immobilized caseinases for the restoration of frescoes (Beutel et al. 2002); ii) alpha-amylases either in solution or as a poultice for the removal of starch from cotton, linen and silk fabrics (Ahmed and Kolisis 2011); iii) alpha-amylase immobilized on a methylcellulose matrix to restore linen and wool tapestry (Whaap 2007). The here reported strategy is in fact the first example of the application of gellan-immobilized alpha amylase to a wool textile.

In conclusion, the multidisciplinary approach used here has proved to be a valuable strategy to obtain interesting information concerning an artwork of great historical interest. ^{14}C dating has allowed an object to be collocated precisely in a very different period from the one assumed

previously on the basis of its artistic features. Analyses on the nature of the textile and the pigments used for the colored parts, as well as the stains contaminating the linen, have given us new insights into the manufacturing techniques and the actual utilization of the tunic in the Egyptian Coptic period. The Coptic tunic, which had been "hidden" from the community sight for a long period, whilst/while awaiting suitable restoration (that would not damage its precious historical value), has now been restored to its ancient beauty, and is currently exhibited at the Egyptian Museum of Turin (Italy) (Fig. 6c). The mildness, specificity and efficacy of the treatment suggest that the enzymatic approach could be employed on a wide variety of art-works, especially for archaeological textiles, without the risk of damage.

Acknowledgements

The present study is an extension of the Master thesis in Conservation and Restoration of Cultural Heritage of the University of Turin (Ferrari 2013) which won the IGIIC Award for the Best Thesis in Conservation and restoration of Cultural Heritage 2014. We would like to thank the textile restorer Susanna Conti for her technical suggestions, the Egyptologist Rosa Boano for her contribution on the historical aspects and Primo Brachi for his fiber deterioration interpretation. Special thanks are due to Daniele Demonte for the IRFC analyses and photographic documentation. Particular thanks to Monica Gulmini for her helpful suggestions and to all the technicians of the scientific laboratories of the Restoration and Conservation Centre "La Venaria Reale" (Turin). We are indebted to Alessandro Lo Giudice and Alessandra Romero for encouraging this project.

Compliance with Ethical Standards

Funding: this work was supported by a research project of Turin University, the Egyptian Museum of Turin and the Restoration and Conservation Centre "La Venaria Reale".

Conflict of interest: all the Authors declare that they do not have any conflict of interest.

Ethical approval: this article does not contain any studies with human participants or animals performed by any of the authors.

No strain deposited in a public strain collection has been employed in this study.

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Figure legend

Fig. 1. (a, b) The original Coptic tunic (Inv. S. 17490) from the Egyptian Museum in Turin (Italy) before the biocleaning intervention. (c, d) Contrast-highlighting graphic diagrams, obtained with AutoCad software, of the two fragments of the tunic. (e, f) Graphic documentation (Autocad software) of the state of conservation of the two fragments showing the extension of the stains due to the presence of organic liquid (in color) and other surface precipitates. (g, h) Multispectral UV image of the tunic showing evident organic deposits.

Fig. 2. Images of woolen (samples 1-5) and linen (sample 6) threads obtained by means of optical microscopy in reflected (left column, ROM) and transmitted (middle column, TOM) light mode. Dye identification, by means of FORS, is reported in the column on the right.

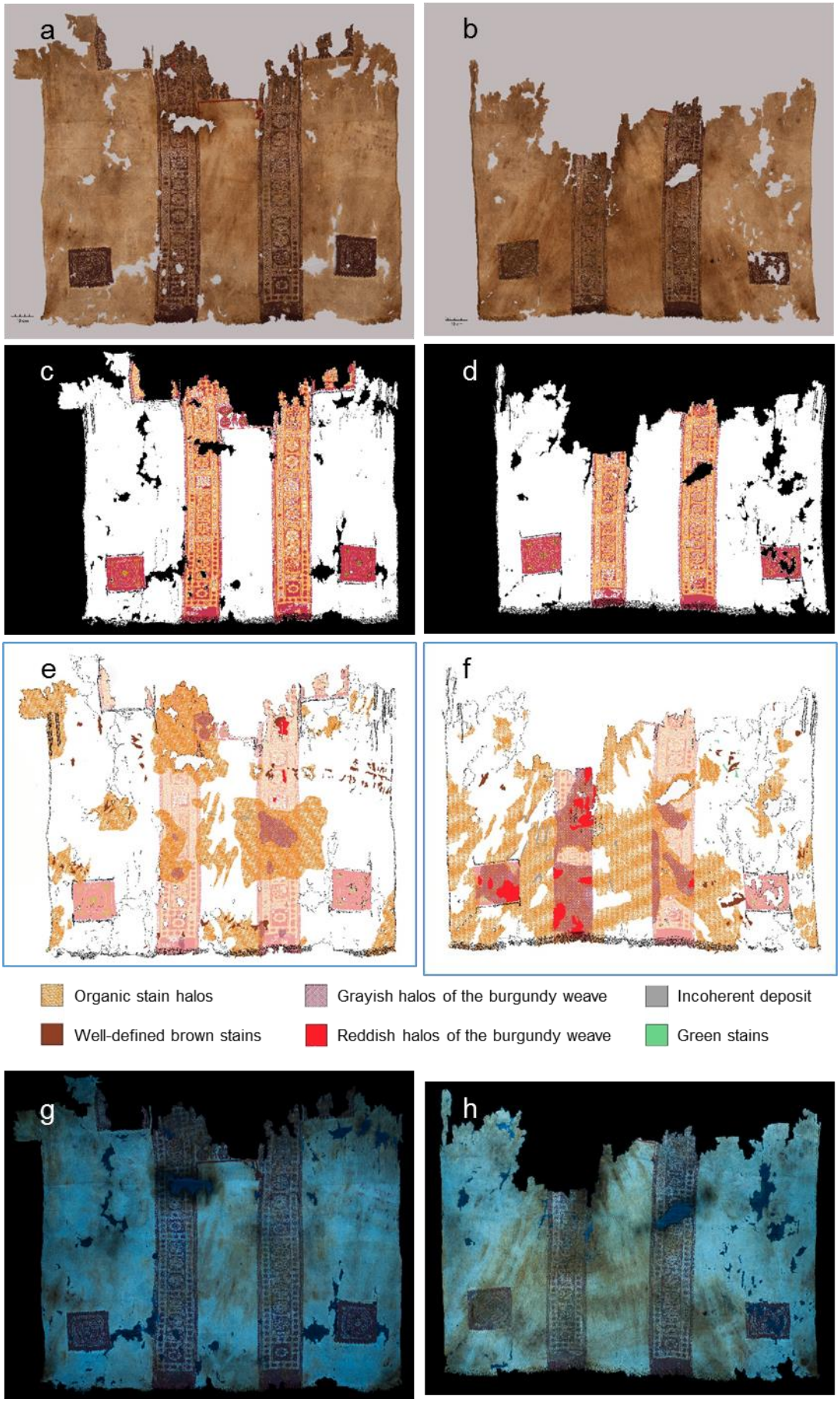
Fig. 3. (a, b) Video microscope photographs of the textiles before cleaning; glue residues are evident. Wool textile fibers before (c) and after (d) the Lugol Spot Test; the dark blue color taken on by the adhesive (d) on the wool fiber indicates the presence of starch.

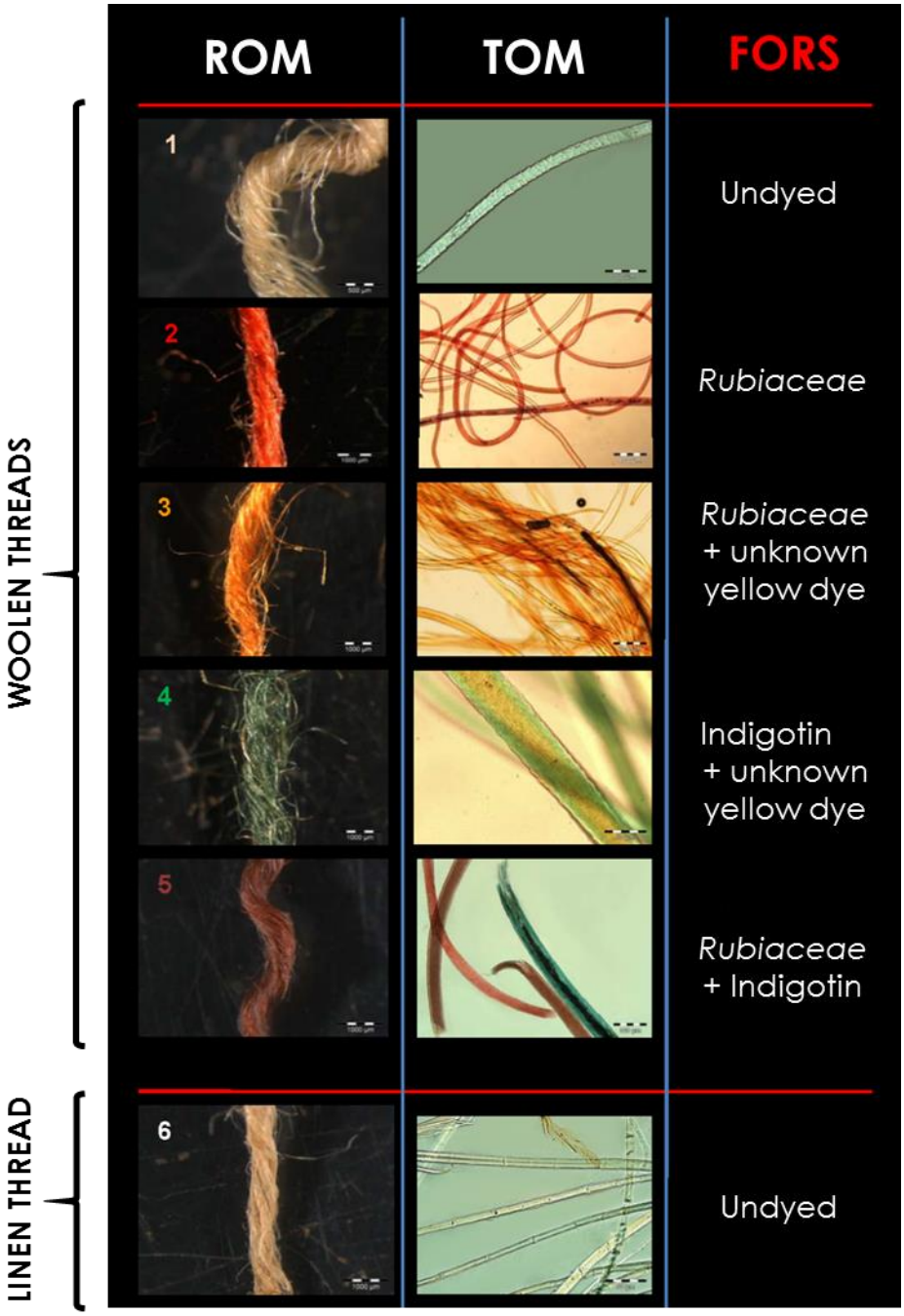
Fig. 4. FT-IR spectrum of the adhesive. Signals corresponding to polysaccharides are clearly visible (1151, 1077, 1039, 933 cm^{-1}) and are highlighted with arrows. Contamination by oxalates (signals at 1651 and 1322 cm^{-1}) and proteinaceous material of the wool are also evident (signals at 1651, 1549, 1454, cm^{-1}).

Fig. 5. (Top) Schematic representation of the used enzyme poultice. 0, non-acidic blotter paper used to verify the amount of water released from the fabric during the cleaning procedure. 1, Coptic tunic. 2, Low basis weight (6 g/m^2) Japanese paper used to prevent the gel from seeping into or migrate onto the textile. 3, gellan-immobilized amylase tablet. 4, Melinex layer used to create a wet room to prevent water evaporation from the tablet. 5, glass weighting to ensure uniformity of the gel-textile contact and the gradual release of water from the gel. (Bottom) Application phases of the enzymatic poultice to the back of the tunic.

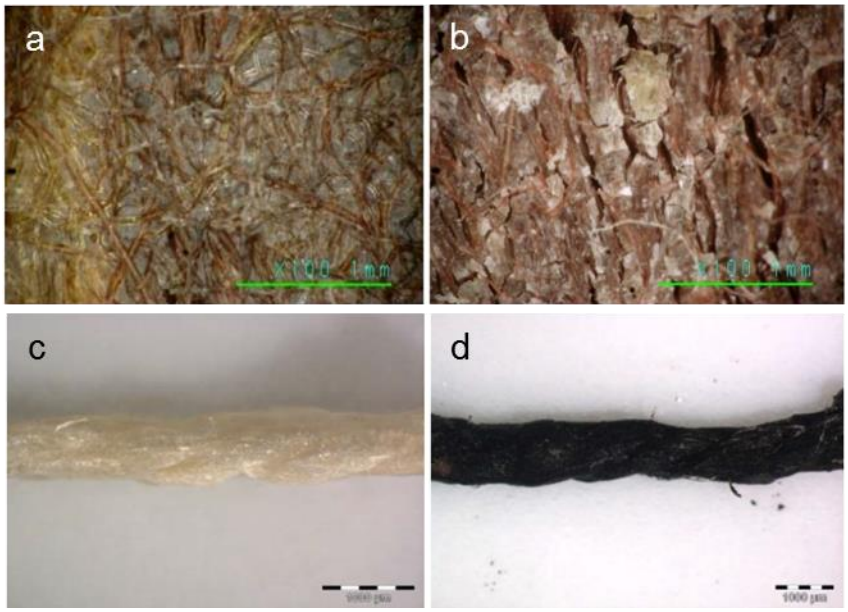
Fig. 6. (a) Cleaning tests with gellan and agar (without enzyme supplementation) and gellan- or agar-immobilized alpha-amylase. The video-microscope photographs show that, after an application time of 15 minutes, the removal of the glue with a moistened cotton swab was very satisfactory for the gellan-immobilized alpha-amylase. (b) Details of the back of the tunic before and after the cleaning intervention with gellan-immobilized alpha-amylase. (c) The restored Coptic tunic on show at the Egyptian Museum of Turin (Italy).

1


a
b
c
d
e
f
Organic stain halos
Grayish halos of the burgundy weave
Incoherent deposit
Well-defined brown stains
Reddish halos of the burgundy weave
Green stains
g
h


ROM
TOM
FORS
1
2
3
4
5
6
Undyed
Rubiaceae
Rubiaceae + unknown yellow dye
Indigotin + unknown yellow dye
Rubiaceae + Indigotin
Undyed
WOOLEN THREADS
LINEN THREAD

1



2

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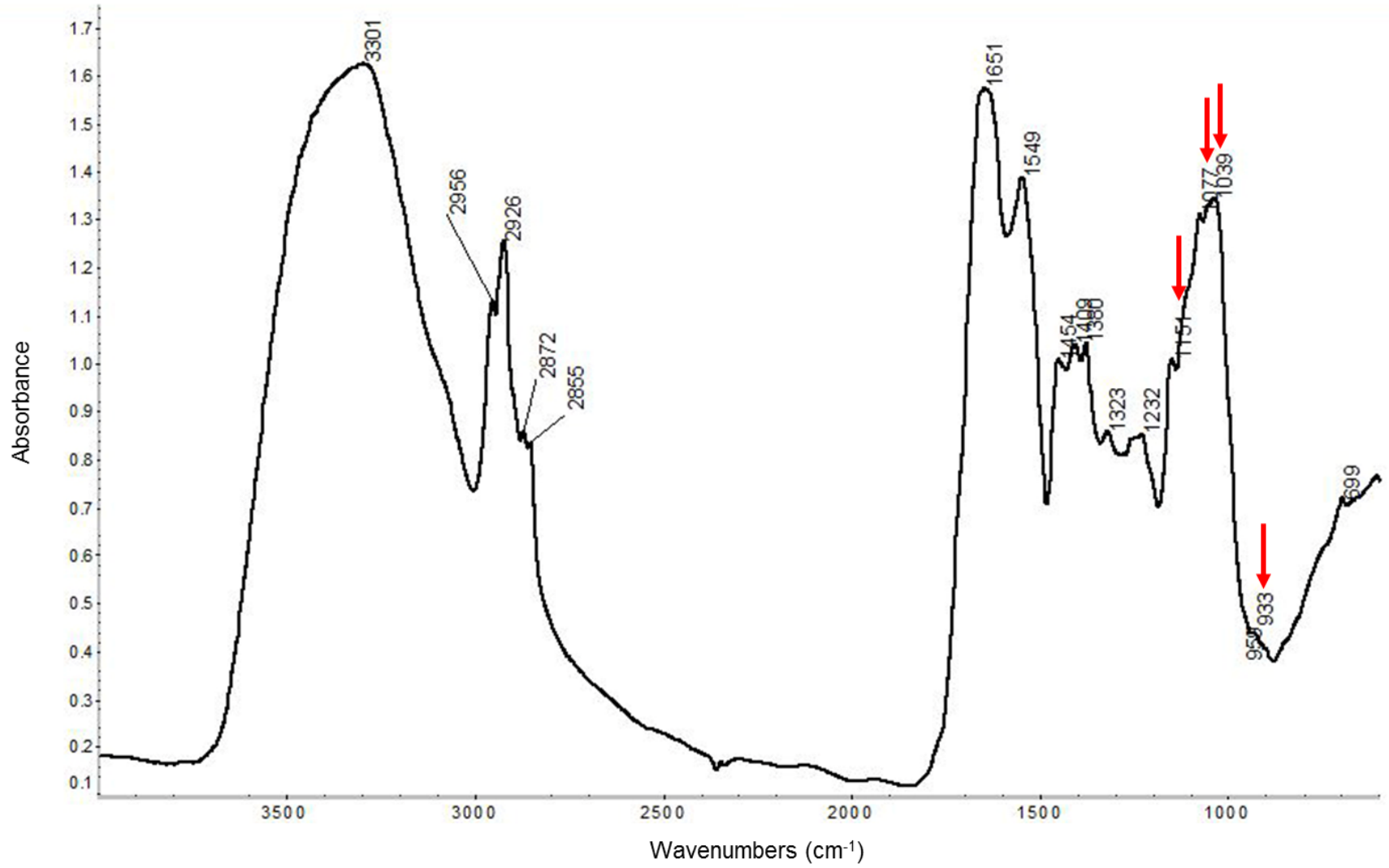
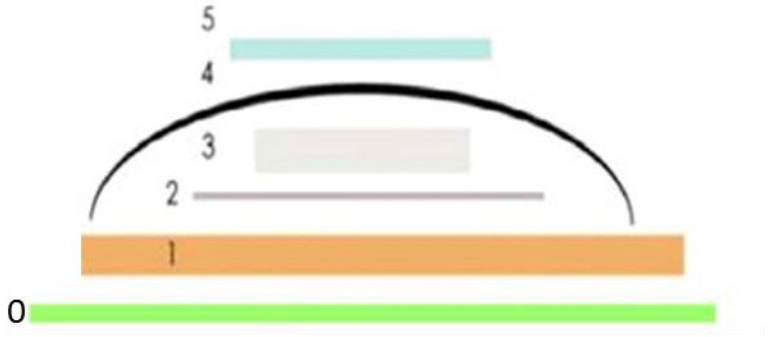
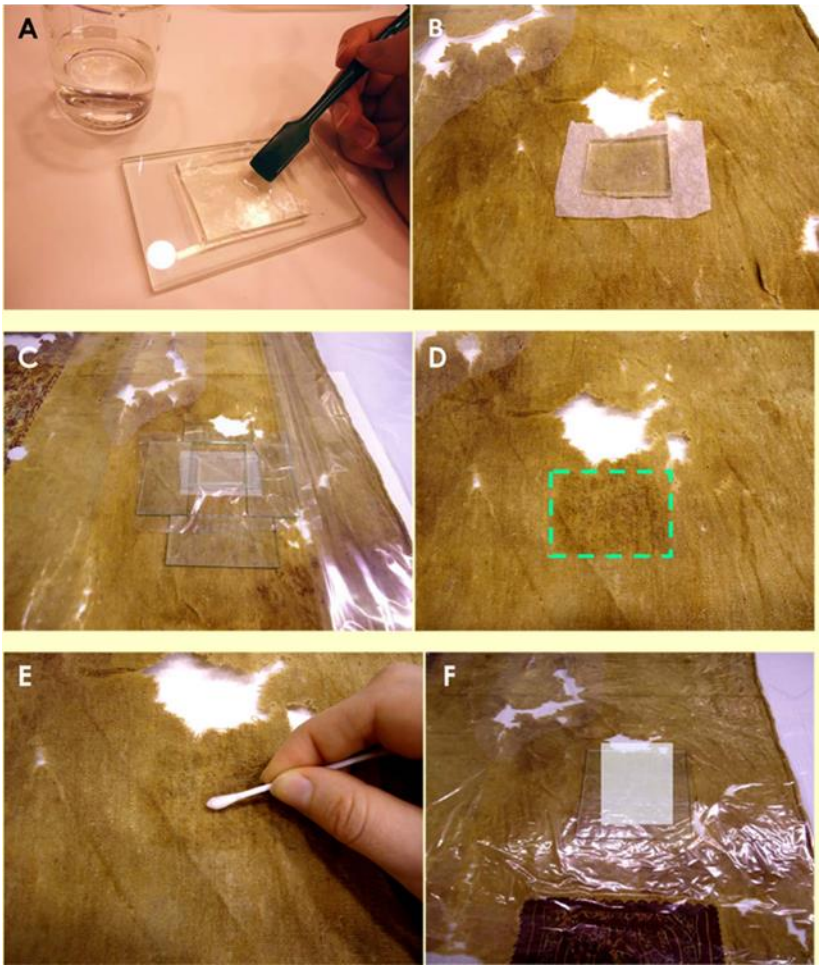

Absorbance
Wavenumbers (cm-1)
3301
2956
2926
2872
2855
1651
1549
1454
1408
1380
1323
1232
1151
1077
1039
933
699
3500
3000
2500
2000
1500
1000

2





1

1

a

Cleaning method	Before	After
Gellan		
Agar		
Gellan plus α-amylase		
Agar plus α-amylase		

b

Cleaning method	Before	After
Gellan plus alpha-amylase		

c

2

3

Supplementary Online Material

Applied Microbiology and Biotechnology

Immobilized alpha-amylase from *Bacillus sp.* for biocleaning an ancient coptic tunic

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Description of Coptic Tunic and its state of conservation. The wool-linen tunic (shown in the Egyptian Museum of Turin, Italy, inventory number INV. S. 17490) was found in Egypt, probably in the second half of 1800s. It was a gift by the Egyptian Museum in Cairo to the Egyptian Museum of Turin between 1888 and 1903 (ASTO). The fifth-century tunic is a typical example of daily wear in byzantine Egypt.

The tunic was woven in one big rectangular piece provided with sleeves, folded in two at shoulder height, with an opening at the center to allow the passage of the head (Fig. S5). The vertical sides were stitched. It is decorated with fine tapestry squares (*tabulae*) and bands (*clavi*) woven with wool warps and wool weft of red, green, yellow, purple depicting animals and stylized men and patterned in a sort of "*horror vacui".* The white linen is present only in the smallest details obtained with flying shuttle decoration.

With the spread of Christianity in Egypt (during the 3th and 4th centuries A.D.) many bodies were simply buried in the clothes they would have worn in life. A phenomenon commonly seen on the backs of excavated Coptic tunics (Bonnard et al. 2013) is the presence of deposits that obscured the wave of the canvas. The back of the tunic in question is severely fragmented, fragile and friable, and it is impregnated with organic deposits that had caused extensive staining.

There are no data on the old restoration: probably the last was by Erminia Caudana (a restorer collaborator of the Turin Egyptian Museum specialized in papyri) who would have applied the techniques of restoration of paper and papyrus to the restoration of textiles in the 50s of the last century. On the entire back surface an adhesive glue was applied to a silk voile. The overall intervention resulted in stiffening of the fabric and accentuation of notable deformation of a structural type. Furthermore, portions of torn textile were folded and glued overlapping, without respecting the squareness of the textile interlacing and the correct repositioning of the flaps.

Turin Egyptian Museum. The birth of Egyptian Museum of Turin is traditionally ascribed to 1630, when Carlo Emanuele I enlarged the Savoia collection by buying the collection of the Dukes of Mantua (Gonzaga). The latter included the famous "Mensa Isiaca". In 1723, Vittorio Amedeo II established the "University Museum" exhibiting several classical and Egyptian items from the Savoia collection. In 1759, the botanist Vitaliano Donati (appointed by Carlo Emanuele III) was travelling in Egypt and Middle East collecting archeological and botanical items with the aim of enlarging the Savoia collection. After his death while he was sealing to Calcutta, a part of his collection (around 600 items from Egypt mainly recovered from Karnak and Coptos, including three monumental statues) was sent to Turin. During the Napoleonic military campaign in Egypt at the beginning of the 19th century, Bernardino Drovetti, the French General Consul of piedmont origin, brought to Torino more than 7000 items, including mummies, jewels, papyri and stelae and the Turin collection became significant. In 1833, the Piedmontese Giuseppe Sossio added more than 1000 items belonging to its own collection. The museum was further enriched and completed at the beginnings of the 20th century by the excavation campaigns of the piedmontese egyptologist Ernesto Schiaparelli (1903-1914, 1920), director of the Egyptian museum, and his successor Giulio Farina (1930, 1935, 1937). As a consequence of their excavations, more than 30000 items were available in the Museum, and among them also mummified animals (Moiso 2016).

Since 2013, the Egyptian Museum of Torino has been classified among the best 50 collections in the World and, as far as Egypt is concerned, it is for sure the oldest. In April 2015, the exhibition area was enlarged and completely renewed. In this year, more the 700000 people visited this site. The Egyptian Museum of Torino is one of the largest museums in Italy. For the high number and historical value of the items exhibited, it is considered the most important Egyptian collection after that of Cairo in Egypt.

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Supplementary Figure legend

Fig. S1. (a) Cover of the January 10th 1904 "Petit Journal" showing the Albert Gayet excavation in Antinoe; on the right there is a mummified body from Pharaonic Era, on the left a Christian mummy dressed with every day life clothes. (b) "Euphemia" Mummy ^{14}C dated 488 ± 25 A.D., in full Christian Period; she was buried with seven layers of superimposed "coptic" tunics (Royal Museums of Art and History, Brussels, Belgium); (c) "Byzantyne Knight" Mummy ^{14}C dated 580-663 A.D.; layers of woolen and linen tunics superimposed are evident (Musée Des Tissus de Lyon, France).

Fig. S2. Scanning electron microscopy (SEM) images of the wool fibers. (a) Fibers taken in correspondence with the brown stains of the fabric; the fibers are affected by accumulations of particulate deposit (the presence of silicon, aluminum, phosphorous was detected by EDX). (b) Coarse fiber (about 60 μm diameter); the scales of epicuticola are degraded and the surface looks smooth, a transversal lesion is evident. (c) Fine fibers (15-20 μm diameter) with the characteristic overlapped surface (their structure is intact). (d) Longitudinal tears in the fibers.

Fig. S3. Gellan and agar gel sheets on a simulated textile sample treated with starch glue. Gellan matrix appears significantly more transparent.

Fig. S4. Cleaning tests on a simulated textile sample. Starch-glue treated textile before (a) and after (b) glue removal by immobilized alpha-amylase. Starch-glue treated textile stained with Lugol iodine solution before (c) and after (d) glue removal immobilized alpha-amylase.

Fig. S5. (a) Reconstruction of the Coptic tunic with the vector drawing software Autocad. Its width was 2.60 m. It was folded in two to shoulder height. (b) Details of the *tabulae*.

Fig. S1

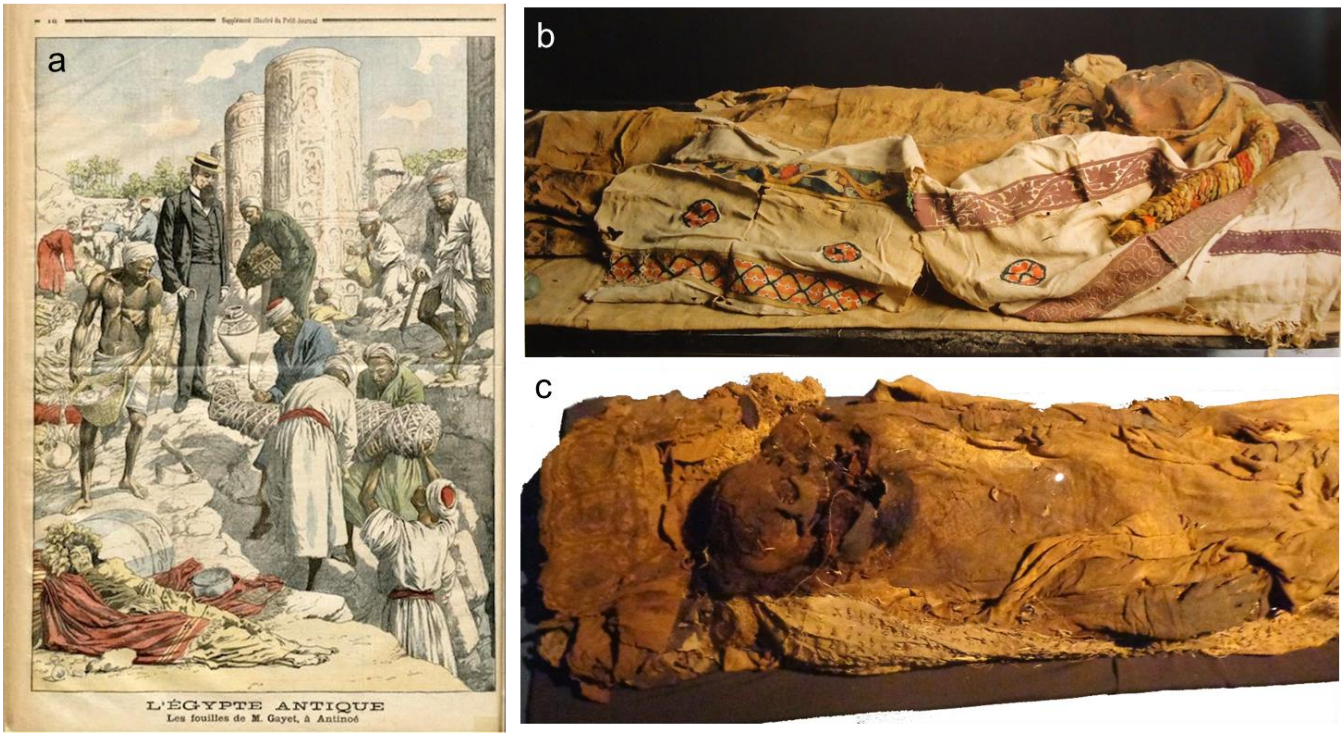
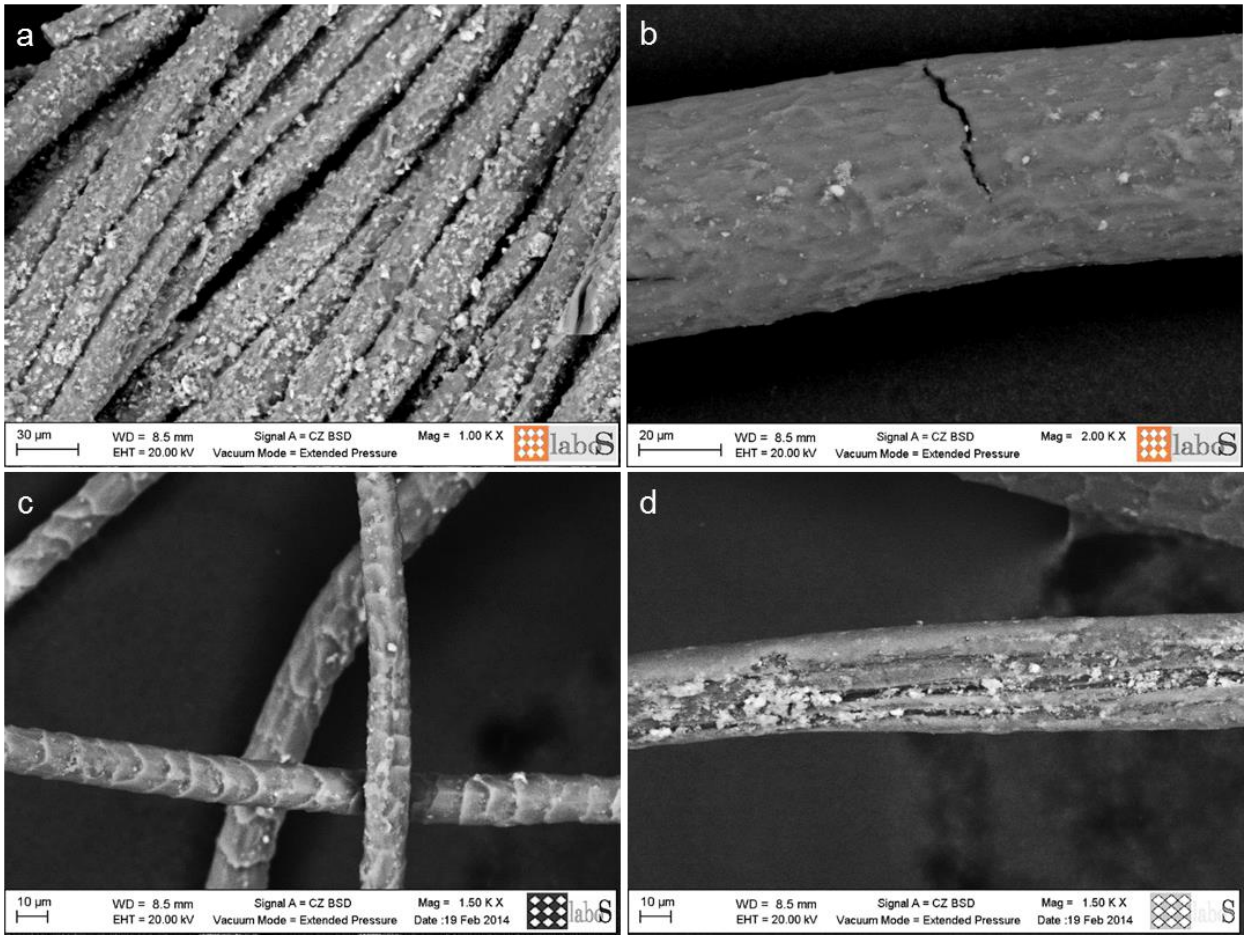


Fig. S2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30 µm
WD = 8.5 mm
EHT = 20.00 kV
Signal A = CZ BSD
Vacuum Mode = Extended Pressure
Mag = 1.00 K X
b
20 µm
WD = 8.5 mm
EHT = 20.00 kV
Signal A = CZ BSD
Vacuum Mode = Extended Pressure
Mag = 2.00 K X
c
10 µm
WD = 8.5 mm
EHT = 20.00 kV
Signal A = CZ BSD
Vacuum Mode = Extended Pressure
Mag = 1.50 K X
Date :19 Feb 2014
d
10 µm
WD = 8.5 mm
EHT = 20.00 kV
Signal A = CZ BSD
Vacuum Mode = Extended Pressure
Mag = 1.50 K X
Date :19 Feb 2014

Fig. S3

Fig. S4

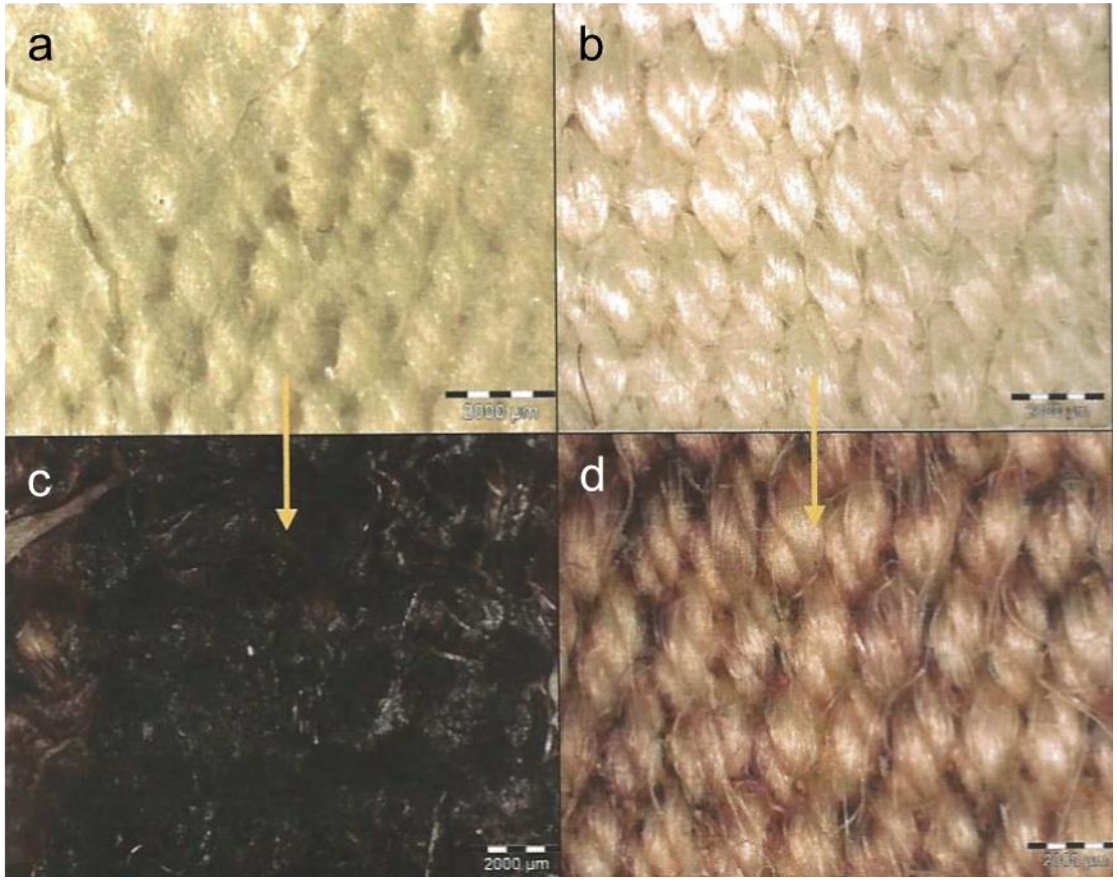

Fig. S5