Cleaning Fungal Stains on Cotton and Wood-containing Paper Using Protease

Mohamed Z. M. Salem,[a,*] Wael S. Mohamed,[b] Olfat Ahmad,[c] Rawan Ahmed,[c] Naglaa Khaled,[c] Nada Abo El-Makarem,[c] Nora Fawzy,[c] Rasha Sadek,[d] Rushdya R. A. Hassan,[c,*] and Maximilian Lackner[e,*]

Protease enzyme at concentrations of 50, 75, and 100 U (μmol/min), in both solution and paste form, was evaluated for cleaning stains caused by *Aspergillus flavus*. This applied study was conducted on paper sheets that had been formed from either cotton or wood-derived cellulose fibers. After cleaning, the infected samples were examined and analyzed to identify any changes and assess the effectiveness of the cleaning process. Color change, digital microscopy, ATR-FTIR, scanning electron microscopy, and pH measurement were employed. The results confirmed that the most effective treatment was the enzyme paste form at 50 and 75 U, as this was able to remove existing fungus spots on the surface or permeate within the fibers. IR spectroscopy confirmed that the chemical composition of both cotton and wood paper remained unchanged. Conversely, there was a significant increase in the characteristic vibrations of water and the crystallization sites of cellulose at the wavenumber of 1300 cm^{-1}.

DOI: 10.15376/biores.20.3.7435-7462

Keywords: Fungal stains; Cotton paper; Wood pulp paper; Protease enzyme; Cellulose

Contact information: a: Forestry and Wood Technology Department, Faculty of Agriculture (El-Shatby), Alexandria University, Alexandria 21545, Egypt; b: Polymer Department, National Research Centre, Dokki, Giza, Egypt; c: Organic Materials Conservation Department, Faculty of Archaeology, Cairo University, 12613, Giza, Egypt; d: Textile Metrology, National Institute of Standards (NIS), Cairo, Egypt; e: Department of Industrial Engineering, University of Applied Sciences Technikum Wien, Hoechstaedtplatz 6, 1200 Vienna, Austria;

**Corresponding authors: maximilian.lackner@technikum-wien.at; rushdyarabii@cu.edu.eg; zidan_forest@yahoo.com*

INTRODUCTION

Ancient books, manuscripts, and archive materials are the best places to find records of our history from the beginning of time. Paper has played an incalculable cultural role in human history (Rylance 1969; Magnússon and Olafsson 2016; Abdel-Maksoud *et al.* 2022). Researchers, scientists, conservators, and heritage managers have been working together more closely in recent decades as a result of growing awareness of the historical knowledge that can still be conveyed by ancient documents (Taylor 2015; Bicchieri and Pinzari 2016). Books from the ancient and medieval periods, in particular, can provide important hints for a correct understanding of the historical, social, and political context in which they were circulated as well as the people who owned and read them (Eriksen 2014). This is just one of the several reasons why maintaining the materiality of archival and book history is crucial, especially in the digital age

(Achachluei and Vatankhah 2010; Claridge 2017).

Documents, manuscripts, and books are predominantly composed of parchment, paper, or both. The cover parts can contain fabrics, leather, wood, and metals, but generally those materials represent a small part of the object (Bicchieri *et al.* 2019). There are, however, also many minor ingredients or compounds accidentally or purposely used or deposited in the materials that can be highlighted with new or particularly accurate analytical techniques, and which might give information on the history, manufacturing, or conservation state of the object (Hassan *et al.* 2021; Afifi *et al.* 2023; Bertrand *et al.* 2023; Koochakzaei and Mallakpour 2023; Hassan *et al.* 2024).

Manuscripts were the main preoccupation of those interested in human heritage and history during human civilization (Assmann 2011; Hassan *et al.* 2020). Manuscripts contain the history of humankind from the earliest ages. For instance, papyrus manuscripts appeared in Ancient Pharaonic civilization (Houston *et al.* 2003; Love 2019; Friedrich 2024; Krawczyk 2024). Furthermore, ancient Egypt was a source of papyrus, which was exported to the rest of the world (García 2014; Moreno García 2017). European civilization also introduced the use of leather treated with alkali materials such as lime to process so-called parchment, which recorded the finest decorative writings (Córdoba 2014).

Artworks and other documents on paper often suffer from the growth of species of fungi (Fouda *et al.* 2022). These fungi usually utilize trace mineral elements in the paper for their metabolism and gradually consume the paper as a carbon source (Pinzari and Gutarowska 2021). During the process, fungi produce stains on paper that may arise from colored organic metabolic waste products, or the fungi may convert colorless metal ions in the paper into visible stains, as occurs in foxing (Sequeira *et al.* 2012; Szulc *et al.* 2018). Moreover, the observed stains can result from colored fungal bodies (Guarner and Brandt Mary 2011).

Several methods have been stated, such as laser stain removal of fungus-produced stains from drawings, prints, and artworks executed on paper (Szczepanowska and Moomaw 1994). Certain stains from selected fungi can be removed by solvents or laser treatment. The stains formed by *Fusarium oxysporum* are eliminated after 24 h of exposure to 1,4-dioxane, while stains from *Penicillium notatum* and *Chaetomium globosum* are greatly diminished. The stains formed by *C. globosum* and *P. notatum* are greatly reduced after a 24-h treatment with N,N-dimethyl-formamide, while stains from *F. oxysporum* are only marginally reduced. The stains caused by *C. globosum* and *F. oxysporum* are partially removed after a 24-h exposure to pyridine (Szczepanowska and Lovett 1992). Deciding what to remove and whether removal is necessary is the first step; understanding what is involved can help make the decision more straightforward (Feilden 2007; Ashurst 2016).

Additionally, a variety of environmental factors may damage archaeological manuscripts, including atmospheric gases, where oxygen and water vapor create an environment conducive to auto-oxidation, which can cause the manuscript paper to turn yellow (Baty *et al.* 2010; Lawman 2011; Bertrand *et al.* 2015; Wiggins 2019; Hassan *et al.* 2020; Chiantore and Poli 2021). Temperature also influences microbial growth, as heat further accelerates the chemical damage of papyrus, parchment, and paper (Franceschi 2011; Cicero *et al.* 2018; Abdel-Maksoud *et al.* 2022). One of the most significant challenges to the longevity of paper is acidic degradation, a problem that has been recognized since the early 19th century (Zervos and Alexopoulou 2015). Acidity principally arises from the degradation of cellulose and lignin and the presence of alum

(Jablonsky *et al.* 2020; Małachowska *et al.* 2020; Yuan *et al.* 2023).

Biocatalysts (enzymes) and biosurfactants are 100% biodegradable, have softer reaction conditions, minimal toxicity, and are environmentally friendly compared to chemically manufactured catalysts and surfactants (Meng *et al.* 2023). For example, the enzyme α-amylase enhances paper quality by eliminating starch stains from paper artifact surfaces (Abdel-Nasser *et al.* 2022). Laccase, another enzyme, has a potential application to bleach fungal pigments on paper and parchment (Abd El Monssef *et al.* 2016) and mediate the degradation of petroleum hydrocarbons in historically contaminated soil (Diefenbach *et al.* 2024). The enzyme protease can eliminate protein-based stains from plant fiber and silk (Hsieh *et al.* 2023). Large protein molecules can be effectively broken down by protease, which makes the stain easy to remove (Niyonzima and More 2015; Sharma *et al.* 2019).

An important question is: How frequently has protease been applied to cotton and paper to remove stains? Among the most researched enzymes are proteases. Proteases are a class of enzymes with many uses in both physiological and commercial domains. They catalyze the breakage of peptide links in proteins, and they dominate the global market for industrial enzymes (Savitha *et al.* 201; Solanki *et al.* 2021).

Although fungi have a wider range of proteases than bacteria, many bacterial alkaline proteases have been extensively researched and are commercially available (Sharma *et al.* 2019). Furthermore, it is safer to use fungi as an enzyme manufacturing platform instead of bacteria because they are acknowledged as being generally considered safe (Pariza and Johnson 2001). Because of their unique range of action and great diversity in terms of being active in the alkaline pH range, fungal alkaline proteases are attractive models for industrial usage (Pawar *et al.* 2023). The generation of alkaline proteases is not as well studied as that of bacteria. Furthermore, the potential of a group of fungi that succeed in producing commercially viable compounds that are stable at alkaline pH has not yet been investigated.

The literature search in this work concentrated on the thorough categorization of proteases, the fermentation of various fungi to produce alkaline proteases, and their possible uses in the food and pharmaceutical industries. It is clear that these enzymes have been introduced in the wood, textile, and leather detergent industries, as well as in silk degumming, waste management, and silver recovery procedures. In addition, there has been a brief discussion of the potential contribution of alkali-tolerant and alkaliphilic fungi to the synthesis of enzymes (Grum-Grzhimaylo *et al.* 2016; Gurumallesh 2019). However, no research has been done on the use of proteases to clean hardwood or cotton paper.

Building upon the preceding discussion, the current study presents an experimental work on cleaning cotton and wooden paper from various fungal stains using the enzyme protease.

EXPERIMENTAL

Historical Manuscript

The fungal swab was taken from a historical manuscript belonging to an Ottoman collection from the 17th century at the Museum of Islamic Art (Doha, Qatar) as shown in Fig. 1. The manuscript's visible signs of deterioration include dirt, dust residue, and several yellow-green spots, which are attributed to a fungal infection.

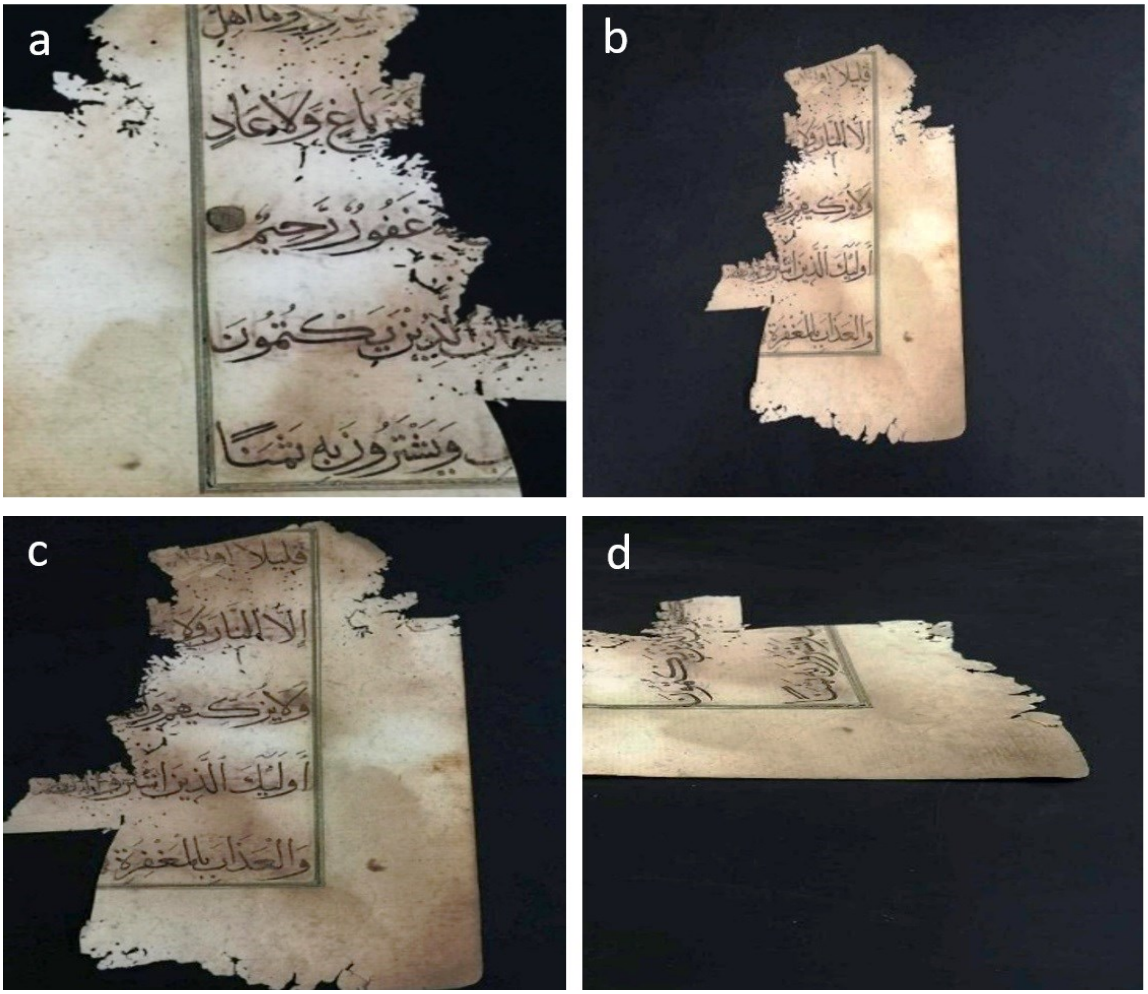


Fig. 1. Degraded paper at the Museum of Islamic Art. The prevalence of insect holes in the hull of the historical paper (a); damaged paper sheets with fading, insect damage, multicolored spots, and other blemishes (b, c, and d). The co-author, Nora Fawzy, took photos.

Paper Samples

Papers used in the present study were cotton linter cellulose (40 g/m²) with a thickness of 180 ± 2 µm and provided by RAKTA (General Company for Paper Industry, Abu Qir – Alexandria, Egypt) and bleached mechanical pulp from *Eucalyptus camaldulensis* wood chips prepared by Thermo-Mechanical Pulping (TMP). This process of refining wood chips under high temperature and pressure to separate the fibers using steam pressure (20 to 40 psi, or 138 to 276 kPa) and temperature (~170 °C). The pulp yield was 96%, and H_2O_2 was used as a bleaching agent and the paper sheets were prepared at (100 g/m²).

Isolation of Fungi from Historical Manuscripts

The isolation was done on two paper samples in the Microanalytical Center, Faculty of Science, Cairo University, Egypt. Fungal isolation was carried out directly in the laboratory following swab collection. The swabs were gently rubbed onto two different culture media: M40Y (composed of 400 g sucrose, 20 g malt extract, 5 g yeast extract, and 20 g agar) and potato dextrose agar (PDA), which contains 200 g potato, 20 g agar, and 20 g dextrose. The inoculated Petri dishes were incubated at 25 ± 2 °C for 7 days. The resultant cultures were purified using the hyphal tip and/or a single spore technique (Senanayake *et al.* 2020). Macroscopic and microscopic characteristics of the obtained isolates, as well as the color, size, and morphology of the vegetative and reproductive structures, were examined and identified using the taxonomic keys (Naranjo-Ortiz and Gabaldón 2019; Senanayake *et al.* 2020).

A fungal infection was induced using the fungus obtained from the isolation results of the historical sample in Fig. 1. After infection, the samples were stored at 18°C and 70% relative humidity, with detection being carried out every three days to monitor fungal growth. After 30 days, growths and spots were found similar to the historical sample from which the isolates were taken (Fig. 2).

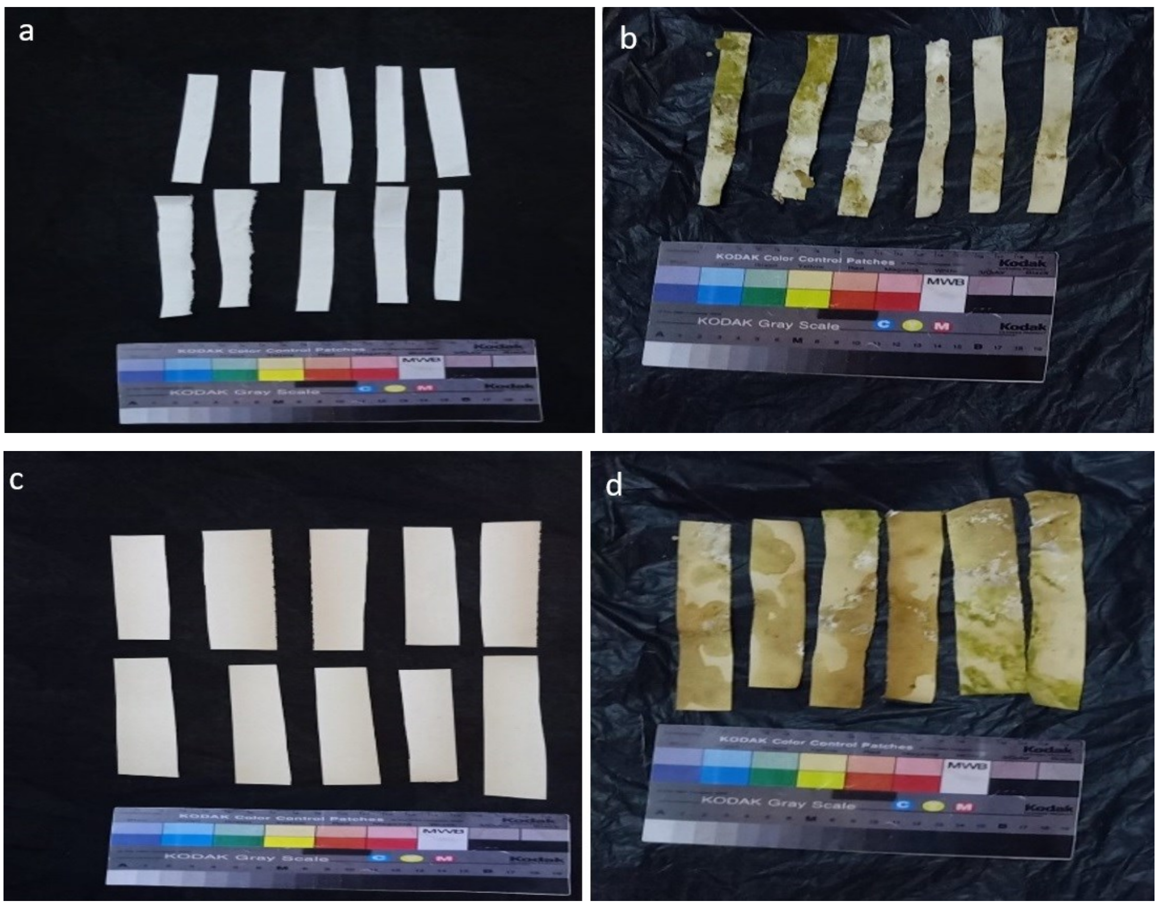


Fig. 2. (A) Standard cotton samples, (B) Cotton samples after fungal damage, (C) Standard mechanical pulp samples, (D) Mechanical pulp after fungal damage

Enzyme Preparation (Solution of Protease - Paste of Protease)

Different types of proteases, which are enzymes that break down proteins, exist. Based on the mechanism of catalysis, proteases are classified into six classes: aspartic, threonine, glutamic, cysteine, metalloproteases, and serine proteases. Protease enzymes used for cleaning under the current study belong to subgroup 4 (Yang *et al.* 2023). In practical applications, the term enzyme unit (U) is more commonly used, which expresses the turnover of 1 micromole of substrate per minute (µmol/min), or which can be defined as the amount of enzyme that catalyzes the transformation of 1 µmol of substrate per minute (Valls *et al.* 2011).

By adding water, hydrolase enzymes hydrolyze molecules; they catalyze the breaking of a variety of chemical bonds, including phosphodiester, ester, glycosidic, and peptide bonds. Usually, a water molecule attacks the substrate in this process, breaking a bond and producing two smaller molecules. The treatment was done in two methods. The first method was with protease enzyme solution with different concentrations, first with a concentration of (50 U) meaning (0.25 g) of enzyme and (100 mL) of sodium bicarbonate 3% (pH=8), and the second with a concentration of (75 U) meaning (37.5 g) and (100 mL) of sodium bicarbonate 3% (pH=8), the third with (100 U) meaning (50 g) and (100 mL) of sodium bicarbonate 3% (pH=8). This material was prepared by using an Ultrasonic sonifier (300 Watt). The second method is by using a paste with different concentrations. Each solution was loaded with hydroxyethyl cellulose (HEC).

Process of Stain Removal

The enzymes must be directly applied to the surface of paper samples that require cleaning once they have been activated. Cotton swabs were used for the solution; they were dipped in it and then moved in a circular motion over the stained region. For the dough, a wooden stick was used to apply a thin layer of paste, which was then removed from the surface after 10 min.

Before the aforementioned application techniques were discovered, the authors carried out numerous first experimental attempts for application, which yielded encouraging results in terms of cleaning efficacy and convenience of use. The authors physically removed the enzyme by thoroughly rinsing the paper samples with water to get rid of both the enzyme and its substrate in order to successfully stop enzyme activity after cleaning. After washing with water, an enzyme inhibitor was used as described by Alsanosi *et al.* (2014).

Fig. 3. Enzyme preparation: (A) preparation of solution, (B) paste concentration of 50 U, (C) paste concentration of 100 U

Examinations and Analyses Before and After Cleaning

Digital microscope

A Compact Video Microscope (CVM, SDL, International Ltd., England) was deployed to investigate the surface of the experimental papers before and after treatment.

Color Change Measurements

The changes in the color values L^*, a^*, and b^* (CIELAB system) were recorded with a MiniScan Model (No. EZ MSEZ0693). The L^* index refers to the black-to-white color scale, ranging from 0 (black) to 100 (white). The a^* index represents the green-to-red axis, typically ranging from –128 (green) to +127 (red). The b^* index corresponds to the blue-to-yellow axis, also ranging from –128 (blue) to +127 (yellow). The overall change in color indices due to aging was expressed as ΔE according to the following formula (Eldeeb *et al.* 2022),

$$\Delta E = \sqrt{(\Delta L^*)^2 + (\Delta a^*)^2 + (\Delta b^*)^2} \quad (1)$$

where $(\Delta L^*)^2$, $(\Delta a^*)^2$, and $(\Delta b^*)^2$ are the squared differences between the values of the color indices before and after damage.

Measuring the pH

The pH values were measured using the hot extraction method (ASTM D778-97 2007) at room temperature using a 55 Digital Mini-pH-Meter (VWR Scientific). A total of 0.5 g of each sample was placed in 50 mL of deionized water for 1 h before the measurement.

ATR-FTIR Spectral Analysis

FTIR-ATR (attenuated total reflection) was deployed to characterize the chemical composition and any changes after cleaning. The samples were analyzed with an FTIR spectrometer (Model 6100; Jasco, Tokyo, Japan). The spectra were obtained in the transmission mode with a TGS detector using the KBr method and represent (2 mm/s) co-added scans within the spectral region ranging from 4000 to 400 cm^{-1} with a resolution of 4 cm^{-1} (Abdel-Hamied *et al.* 2024).

Scanning Electron Microscope

To examine the morphology of both treated and control samples, a scanning electron microscope (Zeiss LEO 1550VP; Carl Zeiss AG, Oberkochen, Germany) equipped with an Edwards Scan Coat K550X sputter coater (Gordon Brothers, Boston, MA, USA) was employed at Asyut University.

Statistical Analysis

The pH measurements' data were subjected to one-way ANOVA. Duncan's Multiple Range Test was used to compare means.

RESULTS AND DISCUSSION

Isolation of the Fungal Strain

Figure 4 shows that *Aspergillus flavus* was found to be the dominant fungus on the historical paper, which caused the spots to appear. *A. flavus* is capable of producing a large number of extracellular hydrolases. The majority of research on *A. flavus* hydrolases has concentrated on amylase, pectinase P2c, and serine and metalloproteinases. Although the control of hydrolase secretion is intricate and substrate-dependent, many hydrolases are thought to have a role in nutrient uptake and polymer breakdown (Mellon *et al.* 2007).

Digital Microscopic Examination

Figure 5 shows that the fungal spots on cotton were lighter than those on wood. The stains had infiltrated the pores of the wood-containing paper but were calcified on the cotton paper's surface. The fungal spots over cotton paper appeared yellow or yellow-brown, while in the case of wood-containing paper, they appear green and black. This could be due to the presence of lignin in the wood-containing pulp, which can interact in a different way with the metabolites of fungi, leading to a different color of the spots between wood-containing paper and cotton paper, which is lignin-free.

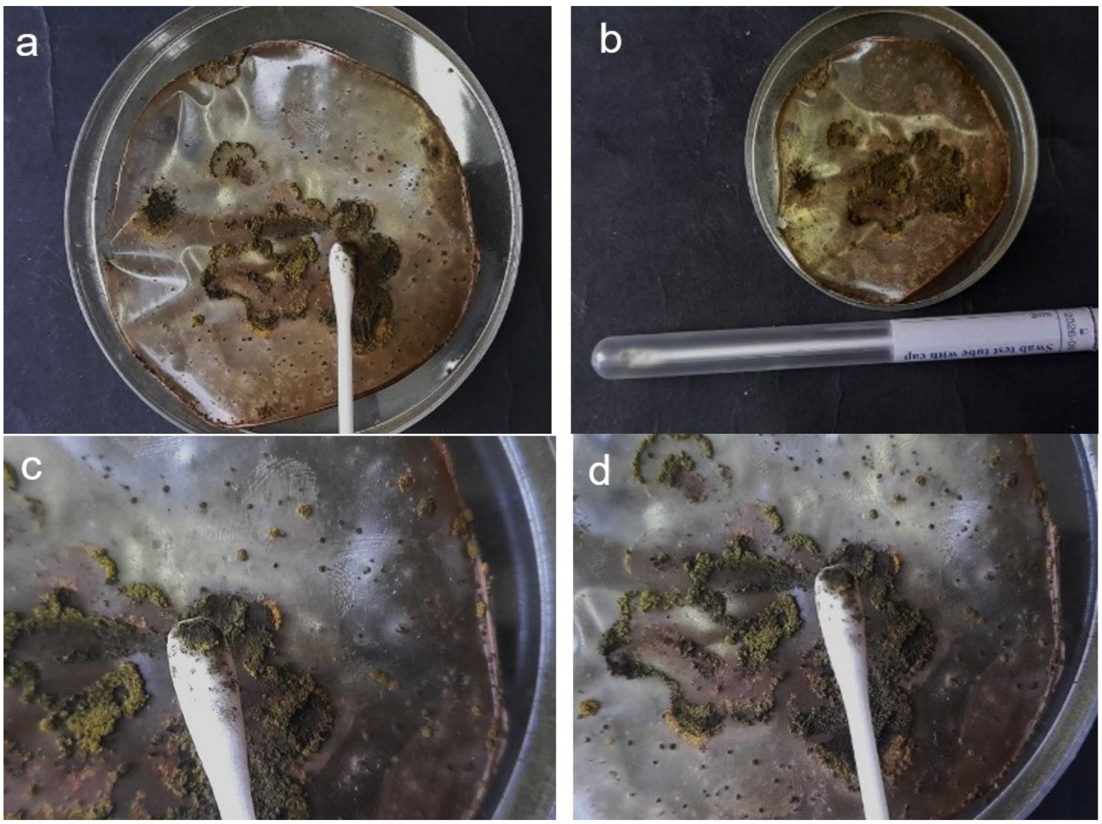
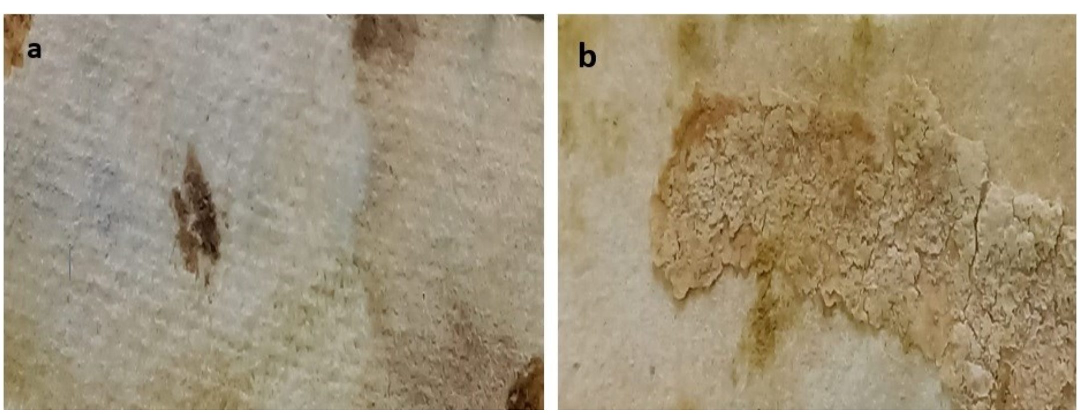


Fig. 4. The stages of isolation and development of fungi within the agar environment are shown in parts a to d



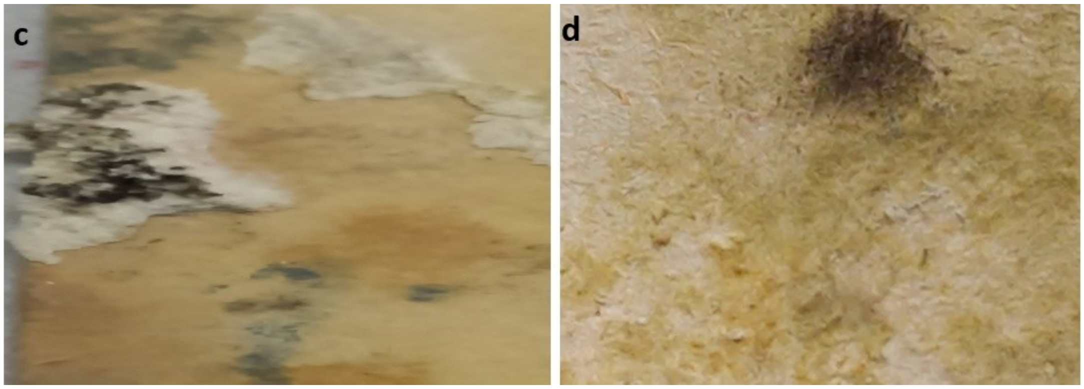


Fig. 5. The stained cotton paper (a,b) and wood-containing paper (c,d)

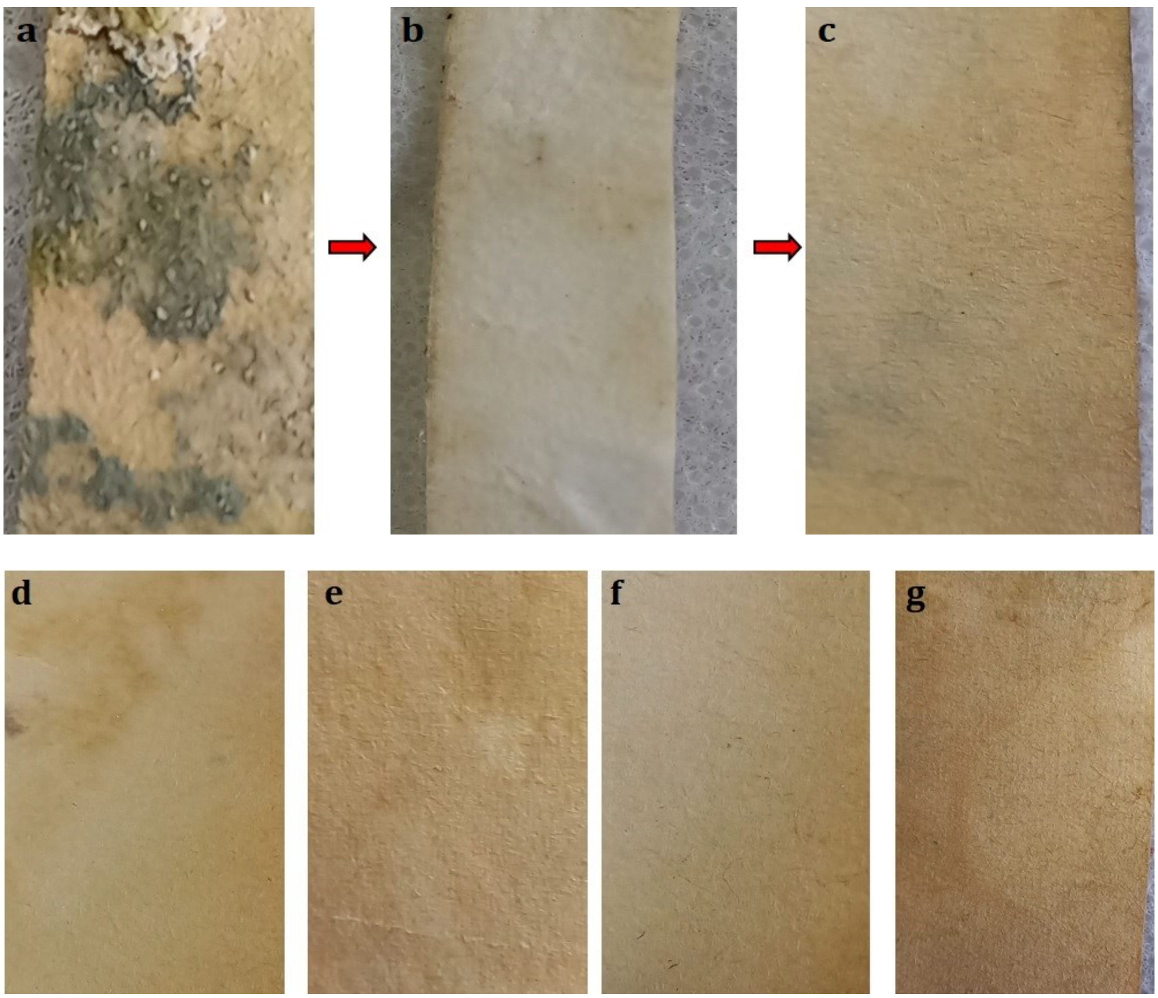
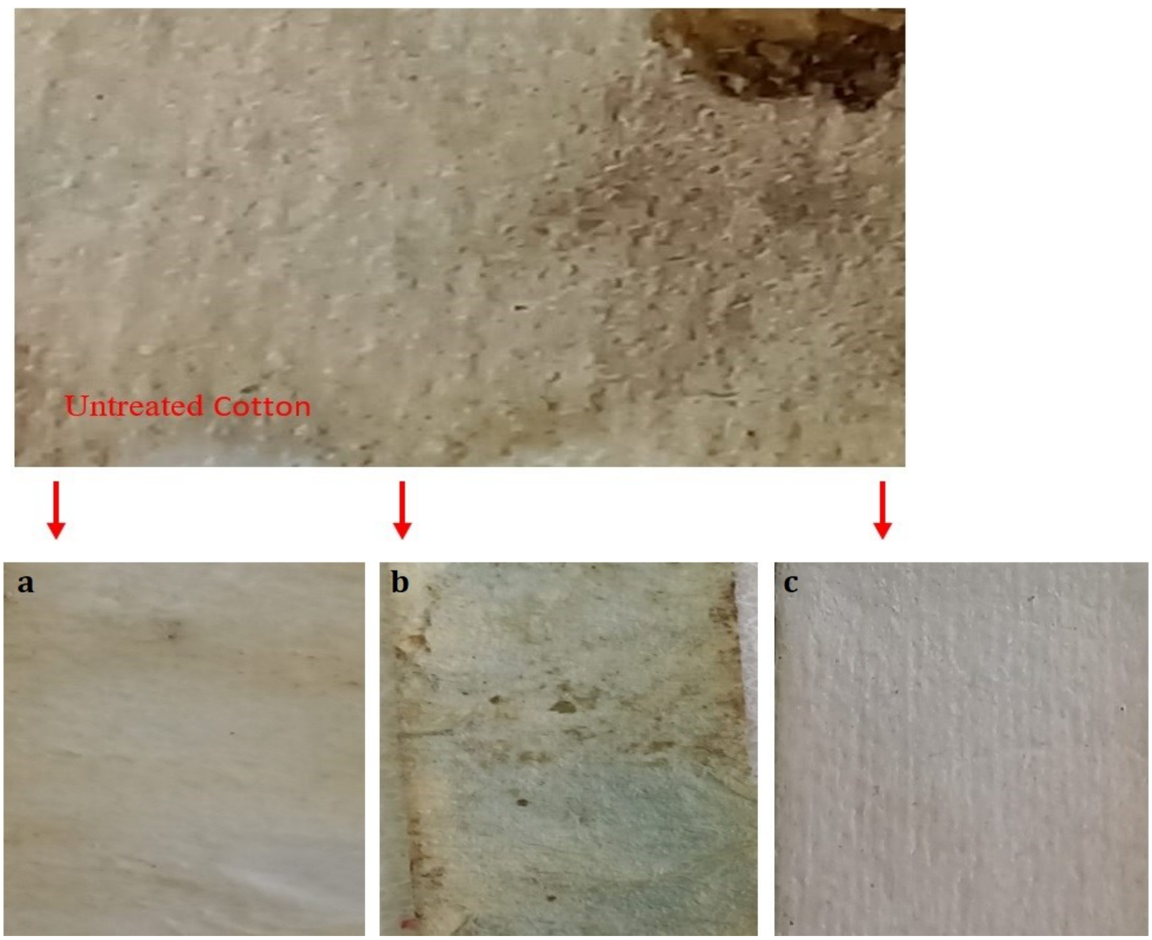


Fig. 6. The fungal stain spots on wood-containing papers before and after cleaning. (a) Stained wood-containing paper; (b) Treated wood-containing paper with the paste of protease enzyme loaded at hydroxyethyl cellulose with a concentration of 100 U of protease; (c) Treated wood-containing paper with protease enzyme solution with concentration of 100 U; (d) Treated wood-containing paper with paste of protease enzyme loaded at hydroxyethyl cellulose with concentration 75 U of protease; (e) Treated wood-containing paper with protease enzyme solution with concentration 75 U; (f) Treated wood-containing paper with paste of protease enzyme loaded at hydroxyethyl cellulose with concentration 50 U of protease; (g) Treated wood-containing paper with protease enzyme solution with concentration 50 U.

Figure 5 shows the calcification of fungal spots of the *A. flavus* fungus on control wood-containing and cotton paper samples. *Aspergillus* fungus produces yellow, yellow-brown, green, and black spots (Christensen 1981; Horn *et al.* 1996; Summerbell 1998). The spots are caused by the fungus's metabolism while it feeds on the cellulose and lignin that make up paper's organic substance (Meyer *et al.* 2020; Pavlović *et al.* 2023). Furthermore, air pollution or pollutants that were present throughout the production process are the cause of some free radicals, such as iron and copper (Pan *et al.* 2019; Yuan *et al.* 2020). The paper is discolored by the metabolic process, which also turns the invisible iron ion dots into colored ones (Pavlović *et al.* 2022; Szczepanowska 2023).

Figure 6 shows the stained wood-containing paper before and after cleaning. The difference showed the protease enzyme paste's ability to remove the fungus stains. In contrast to the enzyme solution, the paste effectively removed stains and improved the paper's lightness and whiteness without harming the paper's fibers. The brighter color after treatment with the paste may be attributed to the fact that it removes all fungi without significant penetration of the enzyme into the pores of the paper (Decoux 2002; Mazzuca *et al.* 2017).



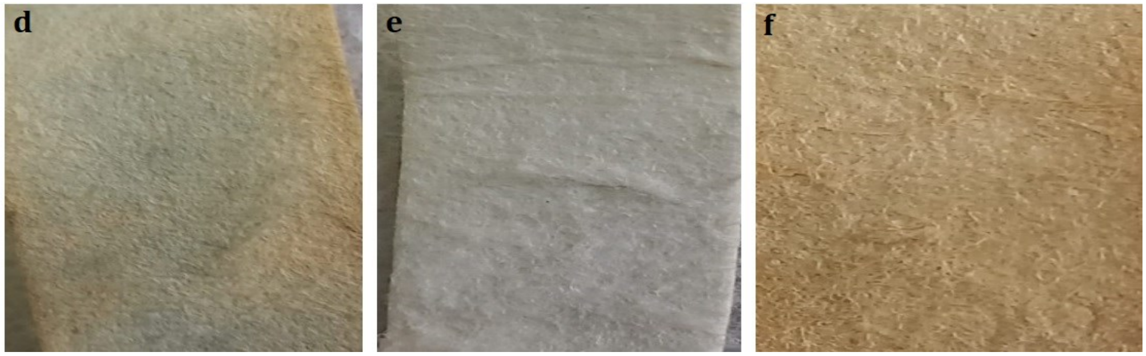


Fig. 7. Stained cotton paper before and after cleaning. (a) Treated with 100 U protease paste; (b) Treated with 100 U protease solution; (c) Treated with 75 U of protease paste; (d) Treated with 75 U of protease solution; (e) Treated with 50 U of protease paste; (f) Treated with 50 U of protease solution

Furthermore, the homogeneity of the paste's composition may be the reason for its efficiency. In addition, the enzyme was loaded onto hydroxyethyl cellulose, which shares a chemical structure with paper. A proportion of water in the solution state caused the paper's fibers to become corroded and to fray and wrinkle, increasing the paper's water content and contributing to the fibers' weakening. Additionally, lightness values increased in every cleaning case; however, the increase was less pronounced at solution and paste concentrations of 100 U than at lower concentrations.

Figure 7 shows the ability of the protease enzyme paste to remove the fungal stains from cotton fibers. However, the protease enzyme solution, in all concentrations, was not effective enough because there were remaining fungal stains on the paper's surface. Additionally, in contrast to the solution, which increases the paper's water content and causes the fibers to deteriorate, the paste improved the color and optical qualities of the paper. In terms of stain removal effectiveness, the paste at a concentration of 100 U was less effective than the lower concentrations. This also applies to the solution with a concentration of 100 U, meaning that higher concentrations are less operative.

Color Change

According to the previous understanding of color change values, fungal green stains on the paper were confirmed, as shown in Table 1, where the (a^*) value of the untreated sample was negative. Additionally, the presence of yellow spots was verified by examining the values of (b^*) for the standard sample, which shows positive values (Table 1). After treatment, the b^* and a^* values of treated samples decreased dramatically, indicating that all concentrations in both cases (solution or paste) effectively reduced yellow and green stains except for concentrations of 75 U (solution) and the paste with a concentration of 50 U. The L^* values, which indicate lightness the paper, showed that only the paste form, in its various concentrations, was effective in increasing the lightness values of treated paper.

Literature states that when fungal growth is present, paper documents typically experience color changes, yellowing, and a decrease in lightness over time (Sterflinger and Pinzari 2012; Kim *et al.* 2019). Acids secreted by fungi cause the paper to become stained and change color. Additionally, they decrease the paper's tensile strength and thereby reduce its mechanical properties (Pessanha *et al.* 2012).

Table 1. Color Changes (Δ*E*) for the Control Cotton Paper and the Treated Ones

Samples	*L**	*a**	*b**	Δ*E*
Control: Stained cotton paper	75.66	-0.11	23.85	-
Treated with solution 50 U	72.78	5.58	19.51	7.68
Treated with solution 75 U	74.23	-1.43	10.00	13.19
Treated with solution 100 U	71.93	1.74	7.51	16.84
Treated with paste 50 U	85.16	-0.09	6.08	20.15
Treated with paste 75 U	88.1	0.48	9.97	18.95
Treated with paste 100 U	87.40	0.59	9.78	18.34

Additionally, the values of *a** and *b** for the treated sample were slightly lower than those for the reference. This change is apparent in Table 2, which represents the wood-containing sample. The enzyme treatment with a 100 U concentration and the paste treatment with a 75 U concentration produced the best results for wood-containing samples because, in contrast to the other treatments, they were able to lessen the yellowness of the treated papers *vs*. the control.

All treatments are successful in raising paper lightness, according to the *L** values in Table 2, which represents paper lightness. In the case of wood-containing paper, the paste in varying concentrations was more successful than the solution at raising the lightness values.

Table 2. Color Change (Δ*E*) for the Control Wood-containing Paper and the Treated Paper

Samples	*L**	*a**	*b**	Δ*E*
Control: Stained wood-containing paper	72.58	3.20	15.14	-
Treated with solution 50 U	74.26	4.21	19.63	4.90
Treated with solution 75 U	75.64	4.83	22.08	7.76
Treated with solution 100 U	72.79	0.14	14	3.09
Treated with paste 50 U	78.94	3.63	19.71	7.84
Treated with paste 75 U	80.19	2.83	18.11	8.21
Treated with paste 100 U	78.87	3.87	19.10	7.46

Measurements of pH

The pH value appropriate for fungal growth lies between 4 and 7 (Pichler *et al.* 2021), and the fungal infection causes the fungus to release a variety of acids, which progressively raises the paper's acidity. For instance, citric acid, which is produced by the *Aspergillus* fungus utilized in the study, effectively raises acidity.

Upon closely examining the pH values in the following Tables 3 and 4, the stained samples' pH values, whether made of cotton or wood-containing pulp, ranged from 4 to 5. Following treatment with protease, the enzyme decreased the acidity of the treated paper observably. This can be attributed to the effectiveness of the enzyme in removing fungal stains, along with the fact that enzyme preparation requires the addition of alkaline sodium bicarbonate, which enhances the enzyme's ability to deacidify the treated paper. The pH values of the treated paper rose, particularly for cotton, indicating direct relationships between the concentration of the enzyme and the rise in the samples' alkalinity. The composition of wood-containing paper, which is naturally more acidic than cotton because of its lignin content (Pinzari *et al.* 2012), is the reason why the level of improvement in cotton's acidity was more than that of wood-containing paper. Ultimately, it is important to highlight how well the enzyme neutralizes the acidity of the treated paper using its concentrations and various techniques (whether paste or solution).

Table 3. The pH Values of Stained Cotton Paper after Cleaning

Treatment	pH value
Control: Stained cotton paper	4.62±0.012^{e}
Treated sample with solution 50 U	6.94±0.008^{d}
Treated sample with solution 75 U	9.74±0.014^{a}
Treated sample with solution 100 U	9.85±0.023^{a}
Treated sample with paste 50 U	7.11±0.065^{c}
Treated sample with paste 75 U	7.25±0.011^{b}
Treated sample with paste 100 U	7.33±0.088^{b}

Values are means ± SE; SE: Standard error; Means with the same letter are not significantly different.

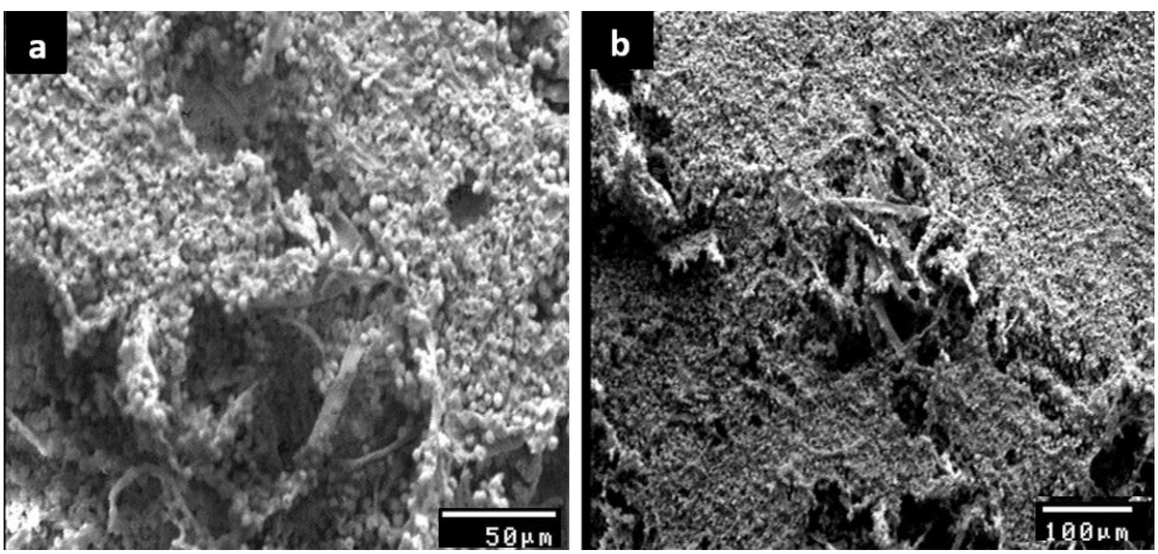
Table 4. The pH Values of Stained Wood-containing Paper after Cleaning

Treatment	pH value
Control: Stained wood-containing paper	4.76±0.032^{e}
Treated sample with solution 50 U	7.50±0.008^{bc}
Treated sample with solution 75 U	7.22±0.014^{d}
Treated sample with solution 100 U	7.65±0.026^{a}
Treated sample with paste 50 U	7.57±0.012^{ab}
Treated sample with paste 75 U	7.44±0.029^{c}
Treated sample with paste 100 U	7.61±0.059^{a}

Values are means ± SE (standard error); Means with the same letter/s are not significantly different.

SEM

Fungal hyphae were able to penetrate and spread within the paper fibers, as shown in the micrographs of stained cotton (Fig. 8).



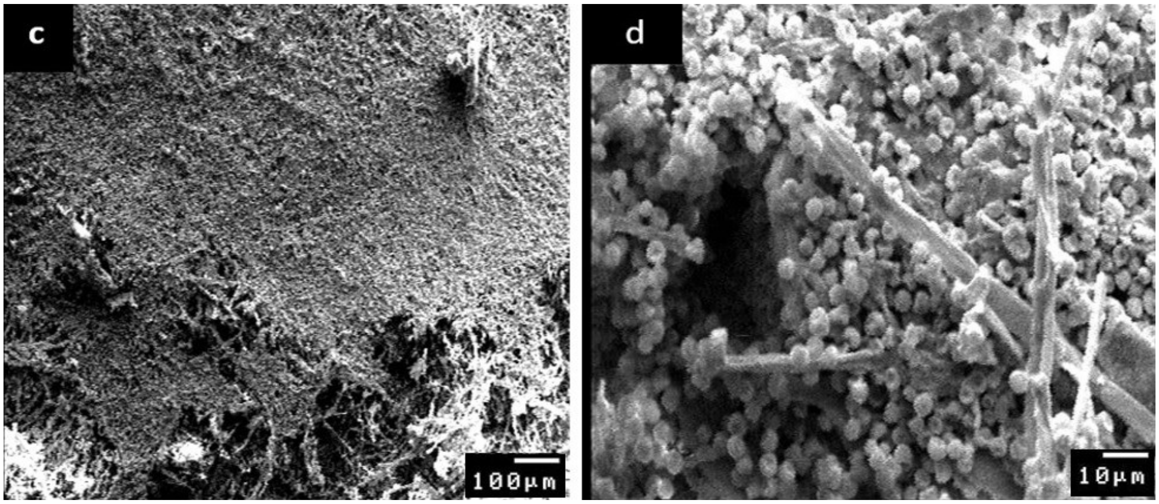


Fig. 8. Cotton paper infected with *Aspergillus flavus* showing spread of fungal spores within paper

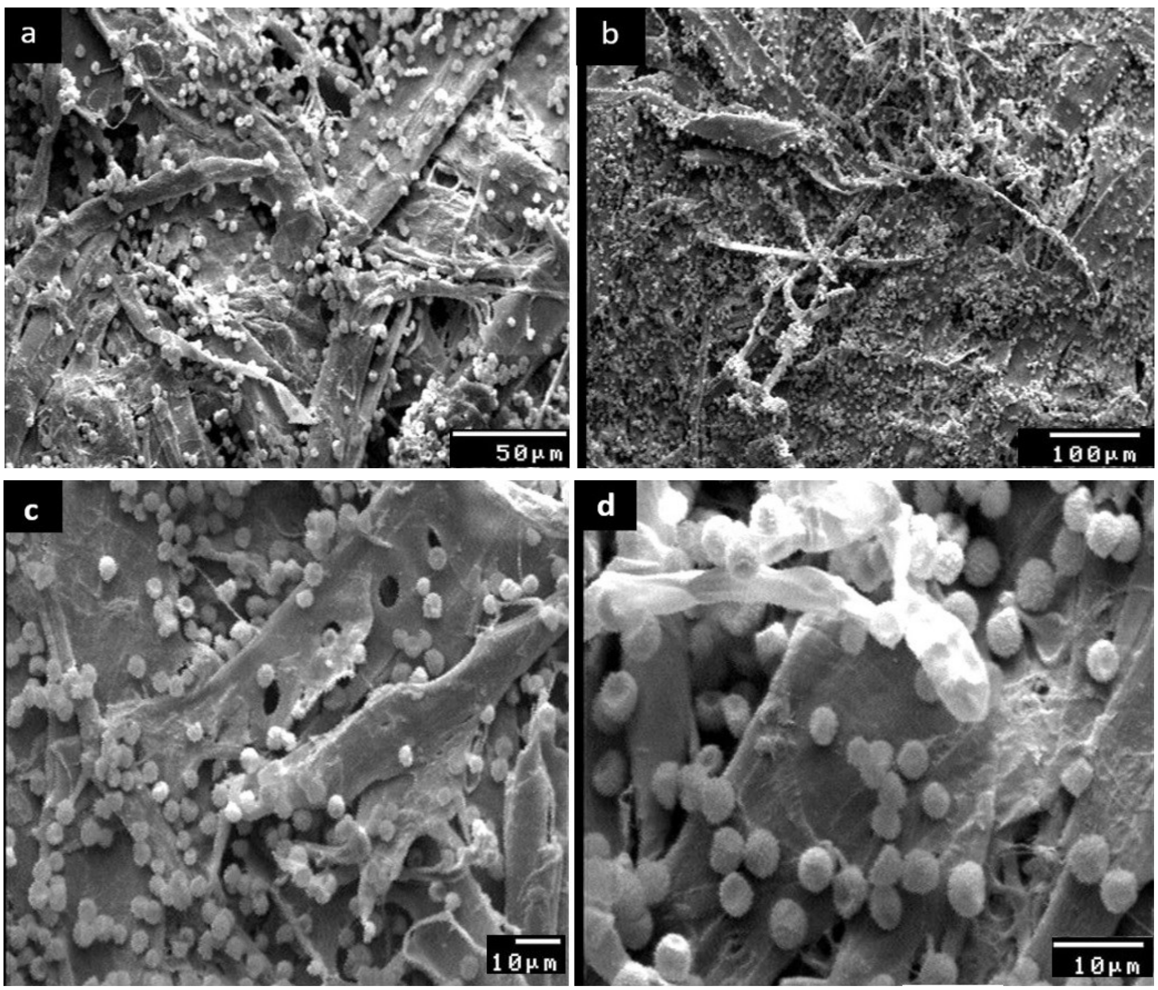
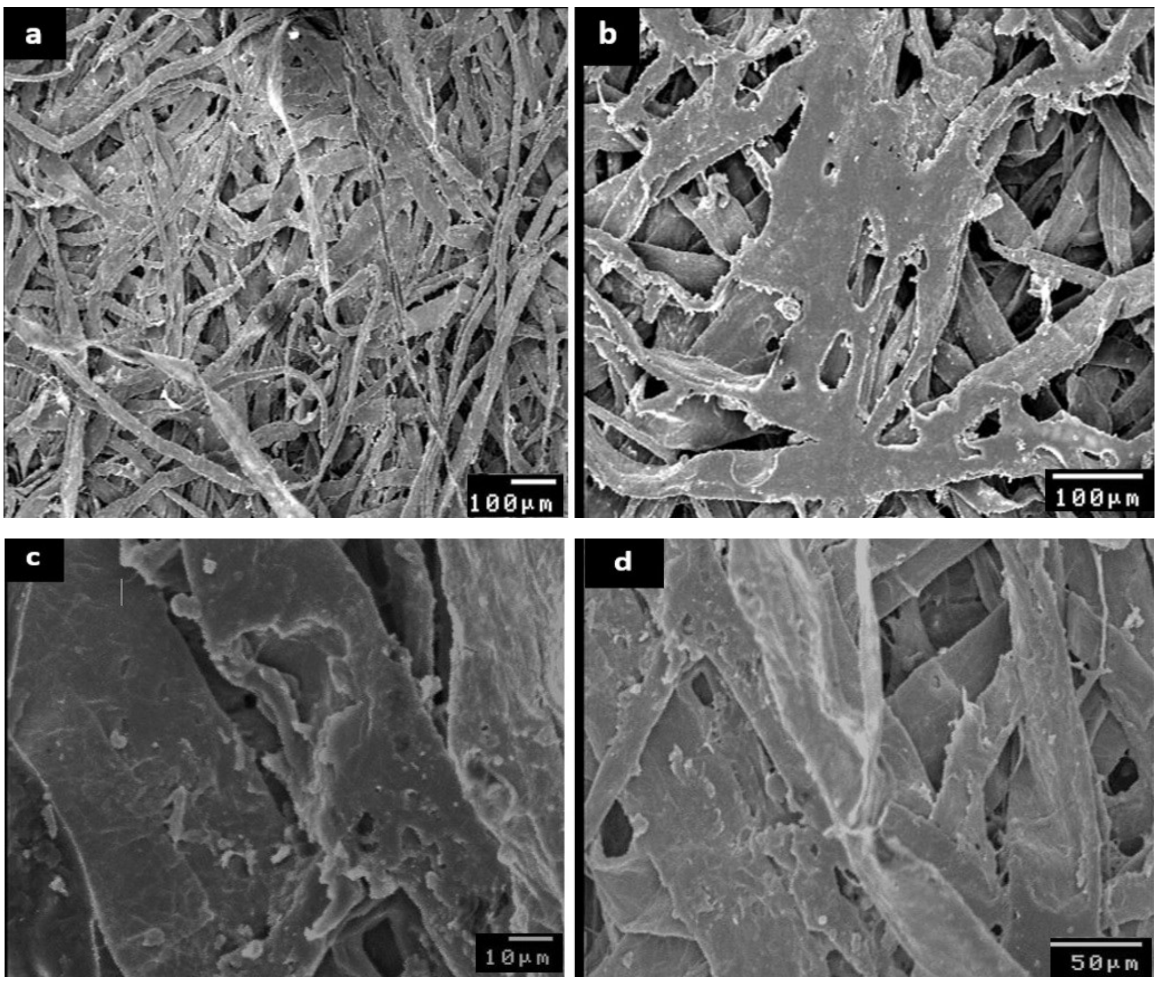


Fig. 9. The wood-containing paper infected with *Aspergillus flavus* with the spread of fungal spores within the paper structure

The impact of fungal infection on paper fibers is apparent, with separations, extensive rips, and tears created in the fiber cells. Additionally, the stained wood-containing paper, as shown in (Fig. 9), reveals the presence of ruptures along with the proliferation of fungal hyphae within the paper fibers, in addition to the holes and breaks inside the fiber cells.

Upon examining treated samples, cotton, and wood-containing papers, with paste 75 U and 100 U (Figs. 10 and 11), the hyphal branches disappeared between the fibers. However, the efficiency of the paste was better in the wood-containing paper as compared to cotton. Despite the obvious success of the paste, it did not negatively affect the fibers. On the contrary, the fibers appeared cohesive and integrated.

The effectiveness of protease in eliminating the fungus from the inner paper structure is demonstrated by microscopic analysis. Proteases are crucial enzymes in numerous essential physiological functions and have a broad range of current and potential industrial uses. Thermostable alkaline protease 50a was previously described as having potential as an eco-friendly enzymatic dehairing agent of animal skins and as an ingredient for laundry detergent (Zhou *et al.* 2018; Ibrahim *et al.* 2020).



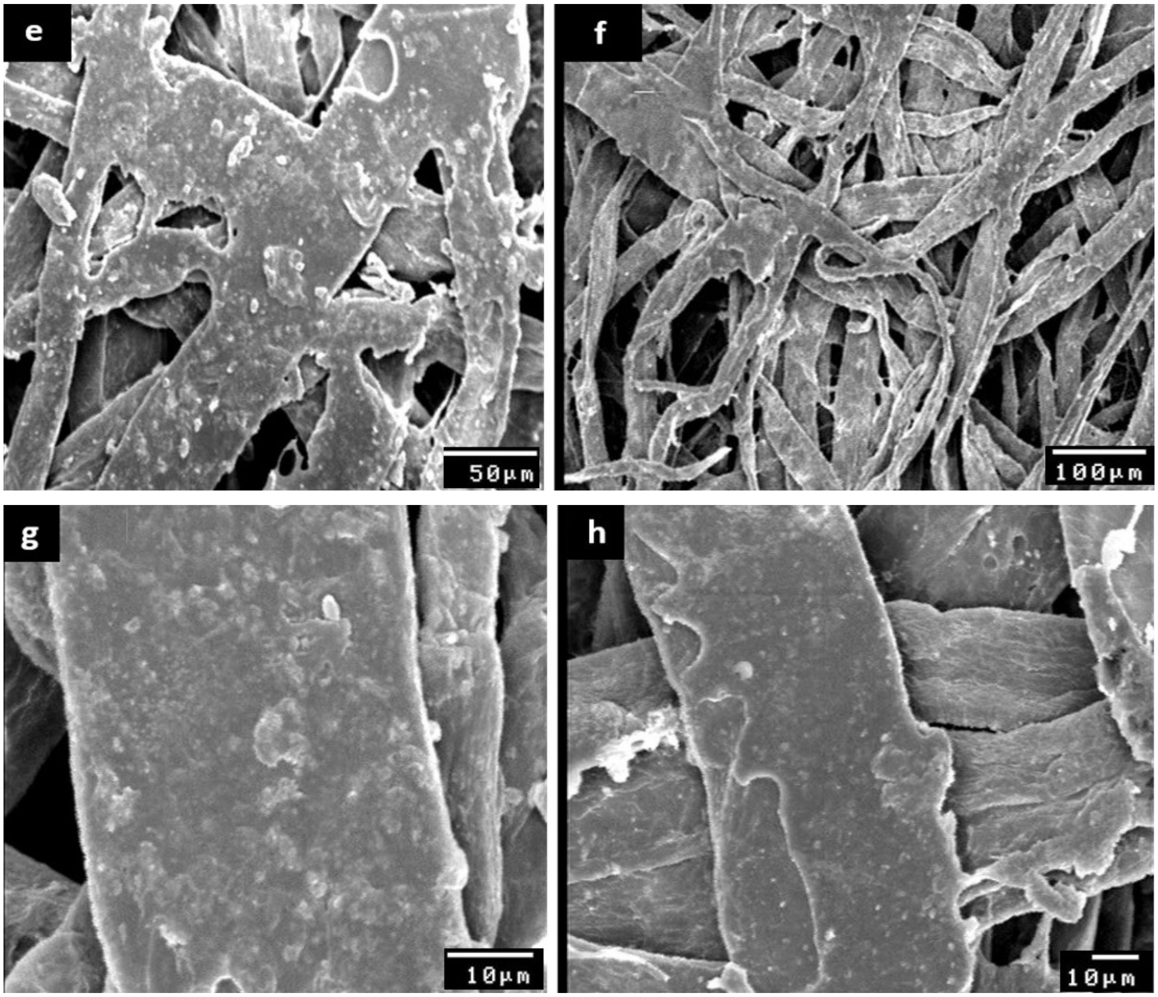
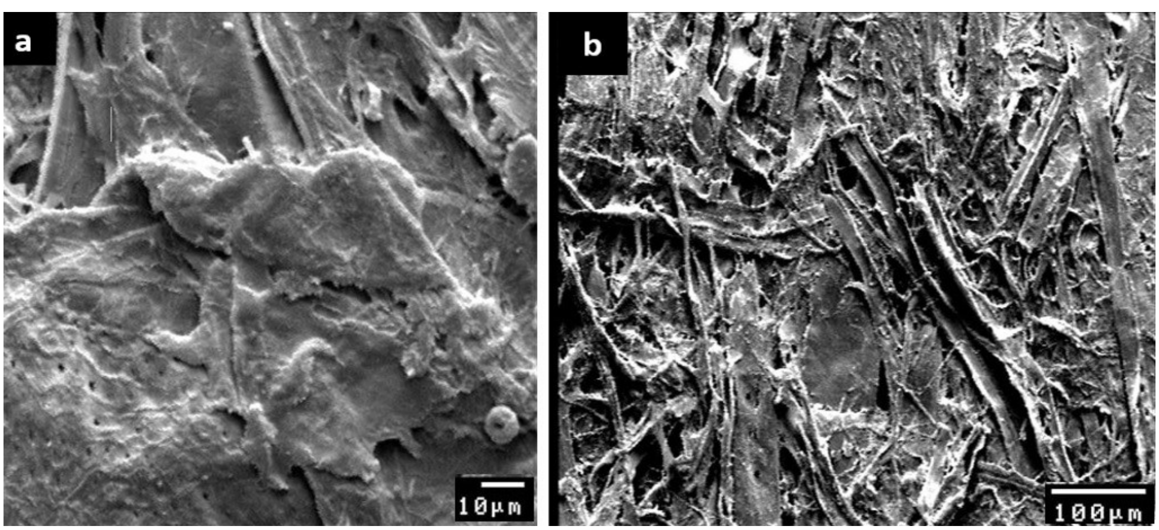


Fig. 10. Cotton paper treated with paste at a concentration of 75 U (a-d); The effect of cleaning is shown by using the paste at a concentration of 100 U (e-h), where the fungi and fungal colonies disappeared entirely without a damaging effect on the structure of the paper.



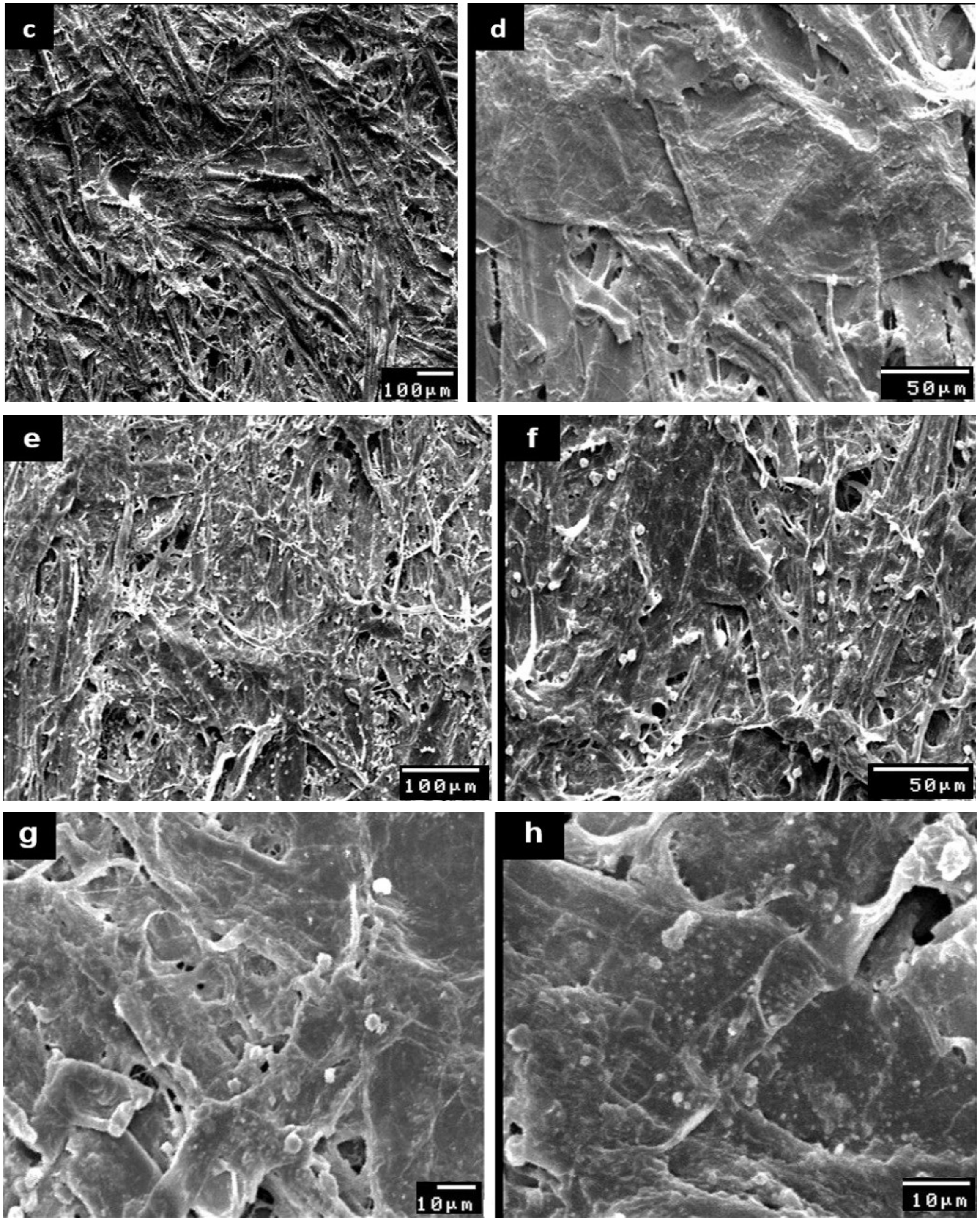


Fig. 11. The wood-containing paper after cleaning with paste at a concentration of 75 U (a-d). The effect of cleaning is shown by using the paste at a concentration of 100 U (e-h), where the fungi and fungal colonies disappeared entirely without a damaging effect on the structure of the wood-containing paper.

Table 5. The Intensities of FTIR Spectra of Untreated, Treated Samples and Un-stained Samples

Functional Group		Un-stained cotton	Stained cotton	Treated cotton with paste 75 U	Treated cotton with paste 100 U	Un-stained wood-containing paper	Stained wood-containing paper	Treated wood-containing paper with paste 75 U	Treated wood-containing paper with paste 100 U
O-H stretching (3400-3409 cm^{-1})	Wavenumber cm^{-1}	3268.2	3268.0	3334.5	3333.9	3268.0	3268.2	3340.4	3334.7
	Intensity	692	655	804	837	655	692	851	870
O-H bending (1650 cm^{-1})	Wavenumber cm^{-1}		1633.9	1635.3	1635.9	1633.9	1633.5	1634.8	1644.7
	Intensity		672	853	927	672	684	925	932
Adsorbed water (weak absorption) of the sample at 1630 cm^{-1}.	Wavenumber cm^{-1}	1633.6	1633.9	1634.5	1558.8	1547.9	1546.5	1559.7	1595.6
	Intensity	684	672	903	940	745	743	928	933
HOH (intramolecular water, 1281 cm^{-1})	Wavenumber cm^{-1}	1376.6	1317.2	1280.5	1280.7	1243.4	1238.9	1265.2	1263.3
	Intensity	738	743	876	894	755	763	884	889
CH bending band (2894-3000 cm^{-1})	Wavenumber cm^{-1}	2923.8	2924.2	2899.6	2900.1	2924.2	2923.7	2895.2	2901.9
	Intensity	763	757	885	907	757	763	913	924
C-O vibrations (1107-1031 cm^{-1})	Wavenumber cm^{-1}	1075.2	1075.5	1052.5	1052.9	1075.5	1075.1	1052.6	1052.6
	Intensity	558	520	556	607	520	558	625	666
	Wavenumber cm^{-1}	1149.0	1148.9	1105.3	1104.8	1149.0	1149	1104	1105.5
	Intensity	702	712	687	735	712	702	742	774
200–900 cm^{-1} (C–O and C–O–P vibrations dominated by polysaccharides and nucleic acids)	Wavenumber cm^{-1}	927.9	929.3	1158.9	1160.1	929.3	927.8	1159.3	1158.7
	Intensity	719	694	794	823	694	719	831	848
	Wavenumber cm^{-1}	1239.0	1243.4	1204.3	1203.9	1019.5	1019	1205.8	_
	Intensity	763	755	891	911	433	460	900	_
Stretching vibration mode of C-O-C in cellulose β-(1-4)- (895-899 cm^{-1})	Wavenumber cm^{-1}	859.1	883.7	894.5	895.7	883.7	859	896.2	898.1
	Intensity	732	720	788	821	720	732	837	848
C-H stretching vibrations Methylene –CH asymm. (2915 cm^{-1})	Wavenumber cm^{-1}	2853.8	2916.8	_	_		2916.7	_	_
	Intensity	810	.811	_	_		.810	_	_
C-H (1600 cm^{-1}) (1048 cm^{-1})	Wavenumber cm^{-1}	1075.2	1019.5	1028.2	1029.4	1019.5	1019	1028.9	1029.1
	Intensity	558	433	538	588	433	460	602	640

ATR-FTIR Spectral Analysis

Table 5 presents the ranges and intensities of the typical functional groups found in paper, including unstained cotton and wood-containing paper, as well as in stained and treated samples. In the stained samples, the intensities of these functional groups were lower than their typical levels, while the treated samples showed increased intensities.

Figure 12 illustrates the characteristic absorbance bands of the unstained wood-containing paper, including the band at 1546 cm^{-1}, which is attributed to aromatic C=C deformation; the band at 3268.2 cm^{-1}, corresponding to O–H stretching vibrations; and the band around 1633.5 cm^{-1}, associated with the C=C bond in lignin molecules (Davis and Mauer 2010). Additionally, the cellulose absorbance region can be observed between 1376 cm^{-1} and 859 cm^{-1}. This includes absorbance peaks corresponding to O–H at 900 cm^{-1}, C–H at 1075 cm^{-1}, C–OH at 1238 cm^{-1}, C=O at 1165 cm^{-1}, and =CH_2 at 1200 cm^{-1}.

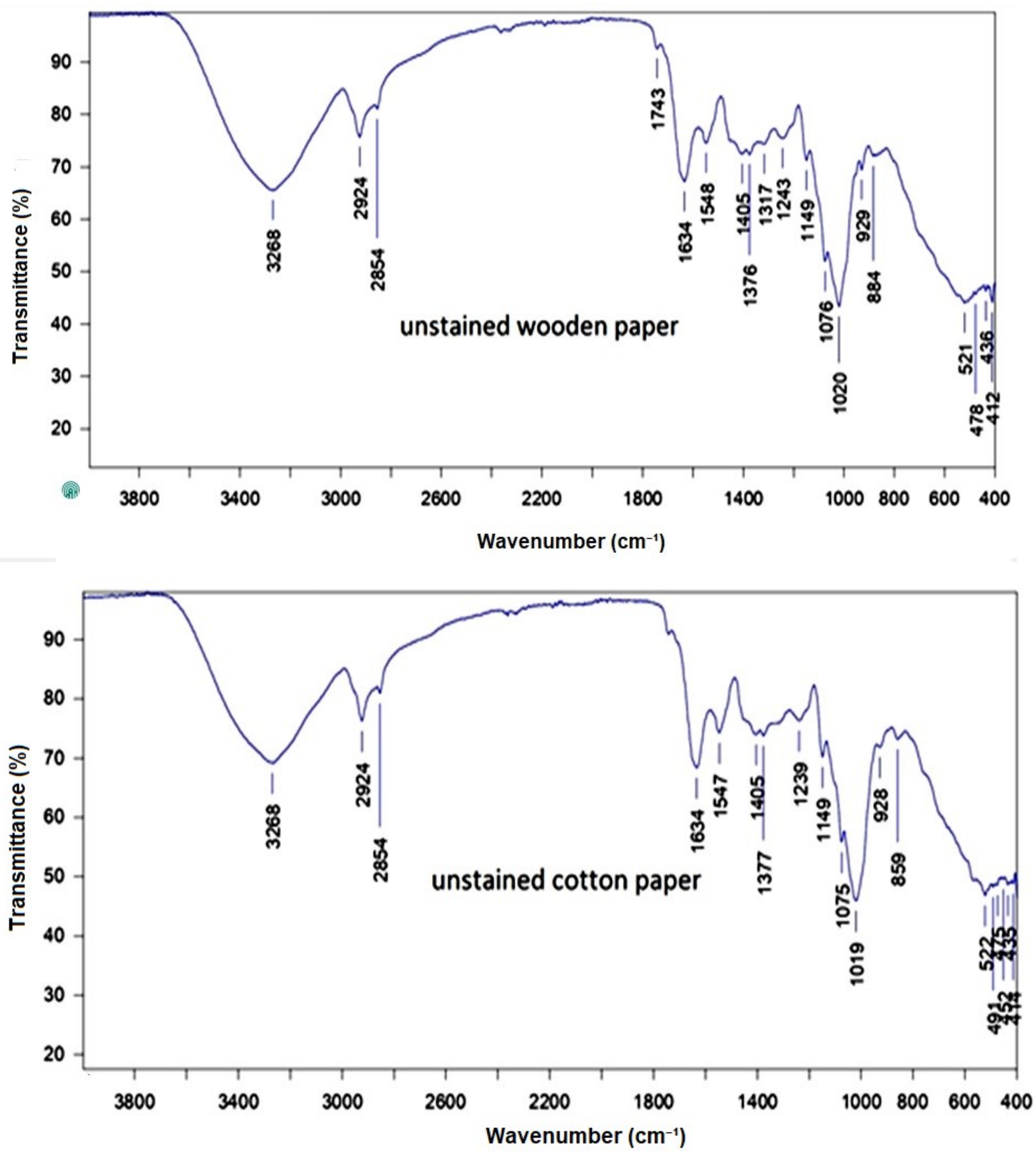


Fig. 12. The spectra for never-stained cotton paper and wood-containing paper

These functional groups are all characteristic of cellulose and are present in cotton paper. In addition to O-H bending at 1650 cm^{-1} shifting to 1633 cm^{-1} for stained cotton and wood-containing paper, the location of O-H stretching (3400 cm^{-1} for cellulosic papers) drastically fell to 3268 and 3268 cm^{-1} for the stained cotton and wood-containing samples, respectively. After cleaning, the intensity of O-H stretching increased to 804 cm^{-1} and 837 cm^{-1} for the treated cotton at concentrations of 75 U and 100 U of paste, respectively; in the wood-containing sample, the intensity of O-H stretching dramatically increased to approximately 851 and 870 cm^{-1} *vs*. 692 cm^{-1} for the stained wood-containing paper samples as humidity increased (Lyu *et al.* 2019; Mansour *et al.* 2021; Eldeeb *et al.* 2022).

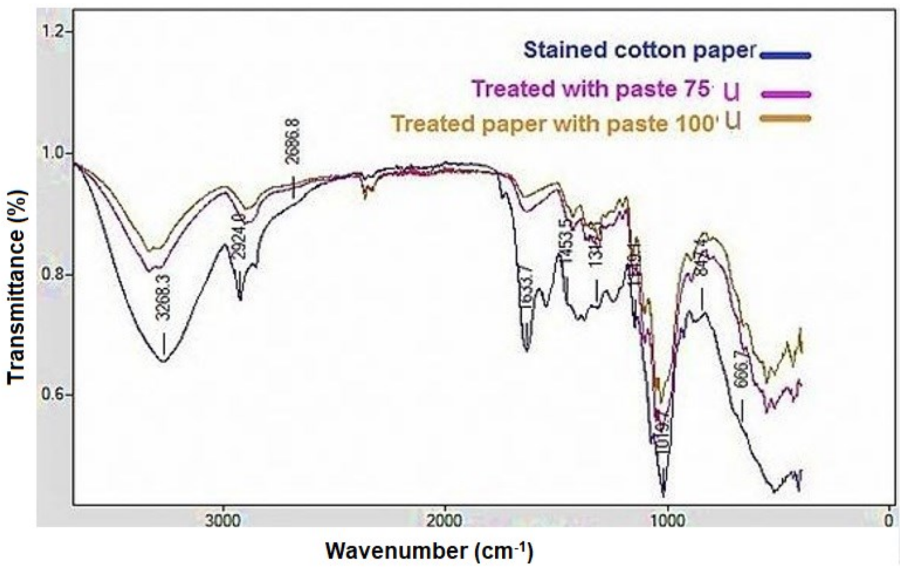


Fig. 13. FTIR spectra of stained cotton paper and the treated paper with paste at 75 U and 100 U

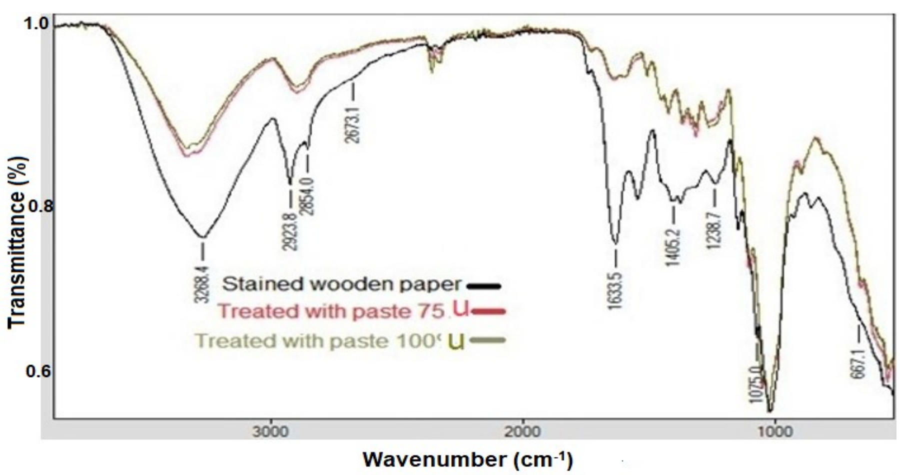


Fig. 14. FTIR spectra of stained wood-containing paper and treated paper with paste 75 U and 100 U

A noticeable intensity increase of the O-H vibration at 1633 cm^{-1} was observed in the amorphous region, as shown in Table 5 and Figs. 12 and 13; after cleaning, there was an increase at 1107 and 1031 $cm^{-1,}$ which belongs to crystalline cellulose (cellulose I).

It was reported that the infection by fungi promotes the breakage of the C-O-C bonds of crystalline cellulose I in the –(1-4)-glycosidic linkage at 895 to 899 cm^{-1}

(Lockington and Kelly 2002). However, following treatment, this peak's intensity increased, which may indicate that amorphous cellulose is changing and ready to crystallize.

Additionally, the figures show a dramatic decline in C–O and C–O–P vibrations dominated by polysaccharides and nucleic acids at 1200 to 900 cm^{-1} for *A. flavus* (Lu *et al.* 2017), which indicates the effectiveness of the enzyme in reducing the spectrum of C–O of *A. flavus*.

The elimination of multiple distinctive bands of *Aspergillus flavus* is one of the strong signs that the enzyme paste is effective at cleaning. For instance, the O-H stretching band completely vanished for treated samples while appearing at 2853.8 and 2853.7 cm^{-1} for stained cotton and hardwood-containing paper, respectively. One of the noteworthy observations is the disappearance of the C-H (sp3 C-H) bond of alkanes giving absorption at about 2850 cm^{-1} which can be attributed to the protease enzyme's ability to remove the methylene group of filamentous fungi (O'Brien Heath *et al.* 2005).

According to previous infrared spectra studies, the treatment utilizing both concentrations removed fungal growth from their bases in addition to stains. Interestingly, after cleaning, the cellulose structure was reinforced, and the internal wetness of the fibers increased, while the chemical structure of cellulose remained unaltered.

CONCLUSIONS

1. The protease treatment of paper was evaluated, and the results showed that it is a promising technique that could have a bright future in the field of conserving paper artwork.
2. The fungus had a significant effect on the fibers of the papers, and the fungal spread was visible both within the fibers and on the surface of the papers. The type of chemical makeup of the spot's carrier and the medium that the fungus feeds on determines the color of the spot that *Aspergillus flavus* produces. Although the fungus creating the spots is the same and the surrounding conditions were the same, the light microscope investigation revealed that the *A. flavus* stains on cotton paper appeared green, while those on wood-containing paper seemed brown. Additionally, the optical microscope demonstrated that, in contrast to the paste, which was successful in removing the fungal stains that penetrated the paper fibers, the enzyme treatment in the form of a solution was successful in removing the fungal stains from the surface without entering the fibers.
3. Following treatment, the fibers were white, as evidenced by the full disappearance of the fungal spot trace under a digital microscope. The enzyme paste with concentrations of 50 U and 75 U produced the best results. By measuring the pH values, it was discovered that the fungus-infected paper had a high level of acidity because the fungus produced some acids. However, after cleaning, whether the enzyme had been supplied as a solution or paste, the protease greatly decreased the acidity, with the best results occurring in the case of the paste with concentrations of 50 U and 75 U rather than 100 U.
4. The color shift demonstrated that the fungus was effective in lowering the damaged paper's lightness values, but the treatment significantly increased them, particularly in

the paste treatment with concentrations of 50 U and 75 U as contrasted to 100 U. Also hyphae and fungal colonies vanished after enzyme treatment, confirming that the removal was not only superficial but also eradicated the internal fungal colonies in treated paper (both cotton and wood-containing paper).

5. A scanning electron microscope analysis verified the effectiveness of the protease in getting rid of internal fungi. Employing the protease to clean paper artwork that has fungal diseases shows promise, warranting more studies in the field of paper conservation.

REFERENCES CITED

Abd El Monssef, R. A., Hassan, E. A., and Ramadan, E. M. (2016). "Production of laccase enzyme for their potential application to decolorize fungal pigments on aging paper and parchment," *Ann. Ag. Sci.* 61(1), 145-154. DOI:10.1016/j.aoas.2015.11.007

Abdel-Hamied, M., Hassan, H. M., Mohamed, Y. A., Ismail, M. E. M., Farid, Y., Mohamed, H., Ismail, S. H., Salem, M. Z. M., and Hassan, R. R. A. (2024). "Hybrid nanogels and their roles in eliminating soot stains from historical paper manuscripts," *Heritage Science* 12(1), article 104. DOI: 10.1186/s40494-024-01216-2

Abdel-Maksoud, G., Abdel-Nasser, M., Sultan, M. H., Eid, A. M., Alotaibi, S. H., Hassan, S. E.-D., and Fouda, A. (2022). "Fungal biodeterioration of a historical manuscript dating back to the 14th century: An insight into various fungal strains and their enzymatic activities," *Life* 12(11), article 1821. DOI: 10.3390/life12111821

Abdel-Nasser, M., Abdel-Maksoud, G., Abdel-Aziz, M. S., Darwish, S. S., Hamed, A. A., and Youssef, A. M. (2022). "Evaluation of the efficiency of nanoparticles for increasing α-amylase enzyme activity for removing starch stain from paper artifacts," *Journal of Cultural Heritage* 53, 14-23. DOI: 10.1016/j.culher.2021.11.004

Achachluei, M., and Vatankhah, G. (2010). *Structural degradation in archaeological woods found in Shahr-i Sukhta Iran*.

Afifi, H. A. M., Mansour, M. M. A., Hassan, A. G. A. I., and Salem, M. Z. M. (2023). "Biodeterioration effects of three *Aspergillus* species on stucco supported on a wooden panel modeled from Sultan al-Ashraf Qaytbay Mausoleum, Egypt," *Scientific Reports* 13(1), article 15241. DOI: 10.1038/s41598-023-42028-x

Alsanosi, S. M. M., Skiffington, C., and Padmanabhan, S. (2014). "Pharmacokinetic pharmacogenomics," in: *Handbook of Pharmacogenomics and Stratified Medicine*, S. Padmanabhan (ed.), pp. 365-383.

Ashurst, N. (2016). *Cleaning Historic Buildings: v. 1: Substrates, Soiling and Investigation*, Routledge, Abingdon, UK.

Assmann, J. (2011). *Cultural Memory and Early Civilization: Writing, Remembrance, and Political Imagination*, Cambridge University Press, Cambridge, UK.

ASTM-D778-97 (2007). "Standard test methods for hydrogen ion concentration (pH) of paper extracts (hot-extraction and cold-extraction procedures)," ASTM International, West Conshohocken, PA, USA.

Baty, J. W., Maitland, C. L., Minter, W., Hubbe, M. A., and Jordan-Mowery, S. K. (2010). "Deacidification for the conservation and preservation of paper-based works: A review," *BioResources* 5(3), 1955-2023. DOI: 10.15376/biores.5.3.1955-2023

Bertrand, L., Schöder, S., Joosten, I., Webb, S. M., Thoury, M., Calligaro, T., Anheim, É., and Simon, A. (2023). "Practical advances towards safer analysis of heritage samples and objects," *TrAC Trends in Analytical Chemistry* 164, article 117078. DOI: 10.1016/j.trac.2023.117078

Bertrand, L., Schöeder, S., Anglos, D., Breese, M. B. H., Janssens, K., Moini, M., and Simon, A. (2015). "Mitigation strategies for radiation damage in the analysis of ancient materials," *TrAC Trends in Analytical Chemistry* 66, 128-145. DOI: 10.1016/j.trac.2014.10.005

Bicchieri, M., Biocca, P., Colaizzi, P., and Pinzari, F. (2019). "Microscopic observations of paper and parchment: The archaeology of small objects," *Heritage Science* 7(1), 1-12. DOI: 10.1186/s40494-019-0291-9

Bicchieri, M., and Pinzari, F. (2016). "Discoveries and oddities in library materials," *Microchemical Journal* 124, 568-577. DOI: 10.1016/j.microc.2015.09.028

Chiantore, O., and Poli, T. (2021). "Indoor air quality in museum display cases: Volatile emissions, materials contributions, impacts," *Atmosphere* 12(3), article 364. DOI: 10.3390/atmos12030364

Christensen, M. (1981). "A synoptic key and evaluation of species in the *Aspergillus flavus* group," *Mycologia* 73(6), 1056-1084. DOI: 10.1080/00275514.1981.12021441

Cicero, C., Pinzari, F., and Mercuri, F. (2018). "18th Century knowledge on microbial attacks on parchment: Analytical and historical evidence," *International Biodeterioration & Biodegradation* 134, 76-82. DOI: 10.1016/j.ibiod.2018.08.007

Claridge, C. (2017). "Voices in medieval history writing," *Nordic Journal of English Studies* 16(1), 7-40.

Córdoba, R. (2014). "The myth of El Dorado: Making and applying gold in the Iberian Peninsula (15th-16th Centuries)," Retrieved from http://digital.casalini.it/3097436

Davis, R. and Mauer, L. J. (2010). "Fourier transform infrared (FT-IR) spectroscopy: A rapid tool for detection and analysis of foodborne pathogenic bacteria," in: *Current Research, Technology and Education Topics in Applied Microbiology and Microbial Biotechnology*, Mendez-Vilas, A. (ed.), Volume 2, Formatex Research Center, Badajoz, Spain, pp. 1582-1594.

Decoux, S. (2002). "Enzymes used for adhesive removal in paper conservation: A literature review," *Journal of the Society of Archivists* 23(2), 187-195. DOI: 10.1080/0033798102200000006372

Diefenbach, T., Sumetzberger-Hasinger, M., Braunschmid, V., Konegger, H., Heipieper, H. J., Guebitz, G. M., Lackner, M., Ribitsch, D., and Loibner, A. P. (2024). "Laccase-mediated degradation of petroleum hydrocarbons in historically contaminated soil," *Chemosphere* 348, article 140733. DOI: 10.1016/j.chemosphere.2023.140733

Eldeeb, H. M. A., Ali, M. F., Mansour, M. M. A., Ali Ahmed, M. A., and Salem, M. Z. M. (2022). "Monitoring the effects of fungi isolated from archival document on model albumen silver prints," *Microbial Pathogenesis* 169, article 105632. DOI: 10.1016/j.micpath.2022.105632

Eriksen, A. (2014). *From Antiquities to Heritage: Transformations of Cultural Memory* (Vol. 1), Berghahn Books, New York.

Feilden, B. (2007). *Conservation of Historic Buildings*, Routledge, Abingdon, UK.

Fouda, A., Abdel-Nasser, M., Khalil, A. M. A., Hassan, S. E.-D., and Abdel-Maksoud, G. (2022). "Investigate the role of fungal communities associated with a historical manuscript from the 17th century in biodegradation," *NPJ Materials Degradation* 6(1), article 88. DOI: 10.1038/s41529-022-00296-4

Franceschi, E. (2011). "Thermoanalytical methods: A valuable tool for art and archaeology: A study of cellulose-based materials," *Journal of Thermal Analysis and Calorimetry* 104(2), 527-539. DOI: 10.1007/s10973-011-1343-x

Friedrich, M. (2024). "Introduction: Towards a holistic study of written artefacts in ancient history division of labour during the nineteenth century has not only led to the establishment of professional academic disciplines but also to the disjunction of *The Ancient World Revisited: Material Dimensions of Written Artefacts,* 37, 1.

García, J. C. M. (2014). "Ancient states and Pharaonic Egypt: An agenda for future research," *J. of Egyptian History* 7(2), 203-240. DOI: 10.1163/18741665-12340018

Grum-Grzhimaylo, A. A., Georgieva, M. L., Bondarenko, S. A., Debets, A. J., and Bilanenko, E. N. (2016). "On the diversity of fungi from soda soils," *Fungal Diversity* 76, 27-74. DOI: 10.1007/s13225-015-0320-2

Guarner, J., and Brandt Mary, E. (2011). "Histopathologic diagnosis of fungal infections in the 21st century," *Clinical Microbiology Reviews* 24(2), 247-280. DOI: 10.1128/cmr.00053-10

Gurumallesh, P., Alagu, K., Ramakrishnan, B., and Muthusamy, S. (2019). "A systematic reconsideration on proteases," *International journal of Biological Macromolecules* 128, 254-267. DOI: 10.1016/j.ijbiomac.2019.01.081

Hassan, R. R. A., Ali, M. F., Fahmy, A.-G. A., Ali, H. M., and Salem, M. Z. M. (2020). "Documentation and evaluation of an ancient paper manuscript with leather binding using spectrometric methods," *Journal of Chemistry* 2020(1), article 6847910. DOI: 10.1155/2020/6847910

Hassan, R. R. A., Mahmoud, S. M. A., Karam, Y. A., Salah, S. M., Ebrahim, S. Y., Abdelwahab, M. A., Ahmed, A.-H.M.H., Ali, H.M., Böhm, and Salem, M. Z. M. (2021). "Application of frankincense and rice starch as eco-friendly substances for the resizing of paper as a conservation practice," *BioResources* 16(4), 7180-7204. DOI: 10.15376/biores.16.4.7180-7204

Hassan, R. R. A., Mohamed, W. S., Salem, M. Z. M., ElMajd, A. M. O. A., Ebrahim, E. E. M., Naeem, E. M. A., Elsayed, E.G.A.E., and Hassan, R. A. A. (2024). "Cellulose and gellan gum compresses for cleaning mud and pomegranate stains from a historical printed paper," *Discover Applied Sciences* 6(2), article 43. DOI: 10.1007/s42452-024-05658-y

Horn, B. W., L., G. R., S., S. V., W., D. J., H., P. J., and Layton, R. C. (1996). "Association of morphology and mycotoxin production with vegetative compatibility groups in *Aspergillus flavus*, *A. parasiticus*, and *A. tamarii*," *Mycologia* 88(4), 574-587. DOI: 10.1080/00275514.1996.12026688

Houston, S., Baines, J., and Cooper, J. (2003). "Last writing: Script obsolescence in Egypt, Mesopotamia, and Mesoamerica," *Comparative Studies in Society and History* 45(3), 430-479. DOI: 10.1017/S0010417503000227

Hsieh, P., Xu, Q., and Yu, H. (2023). "A new technique for the removal of red fungal stains on traditional Chinese painting on silk," *International Biodeterioration & Biodegradation* 181, article 105622. DOI: 10.1016/j.ibiod.2023.105622

Ibrahim, N. A., Ibrahim, N., Lizawardi, N. S. R., Fauzi, N. F. I., and Al-Amsyar, S. M. (2020). "Production and application of thermostable protease 50a as liquid protein stain remover," *IOP Conference Series: Earth and Environmental Science* 596(1), article 012012. DOI: 10.1088/1755-1315/596/1/012012

Jablonsky, M., Šima, J., and Lelovsky, M. (2020). "Considerations on factors influencing the degradation of cellulose in alum-rosin sized paper," *Carbohydrate Polymers* 245, article 116534. DOI: 10.1016/j.carbpol.2020.116534

Kim, G., Kim, J. G., Kang, K., and Yoo, W. S. (2019). "Image-based quantitative analysis of foxing stains on old printed paper documents," *Heritage* 2(3), 2665-2677. DOI: 10.3390/heritage2030164

Koochakzaei, A., and Mallakpour, S. (2023). "Identification of surface dyeing agents of two bookbinding leathers from 19th-century Qajar, Iran, using LC–MS, µXRF and FTIR spectroscopy," *Archaeometry* 65(3), 603-616. DOI: 10.1111/arcm.12832

Krawczyk, B. (2024). "The state of the art of selected writing materials: A comparative study based on 19th-century sources and contemporary knowledge," *Polish Libraries* 12, 130-160. DOI: 10.36155/PLib.12.00002

Lawman, S. J. (2011). *Optical and Material Properties of Varnishes for Paintings*, Nottingham Trent University, UK.

Lockington, R. A., and Kelly, J. M. (2002). "The WD40-repeat protein CreC interacts with and stabilizes the deubiquitinating enzyme CreB *in vivo* in *Aspergillus nidulans*," *Molecular Microbiology* 43(5), 1173-1182. DOI: 10.1046/j.1365-2958.2002.02811.x

Love, E. (2019). *Innovative Scripts and Spellings in Roman Egypt: Investigations into Script Conventions, Domains, Shift, and Obsolescence as Evidenced by Hieroglyphic, Hieratic, Demotic, and Old Coptic Manuscripts*, University of Oxford, UK.

Lu, Y., Lu, Y.-C., Hu, H.-Q., Xie, F.-J., Wei, X.-Y., and Fan, X. (2017). "Structural characterization of lignin and its degradation products with spectroscopic methods," *Journal of Spectroscopy* 2017(1), article 8951658. DOI: 10.1155/2017/8951658

Lyu, W., Shi, Y., Zheng, Y., and Liu, X. E. (2019). "XPS and FTIR studies of fungus-stained *Daemonorops margaritae*," *Journal of Forestry Research* 30(2), 739-743. DOI: 10.1007/s11676-018-0598-5

Magnússon, S. G., and Olafsson, D. (2016). *Minor Knowledge and Microhistory: Manuscript Culture in the Nineteenth Century*, Routledge, Abingdon, UK.

Małachowska, E., Dubowik, M., Boruszewski, P., Łojewska, J., and Przybysz, P. (2020). "Influence of lignin content in cellulose pulp on paper durability," *Scientific Reports* 10(1), article 19998. DOI: 10.1038/s41598-020-77101-2

Mansour, M. M. A., Ali Hassan, R. R., Moustafa Amer Mahmoud, S., and Akl, Y. M. (2021). "An analytical study on the relationship between the fungal degradation and multi-component nature of paper manuscripts." *Pigment & Resin Technology* 50(4), 309-318. DOI: 10.1108/PRT-07-2020-0074

Mazzuca, C., Poggi, G., Bonelli, N., Micheli, L., Baglioni, P., and Palleschi, A. (2017). "Innovative chemical gels meet enzymes: A smart combination for cleaning paper artworks," *J. Colloid Interface Sci.* 502, 153-164. DOI: 10.1016/j.jcis.2017.04.088

Mellon, J. E., Cotty, P. J., and Dowd, M. K. (2007). "*Aspergillus flavus* hydrolases: Their roles in pathogenesis and substrate utilization," *Applied Microbiology and Biotechnology* 77(3), 497-504. DOI: 10.1007/s00253-007-1201-8

Meng, Q., Li, X., Geng, J., Liu, C., and Ben, S. (2023). "A biological cleaning agent for removing mold stains from paper artifacts," *Heritage Science* 11(1), article 243. DOI: 10.1186/s40494-023-01083-3

Meyer, V., Basenko, E. Y., Benz, J. P., Braus, G. H., Caddick, M. X., Csukai, M., de Vries, R.P., Endy, D., Frisvad, J.C., Gunde-Cimerman, N., Haarmann, T., Hadar, Y., Hansen, K., Johnson, R.I., Keller, N.P., Kraševec, N., Mortensen, U.H., Perez, R., Ram, A.F.J., Record, E., Ross, P., Shapaval, V., Steiniger, C., van den Brink, H., van Munster, J., Yarden, O., and Wösten, H. A. B. (2020). "Growing a circular economy with fungal biotechnology: A white paper," *Fungal Biology and Biotechnology* 7(1), article 5. DOI: 10.1186/s40694-020-00095-z

Moreno García, J. C. (2017). "Trade and power in Ancient Egypt: Middle Egypt in the late third/early second millennium BC," *Journal of Archaeological Research* 25(2), 87-132. DOI: 10.1007/s10814-016-9097-4

Naranjo-Ortiz, M. A., and Gabaldón, T. (2019). "Fungal evolution: Diversity, taxonomy and phylogeny of the fungi," *Biol. Rev.* 94(6), 2101-2137. DOI: 10.1111/brv.12550

Niyonzima, F. N., and More, S. (2015). "Detergent-compatible proteases: Microbial production, properties, and stain removal analysis," *Preparative Biochemistry & Biotechnology* 45(3), 233-258. DOI: 10.1080/10826068.2014.907183

O'Brien Heath, E., Parrent Jeri, L., Jackson Jason, A., Moncalvo, J.-M., and Vilgalys, R. (2005). "Fungal community analysis by large-scale sequencing of environmental samples," *Applied and Environmental Microbiology* 71(9), 5544-5550. DOI: 10.1128/AEM.71.9.5544-5550.2005

Pan, B., Li, H., Lang, D., and Xing, B. (2019). "Environmentally persistent free radicals: Occurrence, formation mechanisms and implications," *Environmental Pollution* 248, 320-331. DOI: 10.1016/j.envpol.2019.02.032

Pariza, M. W., and Johnson, E. A. (2001). "Evaluating the safety of microbial enzyme preparations used in food processing: update for a new century," *Regulatory Toxicology and Pharmacology* 33(2), 173-186. DOI: 10.1006/rtph.2001.1466

Pavlović, J., Farkas, Z., Kraková, L., and Pangallo, D. (2022). "Color stains on paper: Fungal pigments, synthetic dyes and their hypothetical removal by enzymatic approaches," *Applied Sciences* 12(19), article 9991. DOI: 10.3390/app12199991

Pavlović, J., Puškárová, A., Planý, M., Farkas, Z., Rusková, M., Kvalová, K., Kraková, L., Bučková, M., and Pangallo, D. (2023). "Colored stains: Microbial survey of cellulose-based and lignin rich papers," *International Journal of Biological Macromolecules* 241, article 124456. DOI: 10.1016/j.ijbiomac.2023.124456

Pawar, K. S., Singh, P. N., and Singh, S. K. (2023). "Fungal alkaline proteases and their potential applications in different industries," *Frontiers in Microbiology* 14, Article 1138401. DOI: 10.3389/fmicb.2023.1138401

Pessanha, S., Manso, M., and Carvalho, M. L. (2012). "Application of spectroscopic techniques to the study of illuminated manuscripts: A survey," *Spectrochimica Acta Part B: Atomic Spectroscopy* 71-72, 54-61. DOI: 10.1016/j.sab.2012.05.014

Pichler, G., Candotto Carniel, F., Muggia, L., Holzinger, A., Tretiach, M., and Kranner, I. (2021). "Enhanced culturing techniques for the mycobiont isolated from the lichen *Xanthoria parietina*," *Mycological Progress* 20(6), 797-808. DOI: 10.1007/s11557-021-01707-7

Pinzari, F., Colaizzi, P., Maggi, O., Persiani, A. M., Schütz, R., and Rabin, I. (2012). "Fungal bioleaching of mineral components in a twentieth-century illuminated parchment," *Analytical and Bioanalytical Chemistry* 402(4), 1541-1550. DOI: 10.1007/s00216-011-5263-1

Pinzari, F., and Gutarowska, B. (2021). "Extreme colonizers and rapid profiteers: the challenging world of microorganisms that attack paper and parchment," in: *Microorganisms in the Deterioration and Preservation of Cultural Heritage*, E. Joseph (ed.), Springer, Cham, Switzerland, pp. 79-113.

Rylance, K. (1969). "Archives and the Intangible," *Archivaria,* 62, 103-120.

Savitha, S., Sadhasivam, S., Swaminathan, K. and Lin, F. H. (2011). "Fungal protease: Production, purification and compatibility with laundry detergents and their wash performance," *Journal of the Taiwan Institute of Chemical Engineers* 42(2), 298-304. DOI: 10.1016/j.jtice.2010.05.012

Senanayake, I., Rathnayaka, A., Marasinghe, D., Calabon, M., Gentekaki, E., Lee, H., Hurdeal, V., Pem, D., Dissanayake, L., and Wijesinghe, S. (2020). "Morphological approaches in studying fungi: Collection, examination, isolation, sporulation and preservation," *Mycosphere* 11(1), 2678-2754.

Sequeira, S., Cabrita, E. J., and Macedo, M. F. (2012). "Antifungals on paper conservation: An overview," *International Biodeterioration & Biodegradation* 74, 67-86. DOI: 10.1016/j.ibiod.2012.07.011

Sharma, M., Gat, Y., Arya, S., Kumar, V., Panghal, A., and Kumar, A. (2019). "A review on microbial alkaline protease: An essential tool for various industrial approaches," *Industrial Biotechnology* 15(2), 69-78. DOI: 10.1089/ind.2018.0032

Solanki, P., Putatunda, C., Kumar, A., Bhatia, R. and Walia, A. (2021). "Microbial proteases: Ubiquitous enzymes with innumerable uses," *3 Biotech* 11(10), article 428. DOI: 10.1007/s13205-021-02928-z

Sterflinger, K., and Pinzari, F. (2012). "The revenge of time: Fungal deterioration of cultural heritage with particular reference to books, paper and parchment," *Environ. Microbiology* 14(3), 559-566. DOI: 10.1111/j.1462-2920.2011.02584.x

Summerbell, R. C. (1998). "Taxonomy and ecology of aspergillus species associated with colonizing infections of the respiratory tract," *Immunology and Allergy Clinics of North America* 18(3), 549-573. DOI: 10.1016/S0889-8561(05)70022-9

Szczepanowska, H. (2023). *Biodeterioration of Cultural Heritage: Dynamic Interfaces between Fungi, Fungal Pigments and Paper*, MDPI, Basel, Switzerland.

Szczepanowska, H., and Lovett, C. M. (1992). "A study of the removal and prevention of fungal stains on paper," *Journal of the American Institute for Conservation* 31(2), 147-160. DOI: 10.1179/019713692806066664

Szczepanowska, H. M., and Moomaw, W. R. (1994). "Laser stain removal of fungus-induced stains from paper," *Journal of the American Institute for Conservation* 33(1), 25-32. DOI: 10.1179/019713694806066437

Szulc, J., Otlewska, A., Ruman, T., Kubiak, K., Karbowska-Berent, J., Kozielec, T., and Gutarowska, B. (2018). "Analysis of paper foxing by newly available omics techniques," *Int. Biodeter. Biodegrad.* 132, 157-165.DOI:10.1016/j.ibiod.2018.03.005

Taylor, J. (2015). "Embodiment unbound: Moving beyond divisions in the understanding and practice of heritage conservation," *Studies in Conservation* 60(1), 65-77. DOI: 10.1179/2047058413Y.0000000122

Valls, C., Pujadas, G., Garcia-Vallve, S. and Mulero, M. (2011). "Characterization of the protease activity of detergents laboratory practicals for studying the protease profile and activity of various commercial detergents," *Biochemistry and Molecular Biology Education* 39(4), 280-290. DOI: 10.1002/bmb.20488

Yang, N., Matthew, M. A., and Yao, C. (2023). "Roles of cysteine proteases in biology and pathogenesis of parasites," *Microorganisms* 11(6), article 1397. DOI: 10.3390/microorganisms11061397

Yuan, B., Mao, X., Wang, Z., Hao, R., and Zhao, Y. (2020). "Radical-induced oxidation removal of multi-air-pollutant: A critical review," *Journal of Hazardous Materials* 383, article 121162. DOI: 10.1016/j.jhazmat.2019.121162

Yuan, J.-C., Huang, R., Jiang, L.-Y., Liu, G.-D., Liu, P.-D., and Xu, W.-R. (2023). "Facile production of cellulose nanofibers from raw elephant grass by an aluminum chloride-enhanced acidic deep eutectic solvent," *International Journal of Biological*

Macromolecules 246, article 125687. DOI: 10.1016/j.ijbiomac.2023.125687
Zervos, S., and Alexopoulou, I. (2015). "Paper conservation methods: A literature review," *Cellulose* 22(5), 2859-2897. DOI: 10.1007/s10570-015-0699-7
Zhou, C., Qin, H., Chen, X., Zhang, Y., Xue, Y., and Ma, Y. (2018). "A novel alkaline protease from alkaliphilic *Idiomarina* sp. C9-1 with potential application for eco-friendly enzymatic dehairing in the leather industry," *Scientific Reports* 8(1), article 16467. DOI: 10.1038/s41598-018-34416-5

Article submitted: May 19, 2025; Peer review completed: June 30, 2025; Revised version received and accepted: July 9, 2025; Published: July 22, 2025.
DOI: 10.15376/biores.20.3.7435-7462