Egypt. J. Chem. **Vol. 69**, No. 2 pp. 197 - 209 (2025)

Egyptian Journal of Chemistry

http://ejchem.journals.ekb.eg/

The Reciprocal Role of Inks, Pigments, and Microbial Metabolism in the Biodeterioration of Historical Parchment Manuscripts

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Abstract

Microbial colonization, especially by proteolytic enzymes that break down the collagen matrix, can cause biodeterioration in historical parchment manuscripts. This study examines the mutual relationship between microbial enzymatic activity and writing materials, particularly historically reconstructed inks and pigments. The biodeteriorated Coptic parchment manuscript CG 8001, which is kept at the Egyptian Museum in Cairo, contained fourteen bacterial strains. Two of these strains (N10 and N13) showed high protease activity and were identified as *Serratia liquefaciens* N13 (MT279350.1) and *Bacillus safensis* N10 (PP083218.1) by 16S rRNA sequencing. Using traditional recipes of cinnabar (HgS), orpiment (As_2S_3), verdigris ($Cu(CH_3COO)_2$), carbon black, and iron gall ink (cold method), all bound with gum arabic, mock parchment samples were made in order to assess how particular writing materials affect microbial degradation. These samples were allowed to air dry before being finely ground and added to culture media that had been inoculated with the two proteolytic isolates. The biodegradability of various coatings was evaluated by tracking enzymatic degradation. The findings demonstrated that orpiment had the most potent antimicrobial activity, considerably preventing enzymatic breakdown. Because they contain copper and mercury, cinnabar and verdigris exhibited moderate resistance. On the other hand, uncoated parchment was extremely vulnerable, and carbon black and iron gall ink offered little to no protection. These results imply that some pigments used in the past had biocidal qualities, which had a direct impact on how differently manuscripts were preserved directly impacted how manuscripts were preserved differently. This study emphasizes the significance of material selection in conservation planning and the intricate role that writing materials play in microbial biodeterioration. It offers a scientific basis for preventive conservation measures specific to parchment manuscripts by clarifying how pigment chemistry influences microbial metabolism.

Keywords: Parchment; Inks; Pigments; Bacterial; Enzymatic Degradation; Metabolic System

1. Introduction

Historical parchment manuscripts are invaluable sources of human knowledge, historical continuity, and cultural identity. Parchment, which consists mostly of collagen fibers, has long been esteemed for its durability. Despite that, in environments with variable humidity and temperature, it is still susceptible to biological degradation [1,2]. The organic, protein-rich composition of parchment makes it an ideal substrate for microbial colonizers like fungi and bacteria. These microbes use parchment as a nutritional source, and their enzymatic activity can cause fiber weakening, collagen hydrolysis, and eventually structural disintegration. Beside endangering the material's mechanical constancy, the biodeterioration process also extremely threatens the preservation and legibility of the intellectual content it contains [2, 3]. Thus, the conservation of historical parchment manuscripts is not merely a technical concern but a vital endeavor in safeguarding our collective cultural and intellectual heritage.

Stabilizing the parchment substrate or suppressing microbiological agents is mostly the main concern of conventional conservation techniques. However, becoming more widely recognized are the involved and ever-changing interactions between the microbial communities, the parchment medium, and the chemical nature of the inks and pigments used in writing manuscripts. Although the historical, aesthetic, and technological consequences of inks and pigments have long been investigated, little is known about how they might boost or prevent microbial colonization and enzymatic activity [4,5]. Mineral pigments, metallic salts, such as iron in iron gall ink or copper compounds in verdigris, likewise organic binders (egg white or gum Arabic) commonly are constituents of these writing materials. These ingredients could have a significant impact

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Received Date: 21 June 2025, Revised Date: 26 July 2025, Accepted Date: 16 August 2025
DOI: 10.21608/EJCHEM.2025.396280.11933

on how microorganisms interact with parchment. For example, iron and copper salts are well known to accelerate oxidative reactions that might damage collagen, and they may also change microbial enzymatic pathways [6,7].

Inks and pigments, according to recent research, may be mutually responsible for biodeterioration, representing active agents that affect microbial survival and enzymatic expression rather than passive targets of microbial assault alone [5,7]. Beside containing natural organic additives that may act as additional sources of nutrients, indirectly promoting the growth of microorganisms [8], iron ions in iron gall ink can take part in Fenton-like reactions that alter the metabolic environment for colonizing microbes and increase oxidative stress in parchment [6].

The collagenous structure of the parchment is directly broken down by proteolytic activity, resulting in material disintegration and brittleness [2, 3]. The interruption mechanism is heavily reliant on these microbial communities' metabolic systems, precisely on extracellular enzymes synthesis. Even though protease-producing bacteria have been isolated from ancient manuscripts and associated with substantial material degradation [7], their enzymatic profiles and interaction mechanisms with writing materials are still insufficiently characterized, highlighting a critical need for interdisciplinary investigation.

The Egyptian Museum in Cairo houses a prestigious collection of Coptic manuscripts, which includes CG 8001 (Figure 1). This manuscript is one of the oldest entries (CG 8001–8741) describing Coptic texts in the museum's collection, having been catalogued by W. E. Crum in the Catalogue générale des antiquités égyptiennes du Musée du Caire: Coptic Monuments [9]. The provenance, content, and material origin of the artifacts were briefly described in Crum's catalogue. CG 8001 and other Coptic manuscripts of The Egyptian Museum's collection are an invaluable resource for researchers focusing their studies on the linguistic, religious, and cultural evolution of Coptic Egypt. Vital background information on the social milieu in which they were formed can also be traced.

This study considers the reciprocated relation between the metabolic system of microbes isolated from a deteriorated historical parchment manuscript and writing materials, precisely inks and pigments. To govern the part these components play in accelerating or slowing down degradation processes, the microbial growth patterns in the presence of particular writing materials and enzymatic activities, especially the production of proteases, will be examined and assessed in this work. Former research has confirmed that parchment, as a collagen-based material, is susceptible to microbial attack; consequently, proteolytic enzymes degrade the substrate's structural proteins. Nevertheless, little effort has been directed to clarify the ways in which specific manuscript pigments or inks can affect this enzymatic activity. In our study, bacterial strains have been isolated from impacted areas of a parchment manuscript, and their protease activity was evaluated both qualitatively and quantitatively. Molecular identification was also carried out, facilitating more knowledge about the isolates' taxonomy and possible biodegradative profiles. This method combines material science, enzymatic, and microbiological techniques offer an in-depth analytical protocol for assessing the biological decay of parchment. An understanding of these microbial dynamics in the context of manuscript composition will help improve conservation strategies by enabling more focused preventive and remedial measures.

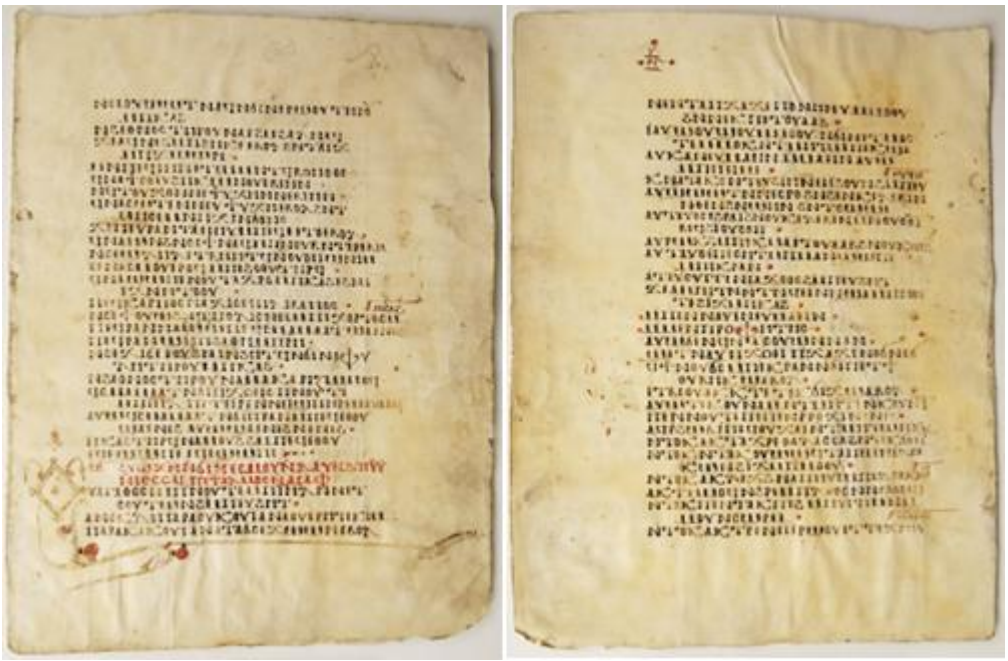

Figure 1: pages 101 on the left and 104 on the right, from Coptic manuscript C.G. num. 8001, Egyptian Museum of Cairo, Egypt

2. Materials and Methods

2.1 Mock-up samples

To evaluate the effect of writing materials on parchment stability under microbial exposure, using historical pigments and inks on a parchment substrate, several mock-up samples were fashioned. The consecutive preparation steps outlined in the workflow illustrated in Figure 2 involves stages of material selection, binding medium formulation, and pigment and ink application. Following these procedures confirms mirroring genuine manuscript conditions.

2.1.1. Parchment Substrate

High-quality sheep parchment was obtained from William Cowley Parchment Makers (London, UK), which is well-known for adhering to traditional parchment-making procedures. To maintain consistency with the experiment, sheets were cut into 2 x 2 cm square parchment samples.

2.1.2. Binding Medium

Gum arabic, a naturally occurring polysaccharide derived from Acacia species, was used as a binding medium for all inks and pigment preparation. According to conformist preparation methods, gum arabic powder, obtained from Kremer Pigmente (Germany), was dissolved in distilled water to make a 10% (w/v) solution [10].

2.1.3. Pigment Preparation and Application

Three historical pigments: verdigris (basic copper acetate), orpiment (As_2S_3), and cinnabar (HgS), simulating the original manuscript CG8001, were purchased from Kremer Pigmente (Germany). To guarantee uniformity, very fine, kindly ground forms were chosen. To obtain an appropriate consistency, the pigments were then combined in a 1:1 weight ratio with the prepared gum arabic solution. A single, even layer was applied on one side of each parchment square using sable brushes (size 0–1). The samples were left to dry in a laboratory setting with ambient conditions (22 ± 1 °C, 50% RH) for 48 hours [11].

2.1.4. Ink Preparation

All inks' ingredients were purchased from Kremer Pigmente (Germany)

2.1.4.1. Carbon Ink (Lamp Black):

Lamp black, a fine carbon pigment made by gathering soot from burning linseed oil, mixed with a 10% gum arabic solution, in a 1:1.5 weight ratio (pigment to binder), forming the carbon ink. This method is consistent with the ink-making techniques used historically and recorded in Persian manuscripts [10].

2.1.4.2. Iron Gall Ink (Cold Extraction Method):

Iron gall ink was prepared according to a cold extraction method.
As a first step, 10 g of crushed Aleppo gallnuts were soaked in 100 mL of distilled water for five days at room temperature. The tannic acid-rich extract was then obtained by filtering the mixture through an extremely fine gauze fabric. After that, a 5 g of ferrous sulfate heptahydrate ($FeSO_4 \cdot 7H_2O$) was dissolved in the gallnut extract using constant stirring. 5 mL of the 10% gum arabic solution was added to the mixture in order to stabilize the ink.
This method of preparation aligns with both contemporary analyses of iron gall ink formulations and historical recipes [12].

2.1.4.3. Mixed Ink (Carbon-Iron Blend):

To formulate the mixed ink, which is very common in Coptic manuscripts, equal volumes of the prepared carbon ink and iron gall ink were blended.

2.1.5. Drying and Storage Conditions

To ensure complete stabilization before enzymatic exposure, after being air-dried for 48 hours in a dust-free fume hood, all treated parchment samples were kept in acid-free envelopes at room temperature (20–22 °C, 50–55% RH) for a week. Precautions were taken during handling and preparation to avoid cross-contamination [10]. To grind the coated parchment squares into uniform powders after drying and storage period, a sterile agate mortar and pestle were used. These powdered samples were subsequently added to culture media, A solidified agar plate medium containing skim milk and casein was used, as described in Section 2.3, then inoculated with bacterial isolates 13 and 10, both of which are known to secrete proteolytic enzymes. Qualitative and quantitative assessment of the effect of different inks and pigments on enzyme secretion was conducted.

2.2 Isolation of bacterial strains from deteriorated historical parchment

Swabs from deteriorated historical parchment from the Coptic era were used for this purpose. Swabs were suspended in 2mL of sterile saline solution (0.85% NaCl) and the resulting suspensions were subjected to serial dilution up to 10-4. 50µl from each dilution was used to inoculate previously prepared nutrient agar medium supplemented with the antifungal cyclohexamide (50mg/l). After incubation at 37 ℃ for 24-48h. The resulting colonies were picked up, purified, and maintained on nutrient agar slants and kept at 4℃ till next used [13].

2.3 Qualitative screening of the protease activity of isolated bacteria

Fourteen bacterial strains were isolated from deteriorated historical parchment. These isolates were screened for their abilities to produce protease enzymes (protein-degrading enzymes). Solidified agar plate medium was used for this purpose.

Skim milk agar plates having the following ingredients (g/l): skim milk (28), yeast extract (2.5), dextrose (1), casein (5) as well as agar (18) was used. The pH of the medium was adjusted at pH 7.0, the plates were incubated at 35 °C for 48 hours to allow observation of clear zones. [14]. The appearance of clear zones around the tested bacterial strains were considered a positive result. For more assurance, amidoblack reagent (0.1%) was used. Amidoblack reagent dye was prepared by dissolving 0.1 g of amidoblack in a reaction mixture of methanol, acetic acid and water (30:10:60 w/w). The plates having bacterial growth were flooded with this reagent at 30℃ . After that, the decolorization was performed using the same solvent mixture [15].

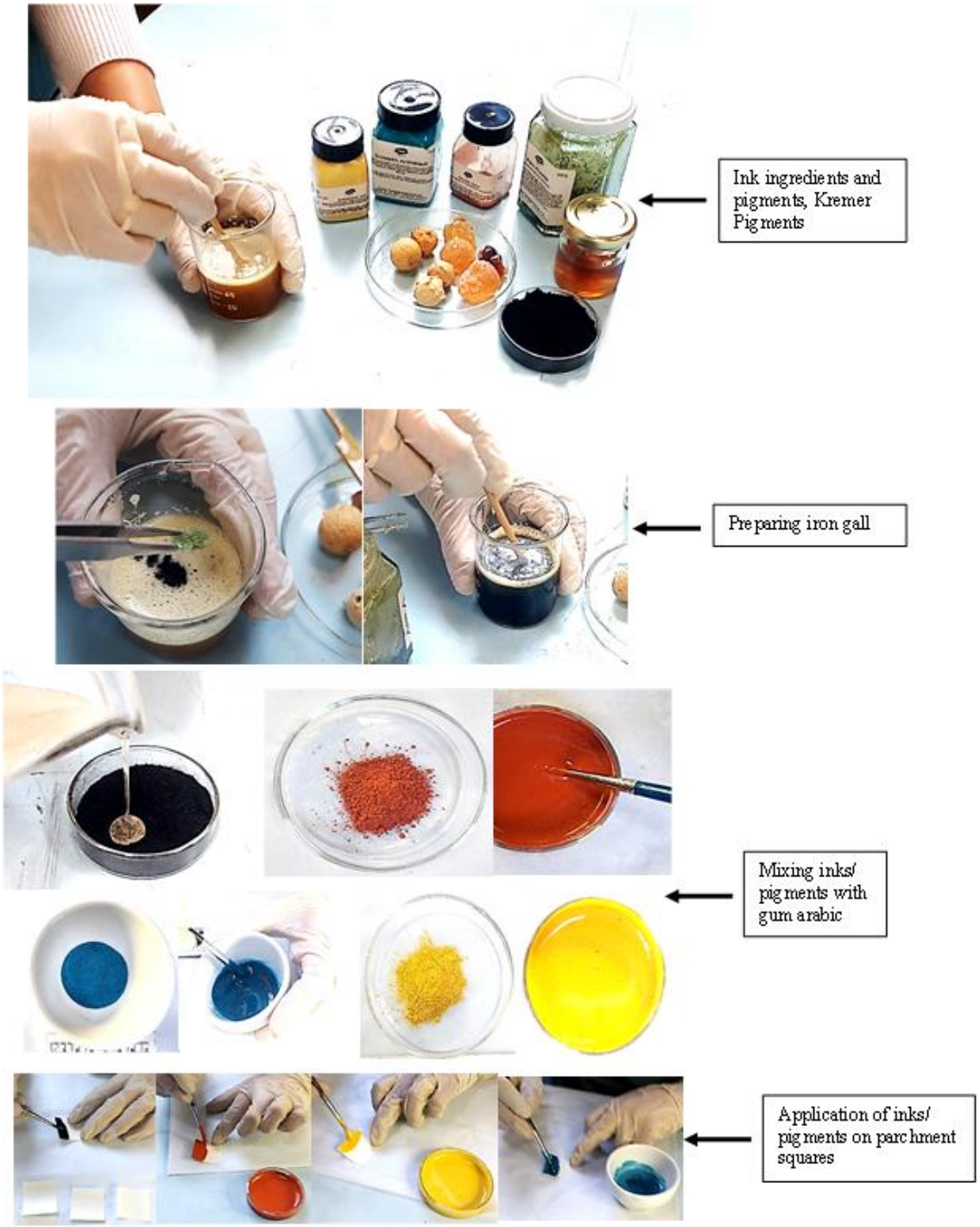

Figure 2: The workflow outlines the sequential preparation steps, from substrate selection and binder formulation to the application of pigments and inks

2.4 Quantitative screening of protease production

2.4.1. Cultivation and culture conditions

Seven bacterial strains that had profound results from the screening test were quantitatively tested for their protease enzyme production. The bacterial strains were cultivated on the following medium (g/l): glucose (10), casein (5), yeast extract (5), KH2PO4 (2), K2HPO4 (2), MgSO4.7H2O (1), and the pH was adjusted to 7. Conical flasks (250 mL volumes) each having 50 mL of the prepared culture medium, were inoculated with 2% of each bacterial stock solution (Abs. of 1 at 600 nm) and the flasks were incubated on a rotary shaker (Model: NB-205QF, N-BIOTEK, Korea) operating at 150 rpm and 35 °C for 4 days. At the end of the incubation period, the culture broth from each strain was centrifuged at 8000 rpm for 20 min using a refrigerated centrifuge (Model: K2015R, Centurion Scientific Ltd., UK). And the produced supernatant was considered as the crude enzyme source [16].

2.4.2. Protease enzyme assay

Cell-free supernatant from each bacterial strain was used as the enzyme source. The protease activity was assessed according to the method described by Takami et al. [17]. Casein (1%) in 50 mM glycine NaOH buffer (pH 9) was used as substrate. The assay was carried out in a mixture containing 0.5 mL of suitably diluted enzyme and 2.5 mL of casein solution, and the reaction mixture was incubated at 40℃ for 60 min. The reaction was terminated by the addition of 2.5 mL of 0.44 M trichloroacetic acid (TCA). After 10 min, the mixture was centrifuged at 8000 rpm (on a cooling centrifuge at 4℃) and 0.5 mL of the produced supernatant was mixed with 2.5 mL of 0.5 of Folin-Ciocalteu's phenol reagent and stood for 30 min for complete color development, and the optical density was measured at 660 nm using a UV-Visible spectrophotometer (Model: UV-2401PC, Shimadzu, Japan). Tyrosine was considered standard. One enzyme unit was defined as the amount of enzyme resulting in the release of 1µg of tyrosine equivalent at 40℃ under the defined assay conditions. All measurements were performed in triplicate to allow calculation of standard deviation

2.5 Identification of the bacterial strains

The bacterial strains that exhibited potent protease activities were subjected to molecular identification using the 16S rRNA sequencing techniques. Bacterial isolates exhibiting potent protease production were identified according to a molecular biological protocol by DNA isolation, amplification (PCR), and sequencing to 16rRNA technique. The forward primers 27F (5'-AGAGTTTGATCMTGGCTCAG-3'), and 518F (5'-CCAGCAGCCGCGGTAATACG-3') and a reverse 1492R (5'-TACGGYTACCTTGTTACGACTT-3'), and 800R (5'-TACCAGGGTATCTAATCC-3') were used. The purification of the PCR products was carried out to remove unincorporated PCR primers and dNTPs from PCR products by using the Montage PCR Cleanup kit (Millipore). Sequencing was performed using the Big Dye Terminator Cycle Sequencing Kit (Applied Biosystems, USA). Sequencing products were resolved on an Applied Biosystems model 3730XL automated DNA sequencing system (Applied Biosystems, USA). Amplification and sequencing of the 16S rRNA gene had been done according to Myer et al. [18]. The Blast program was used to assess the DNA similarities (www.ncbi.nlm.nih.gov/blst). Phylogenetic trees were constructed using the neighbour-joining DNA distance algorithm implemented in MEGA 7 software [19]

2.6 Biodegradability of parchments treated with different pigments/inks using protease from N10 and N13 bacterial strains

The experiment was designed by incubating the parchments with proteases from potent protease-producing bacterial strains (N10 and N13) and measuring the produced amino acids as described in the protease assay [17]. Among isolates with similar colony morphology and presumed taxonomic identity (e.g., N13 and N14), only one representative strain was selected for quantitative enzyme assays. All enzyme activity measurements were performed in triplicate to allow calculation of standard deviation

3. Results and Discussion

3.1. Isolation of bacterial strains from deteriorated historical parchment

Results represented in Figure 3 showed the isolated 14 bacterial strains from an ancient historical parchment from the 9th century. These isolates were isolated using a specific medium described by Nasfi et al. [13].

3.2. Qualitative screening of protease activity

The fourteen isolated bacterial strains were screened for their proteolytic ability. A specified medium containing skim milk and casein was used [14]**.** The appearance of clear zones around the growing bacterial colonies was reported as positive results. Moreover, the plates were flooded with amidoblack dye, and the presence of clear halos around the bacterial colonies, leaving the rest of the plate dark blue, is considered a positive result [15]. Results in Table 1 and Figures 4 and 5 revealed that 9 of 14 bacterial isolates exhibited protease activities. Isolates 2, 10, 13, and 14 exhibited higher protease activities. Isolate 1 showed low activity, but isolate 4, 7 and 9 showed moderate enzyme activities. In the present research, we used crude enzyme extracts produced from bacterial culture sediments without any further purification or concentration processes. This methodological decision is consistent with established approaches in biodegradation research, which seeks to simulate the natural processes of microbial decomposition on cultural heritage objects as accurately as possible [20-23]. Crude extracts

contain a complex mixture of hydrolytic enzymes, secondary metabolites, and cofactors that may act synergistically to produce more realistic hydrolysis patterns [24].

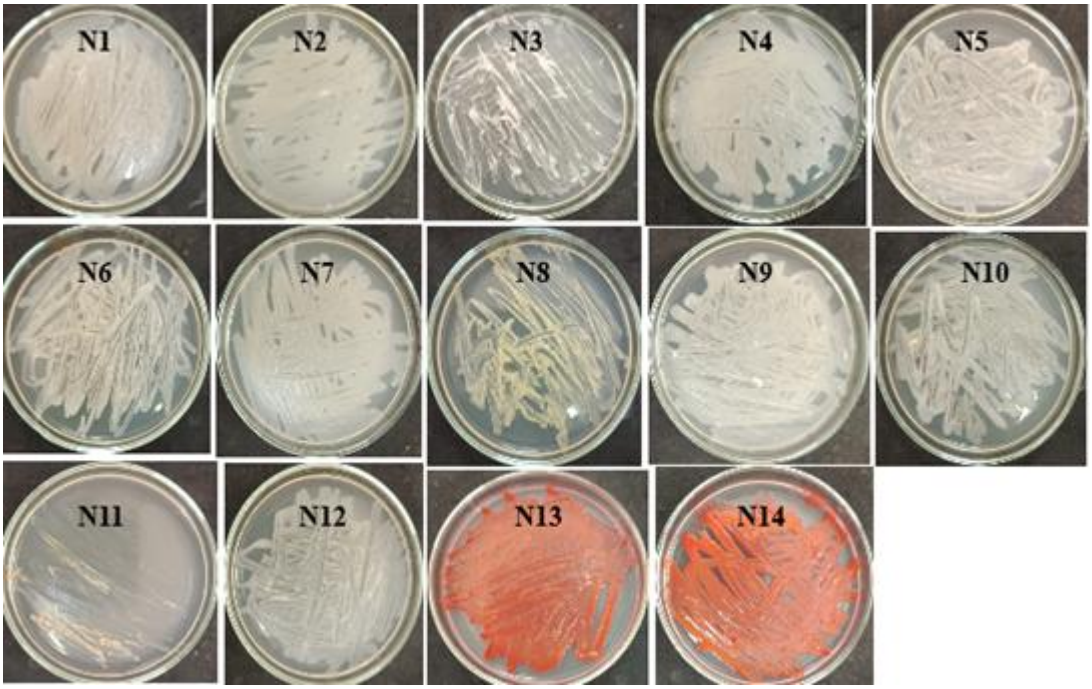

Figure 3: The isolated bacterial strains**.**

While partial purification (e.g., ammonium sulfate or solvent precipitation) can enhance specific enzyme activity [25], it can also remove synergistic co-factors or metabolites essential to real biodeterioration [26]. To focus on historical risk assessment, we followed the practice of several studies that successfully applied crude enzyme extracts from bacteria or fungi directly to cultural materials [21-23]. To focus on historical risk assessment, we followed the practices of several studies that successfully applied crude enzyme extracts of bacteria or fungi directly to cultured materials [21–23].

By adopting this approach, our study aims to capture the complexity of enzyme-mediated deterioration as it might occur naturally on historical parchment, rather than isolating the effect of a single enzyme class. However, future research comparing the effect of partially purified fractions will help identifying the most active components responsible for the degradation. Which will contribute to improving targeted conservation strategies and deepening our understanding of the mechanisms of biological deterioration in historical manuscripts.

Table 1: The qualitative screening of bacterial strains isolated from deteriorated parchment

Isolate number	Proteolytic activity (clear zone)	
	On plate	After amidoblack
N1	+	+
N2	+++	+++
N3	-	-
N4	+++	++
N5	++++	+++
N6	-	-
N7	+++	++
N8	-	-
N9	++	+++
N10	+++	+++
N11	-	_
N12	-	_
N13	+++	+++
N14	+++	++

*(++++) = very high activity, (+++) = high activity, (++) = moderate activity, and (+) = low activity

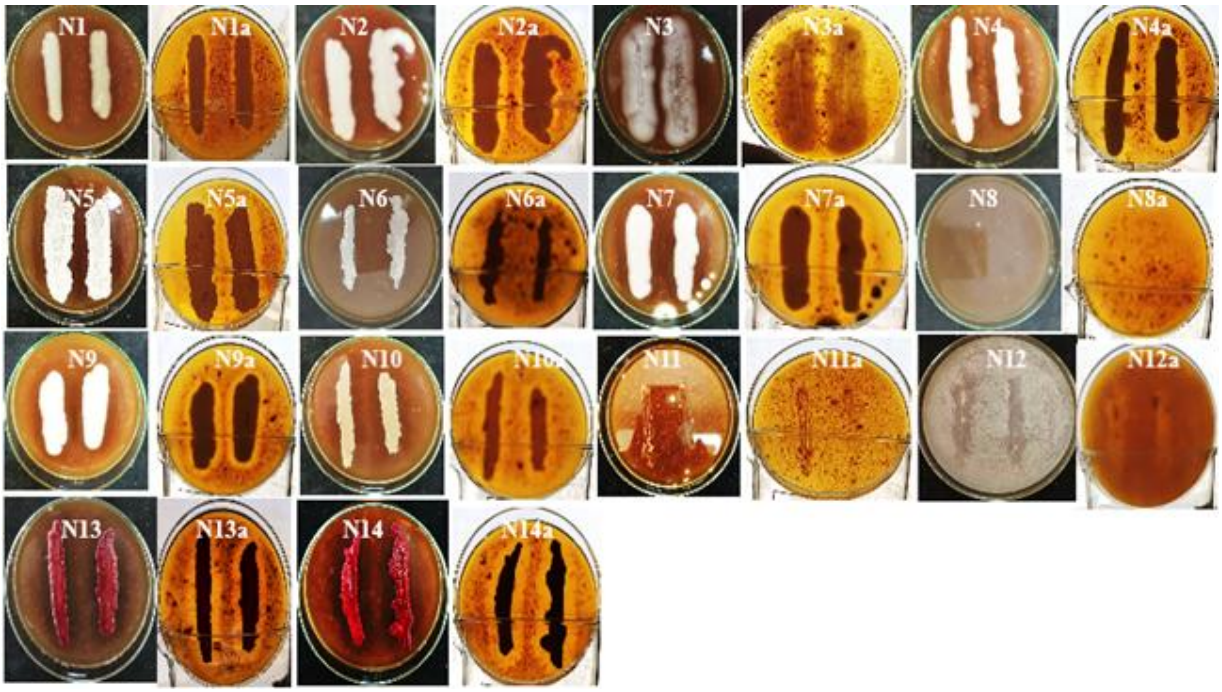

Figure 4: The qualitative screening of bacterial strains isolated from deteriorated parchment grown on skim milk agar plates; Plates were photographed both under direct illumination (without letter "a") and under transmitted backlight (labeled "a") to better reveal the enzyme-induced clear zones around colonies. Backlighting increases contrast, making transparent halos more visible

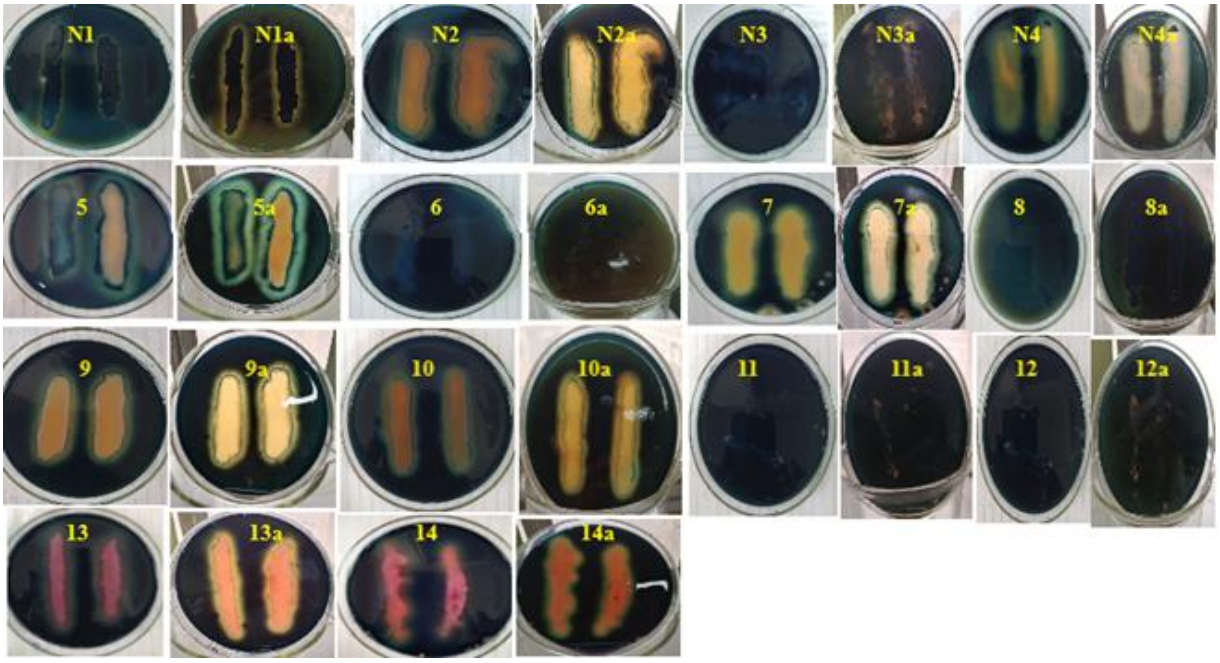

Figure 5: The qualitative screening of bacterial strains isolated from deteriorated parchment grown on skim milk agar plates stained with amidoblack; Plates were photographed both under direct illumination (without letter "a") and under transmitted backlight (labeled "a") to better reveal the enzyme-induced clear zones around colonies. Backlighting increases contrast, making transparent halos more visible

3.3. Quantitative assessment of potent protease-producing bacteria

For this purpose, the bacterial strains exhibiting positive results (moderate and high) from the qualitative screening test were cultivated on broth medium having casein as the sole nitrogen source. The cell-free supernatant from these cultures was considered as a protease source. Results in Table 2 revealed that isolates 10 and 13 exhibited the highest protease activity (41.56 and 40.71 U/ml, respectively). Bacterial strains N2, N4, and N5 exhibited moderate protease activities (28.46, 25.18, and 32.63 U/ml, respectively). On the other hand, isolates 7 and 9 showed moderate to low activities (20.43 and 23.36 U/ml, respectively).

Enzyme activities were assessed on both the third and fourth day of incubation to explore possible temporal variations in enzyme production. The data show that certain isolates, notably no. 5, 7, and 9, exhibited higher hydrolytic activity on the third day compared to the fourth. This finding suggests that peak enzyme secretion may occur before the end of the normal four-day incubation period for some strains, possibly due to metabolic alterations or nutrient depletion impacting enzyme synthesis [27-29].

The temporal dynamics of enzyme production detected in this study are in line with former reports that hydrolytic enzyme secretion by microorganisms can vary through the cultivation cycle [30,31]. The increase in activity on the third day for isolates no. 5, 7, and 9 proposes that optimal enzyme yield might arise earlier than the four-day endpoint, as microbial metabolism transitions from exponential growth to stationary phase can lessen enzyme synthesis [27-29]. While the standard four-day incubation was preferred to align with previous biodeterioration studies [20,23], these results highlight the importance of bearing in mind incubation time as a variable affecting enzyme-based biodeterioration assessments [30,31]. Future work could refine this approach by sampling across multiple time points to construct detailed enzyme production curves for each isolate.

Table 2: Protease activities of different bacterial strains isolated from deteriorated parchment

Bacterial isolate	Protease activity (U/ml)	
	Third day	Fourth day
N2	23.04 ± 1.113	28.46 ± 0.277
N4	21.15 ± 0.171	25.18 ± 0.302
N5	35.13 ± 0.927	32.63 ± 0.609
N7	31.53 ± 0.670	20.43 ± 0.625
N9	33.70 ± 0.348	23.36 ± 0.472
N10	32.96 ± 0.869	41.56 ± 2.165
N13	30.98 ± 1.179	40.71 ± 2.039

*Values represent the mean ± standard deviation of three independent replicates (n = 3) for each bacterial isolate.

3.4. Bacterial identification

The molecular identification of the two potent protease-producing bacterial isolates revealed that a nucleotide sequence of 1260 and 1190bp of the whole 16S rRNA gene of the bacterial isolates N10 and N13, respectively. BLAST search revealed that isolate N10 exhibited 100% similarity to *Bacillus safensis* subsp. safensis strain ZGTB13-1 (accession number OR243865.1) and the phylogenetic tree of this bacterial isolate were also constructed (Figure 6). The gene sequence of bacterial isolate N10 was deposited in theGenBank database as *Bacillus safensis* N10 (Accession number PP083218.1). On the other hand, bacterial isolate N13 exhibited 100% similarity to *Serratia liquefaciens* strain CEA1 (Accession number MT279350.1), and the phylogenetic tree of this strain N13 was constructed (Figure 7). The gene sequences of bacterial isolate N13 was deposited in GenBank database as *Serratia liquefaciens* strain N13 (Accession number PP083185.1). It is well known that the traditional identification techniques are dependent on the culture morphology and some biochemical reactions are known to be time-consuming methods that also need a talented-persons with very high experience [32]. Consequently, the use of DNA sequences in the current study was proved to be an objective, reproducible, accurate and quick tool for identification. Universal primers: 27F (5'GAGTTTGATCATGGCTCAG3'), 1492R (5'GGTTACCTTGTTACGACTT3') [for PCR], and 518F (5'CCAGCAGCCGCGGTAATACG3') and 800R (5'TACCAGGGTATCTAATCC3') were used in the study of the genotypic identification of microorganisms isolated from non-parenteral pharmaceutical formulations. Moreover, the primers 27F and 1492R were used for the identification of bacteria.

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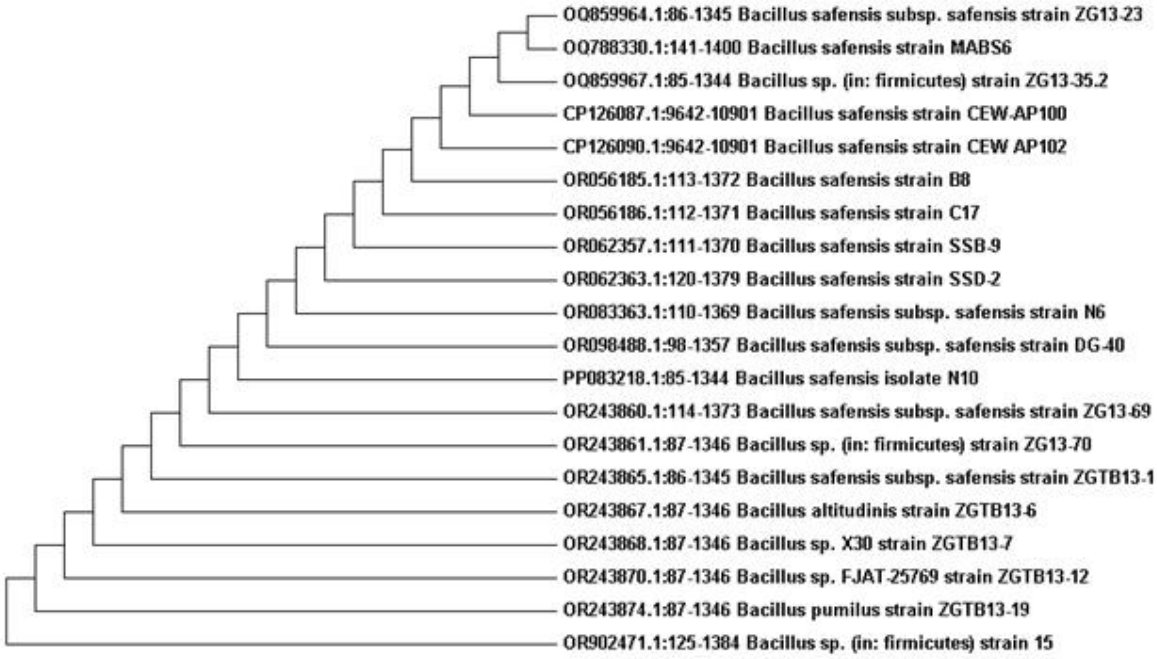

Figure 6: Phylogenetic trees showing the relationship of strain N10 with other related bacterial species retrieved from GenBank based on their sequence homologies of 16S rRNA.

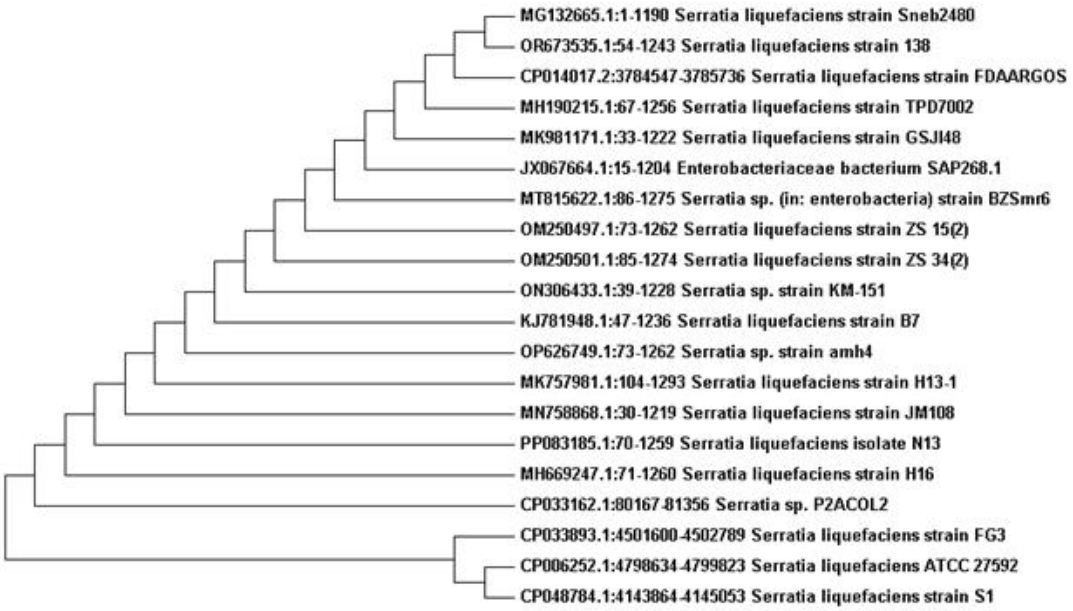

Figure 7: Phylogenetic trees showing the relationship of N13 with other related bacteria species retrieved from GenBank based on their sequence homologies of 16S rRNA.

3.6. Biodegradability of parchments coated with different pigments using proteases from bacterial strains N10 and N13

The effect of different inks/pigments on the enzymatic biodegradability of parchment samples was assessed using two proteolytic enzymes derived from bacterial isolates 13 and 10. The parchment squares—each coated with a different pigment or ink using gum arabic as a medium—were powdered and introduced into culture media to evaluate their enzymatic degradation. The results (see Table 3) revealed considerable differences in degradation susceptibility depending on the surface coating. Sample B, the control untreated parchment sample, demonstrated a high level of degradation by 76.39% and 80.15% with enzymes from strains 13 and 10, respectively. Likewise, sample C, the carbon black ink sample, revealed high susceptibility. Typically, elemental carbon in the form of fine particulates is chemically inert and does not possess antimicrobial or protein-crosslinking abilities [33]. Accordingly, it doesn't considerably prevent enzymes from degrading the substrate. However, Sample Y, the parchment coated with orpiment (arsenic sulfide) yellow pigment, which only degraded at rates of 26.67% and 34.89% for strains 13 and 10, respectively, recorded the least biodegradability. These outcomes are aligned with a study by FitzHugh [34] stated that compounds containing arsenic are extremely detrimental to microorganisms and may also interrupt enzymatic activity [7,34]. In Sample A, parchment samples coated with gum arabic alone, intermediary degradation levels (roughly 60–63%) are shown, revealing that the binding medium does not remarkably influence microbial activity. According to Kolar [12] and Malešič [35], under the right conditions, gum arabic can function as a nutrient source for microbial growth in addition to acting as a stabilizing medium. Sample G, where parchment was coated with verdigris (a basic copper acetate), demonstrated a moderate susceptibility to enzymatic degradation, with degradation percentages ranging from 60 to 63% against both bacterial protease sources. Even copper ions are known for their biocidal properties [36–40], verdigris, a copper-based pigment, validated insignificant antimicrobial effects when compared to orpiment-coated samples, which verified noticeably stronger antimicrobial effects. In this instance, the moderate degradation seen may have been caused by the buffering or limitation of verdigris's antimicrobial activity by the use of gum arabic as a binding medium. Besides, some bacteria and fungi have evolved resistance mechanisms and can metabolize or tolerate copper compounds [41, 42]. This was in accordance with Hofmann et al. [37], who elucidated that verdigris applied to paper can change significantly, as microbial colonization can speed up deterioration by forming organic acids or biofilms that change the stability of the pigment. Also, according to Bette et al. [36], verdigris's crystalline structure is unstable and affected by pH and humidity, both of which can be locally changed by microbiological activity. Iron gall ink-coated samples, sample I, likewise displayed degradation in the 60–63% range. Iron gall inks made of gallic acid and iron salts, it has been demonstrated that some bacteria and fungi can colonize iron gall ink inscriptions, and iron is an essential micronutrient for many microbial species [6,35]. Through metabolic processes that either use iron or break down nearby collagen structures, these microbes can worsen ink corrosion [5]. According to Kip and Van Veen [31], microbes to protect or destroy metals depending on the conditions. Gilbert and Kubacki [44] further demonstrate that biological processes like oxidative stress can increase corrosion in metals. Tiano [45] also highlights how microbial activity contributes to the deterioration of cultural heritage materials through similar mechanisms. Sample M, where parchment coated with a mixed ink showed slightly lower degradation levels for isolates 13 and 10, at 41.71% and 51.01%, respectively. Relying on its ingredients, the mixed ink sample's modest degradability may specify partial protection or enzyme inhibition, possibly resulting from the precipitation of insoluble complexes that somewhat block enzymatic access. Degradation values of 55.15% and 62.08% for both enzymatic sources, respectively, were demonstrated by Sample R, parchment coated with cinnabar (mercury sulfide). The red pigment cinnabar (HgS) has been in use as a painting material for thousands of years in many cultures. It is known for its deep red, special gloss, good covering characteristics, and adhesive strength [46]. Besides, cinnabar is recognized for its biocidal qualities as the mercury ions are known for their toxicity, which in some situations can prevent microbial growth [46]. However, when cinnabar is bound in organic media such as gum arabic, its protective effects are limited because it does not completely prevent enzymatic degradation. In addition, previous studies indicated that certain bacteria, specifically Pseudomonas species, can methylate inorganic mercury and have mercury resistance mechanisms [48, 49]. In long-term deterioration scenarios, the role of mercury-resistant microbial communities should not be disregarded, even though abiotic factors like light and humidity dominate cinnabar degradation [50].

To put it briefly, the results of this study pick out and emphasize the fact that pigments like orpiment, verdigris, and cinnabar, which have historically been supposed to have antimicrobial properties, can deliver different degrees of protection based on their chemical form, binding medium, and bioavailability. Cinnabar and verdigris were only moderately protected, but the most obvious inhibitory effect was exposed by orpiment, based on arsenic. Crude parchment remained significantly susceptible, and neither carbon black nor iron gall ink yielded any real defence against microbial deterioration. These findings forge ahead our knowledge of how historical writing materials in the context of manuscript preservation can affect microbial susceptibility. Several factors, however, interact intricately to commence the biodeterioration of writing materials and support more than just the pigment or ink. The substrate, existing nutrients—organic media, or adhesives—, and ambient conditions all work together to create an Appropriate environment that promotes microbial growth. This emphasizes the essential

requirement of thorough, microbiologically informed conservation plans that are adapted for each artifact's unique material composition.

Table 3: The degradability of different samples by proteases from bacterial isolates 10 and 13

Sample	Isolate 13		Isolate 10	
	U/ml	Activity (%)	U/ml	Activity (%)
A	25.95 ± 0.078	63.74	27.19 ± 0.079	65.42
B	31.1 ± 0.245	76.39	33.31 ± 0.156	80.15
C	29.58 ± 0.200	72.66	33.27 ± 0.108	80.05
G	24.81 ± 0.076	60.94	27.76 ± 0.124	66.79
I	25.15 ± 0.146	61.83	27.33 ± 0.087	65.76
M	16.98 ± 0.076	41.71	21.20 ± 0.154	51.01
R	22.45 ± 0.185	55.15	25.80 ± 0.146	62.08
Y	10.85 ± 0.198	26.67	14.50 ± 0.161	34.89

*Values represent the mean ± standard deviation of three independent replicates (n = 3) for each bacterial isolate.

4. Conclusions

Writing inks and pigments on parchment manuscripts has a considerable impact on the process of their biodegradation. Toxic metals in inks or pigments (such as arsenic in orpiment, copper in verdigris, and mercury in cinnabar) can minimize susceptibility to enzymatic biodegradation by constraining microbial activity or enzyme function. Non-toxic or inert substrates, such as gum arabic and carbon black, afford slight defense. These results shed insights into the role of historical writing materials in the levels of preservation of historical parchment manuscripts.

5. Conflicts of interest

The authors declare no competing interests.

6. Acknowledgments

The author (Nagah S. Saada**)** gratefully acknowledges the generous support of the American Research Center in Egypt (ARCE) through its fellowship program. This research was conducted during the tenure of a Ph.D. fellowship awarded by ARCE, whose funding and academic support were instrumental in the development of this study.

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