The Hydrolytic Activities of Two Fungal Species Isolated from Historical Quranic Parchment Manuscript

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Abstract

Two fungal strains were isolated from a historical parchment manuscript found in Al-Azhar library, Egypt and were identified using the molecular technique 18S rRNA. New parchment samples (see 2.1) were placed on the surface of agar plates and inoculated with the two identified fungi and incubated at 30°C for 10, 20 and 30 days. The parchment samples were checked for degradation by measuring its mechanical properties (elongation and tensile strength), and ATR-FTIR analysis to identify structural changes induced in the collagen triple helix. SEM analysis was also carried out to investigate the change of surface morphology of bio-deteriorated collagen fibres. The colour changes in the fungal-treated parchment samples were also examined in order to determine the proteolytic activity caused by the tested fungal strains and their impact on the parchment properties. The results obtained revealed that *Aspergillus fumigatus* and T*alaromyces spectabilis* denature collagen as a result of their proteolytic activity, leading to loss of mechanical properties and the fibre network, so the parchment becomes brittle and discoloured.

1 INTRODUCTION

Parchments were commonly used as a writing support from the second century BC until the end of the Middle Ages, and evidence of the ideological and cultural changes in the human race can be found in historic documents made of the material. Parchment is a collagen-based biomaterial prepared from calf, sheep and goat skins. Given the historical importance of such documents it is important to understand the degradation processes that may be induced in parchments in order to manage those changes, and preserve the artifacts.[1,2] From the chemical point of view, collagen is the main component of parchment[3-5] and several denaturation processes can result in the degradation of this ordered structure converting it into disordered gelatine. This leads to fragility and brittleness in parchment artefacts.[6]

Collagen is vulnerable to deterioration by various micro-organisms, particularly bacteria and fungi as it serves as an energy and carbon source.[2] Bio-deterioration takes place particularly under conditions of high relative humidity that enable bacteria, actinomycetes or fungi to grow,[7] and ancient parchment provides a good environment for the development of proteolytic fungi which can hydrolyse collagen fibres and other proteinaceous molecules as well as producing pigments and organic acids that discolour the parchment and consequently lead to indirect damage.[2,3] As a result, the parchment can become hard, brittle, distorted and stained.[8] It should, therefore, be of value to look into the microflora responsible for that biodegradation, in order to help preserve this important writing support, which was the main vehicle of culture especially during the Middle Ages.[9] This work aims to isolate fungal strains from deteriorated parchment dating back to the third century AH [816CE–913CE], which are present in the Al-Azhar Library, Cairo,Egypt.

2 MATERIALS AND METHODS

2.1 New parchment samples

Samples of parchment from goatskin were prepared according to (Abdel-Maksoud 2002).[10]

The essential stages in this preparation of parchment are:

Temporary preservation: immediately the skin is removed from the animal, it is vulnerable to attack by micro-organisms. To hold the skin in good condition, it requires some form of temporary preservation. Salt is liberally applied to the raw skin.

Soaking: the skin is immersed in a large volume of cold, clean, and sometimes running water for 24 hours, to extract the salt and re-hydrate the skin.

Liming: the skin is immersed in a suspension of slaked lime for some time, during which time the skin is gently moved in the lime liquor. The alkalinity of the lime loosens the hair and the epidermis.

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Unhairing: the surface of the flesh layer of the skin is unhaired by using an upright conex curved, blunt-edged knife. The skin is usually returned to the lime for some time, after which it is washed in water to remove the remains of lime.

Drying: the skin is suspended within a rectangular wooden frame for drying. The drying process was in the normal temperature.[10]

2.2 Sampling and fungal isolation

The Kufic-written Quran parchment dating back to the third century AH was found in the Al-Azhar Mosque, Cairo, Egypt before being transferred to the Library of Al-Azhar in 1994CE. Fungi on the parchment were isolated using the following method: sterile cotton swabs were wiped across fungal colonies then transferred to the laboratory in sterile tubes and used for fungal isolation.[11] All samples were transferred to the laboratory the same day of collection and immediately processed. Each swab of fungal growth was immersed in a sterile glass vial containing 5ml of sterile distilled water and shaken for 2 hours on a reciprocal shaker. Aliquots (100µl) of spore suspension were spread on 9cm Petri dishes (3 plates per sample) containing Czapek-Dox agar medium, comprising: sucrose (30g/L), $NaNO_3$ (3g/L), $MgSO_4.7H_2O$ (0.5g/L), KCl (0.5g/L), $FeSO_4.7H_2O$ (0.001g/l), K_2HPO_4 (1g/L) and agar (20g/L). This medium has been supplemented with the antibacterial agent (Streptomycin, 0.1%) and Rose Bengal[12] to limit the fungal growth. Plates were incubated for 7 to 14 days at 30°C in the dark and the single colonies were picked up and used to inoculate potato dextrose agar medium. This consisted of peeled potato (200g/L), glucose (20g/L) and agar (20g/L) and was incubated at 30°C for 7 days and maintained at 4°C as pure cultures.

2.3 Identification of the fungal strains (N_1 and N_2)

The strains appearing most were subjected to molecular identification using the 18s rRNA sequencing techniques.

2.4 Identification of fungal cultures

Fungal cultures (N_1 and N_2) were identified according to a molecular biological protocol by DNA isolation, amplification (PCR) and sequencing of the ITS region. The primers ITS2 (GCTGCGTTCTTCATCGATGC) and ITS3 (GCATCGATGAAGAACGCAGC) were used at PCR while ITS1 (TCCGTAGGTGAACCTGCGG) and ITS4 (TCCTCCGCTTATTGATATGC) were used at sequencing. The purification of the PCR products was undertaken to remove unincorporated PCR primers and dNTPs from PCR products by using Montage PCR Clean up kit (Millipore). Sequencing was performed by using 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). *Candida sp.* was used as the control.

2.5 Fungal degradation of the parchment samples

Parchment samples were sterilized with acetone then placed in Petri dishes containing solidified agar medium and inoculated with the two fungal species previously identified.[13] Inoculation of the samples was performed by spread plate method using sterile specialized glass rods to distribute the inoculum over the surface. Then all Petri dishes were covered by plastic lids, incubated at 30°C and checked at regular time intervals (10, 20 and 30 days).

2.6 Mechanical properties

The tensile strength, yield strength and elongation at break were measured with a universal testing machine (Zwick/Roell Germany) at a crosshead speed of 5mm/min. Test specimens were prepared to conform to ASTM D 638 specifications. Three different specimens were sampled from each time interval (10, 20 and 30 days of incubation) for measurement. The three test results were averaged and then reported.

2.7 Fourier transform infrared spectroscopy (FTIR)

A Bruker Vector 33 FTIR spectrometer (Germany) was used for FTIR analysis. The infrared spectra were recorded in the 4000-400cm^{-1} range. FTIR spectra were measured in KBr pellets containing 1% finely ground samples. Fourier transform infrared attenuated total reflection (FTIR-ATR) allows the observation of the conformational variations in the triple helical structure of collagen.

2.8 SEM Examination

The extent of deterioration of modern and aged parchments was examined by scanning electron microscope (SEM). Microphotographs of the parchment surfaces were taken at three magnifications: 100µm, 50µm, and 10µm. SEM revealed significant surface changes. Samples were immersed into liquid nitrogen for 45 seconds. Then, the samples were fractured and coated with a gold-palladium film. A Hitachi S- 3000N SEM instrument was used to examine the fractured surfaces.

2.9 Colour change

The change of parchment colour due to fungal deterioration was monitored prior and after fungal degradation, the reflectance spectra of all samples were developed. The colorimetric parameters were measured using CIE *Lab system commonly used to compare the colours of two samples. The L-scale measures lightness, and varies from 0 (black) to 100 (perfect white). The a-scale measures red-green; (+a) means more red, (-a) measures green; the b-scale measures yellow-blue; (+b) meaning more yellow, (-b) deep blue. The total colour difference (ΔE) is calculated according to the following equation:

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

The measurement was made using Ultra Scan PRO Hunter Lab D65, 10 A.

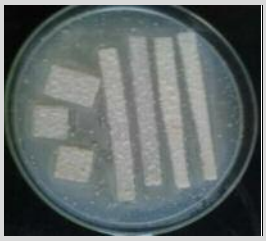
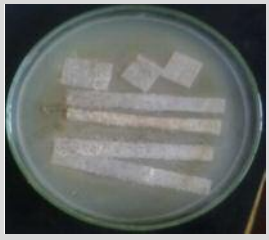
3 RESULTS AND DISCUSSION

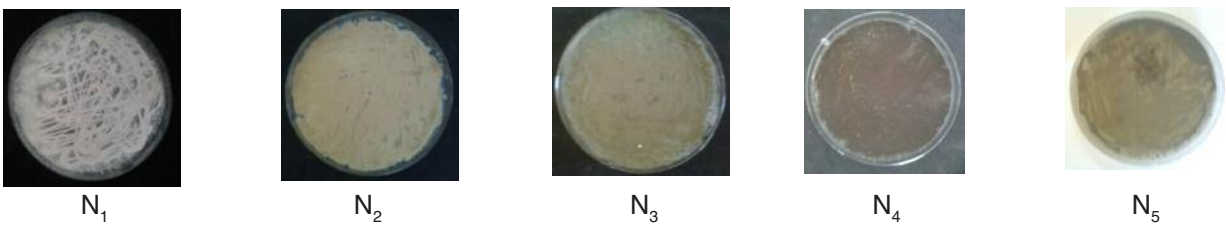
Five fungal strains were isolated from different swabs taken from an ancient parchment of Quran found in the Al-Azhar Library (Fig. 1). These fungi were purified and identified by traditional method according to Barnett and Hunter[14]. These fungi were identified as *Aspergillus sp.* (N_1), *Penicillium sp.* (N_2, N_3 and N_5) and *Aspergillus niger* (N_4). The dominant fungal strains which showed several appearance in several isolating plates (N_1 and N_2) were applied to parchments as hydrolysing microbes and studying the degree of deterioration that takes place for parchments after incubation was carried out for 10, 20 and 30 days at 30°C (Table I). These two fungi were then molecularly identified using 18S rRNA techniques. Deteriorated wooden sculptures and art photographs stored in the quarantine room of the Cultural Centre in Belgrade, Serbia, were subjected to microbiological studies.[15] Twelve fungal sp. were identified including *Absidia, Alternaria, Aspergillus, Chaetomium, Neurospora, Penicillium, Rhizopus, Syncephalastrum* and *Trichoderma* (from wooden substratum) in addition to *Fusarium, Humicola, Paecilomyces, Trichoderma* and *Ulocladium* (from the photographs).

3.1 Molecular identification of the fungal isolate N_1 and N_2

A nucleotide sequence of 1563bp and 1439 of the whole 18S rRNA gene of the fungal sp. isolate N_1 and N_2 (respectively) was determined in both strands. The AB1 chromatograms of DNA sequencing of the isolate N_1 and N_2. BLAST search revealed that isolate N_1 exhibited 97% similarity to *Aspergillus fumigatus* strain VV11 (acc. no. KT031993.1) and N_2 exhibited 97% similarity to *Talaromyces spectabilis* strain CBS 101075 (acc. no. AY526473.2). The phylogentic tree of these fungi were also constructed (Figures 2 and 3). These fungi were identified as *Aspergillus fumigatus* EGY-N1 and *Talaromyces spectabilis* EGY-N2 with the GeneBank accession numbers KU509536 and KU509537 for N_1 and N_2 respectively. Traditional identification of fungi is established by cultivation and microscopic examination including mycelial colour, size and shape as well as conidial shape and the morphology of conidiophores.[16,17] These methods need highly experienced taxonomists and are time consuming.[18] But molecular methods are considered as effective, fast and easy tools for fungal identification.[19] Ma *et al.* (2015)[20] used the molecular techniques (16S rRNA and 18S rRNA) to compare the microbial groups colonising the wall paintings on the ancient cave Magao grottoes there were causing

TABLE I
Parchment samples after incubation with *Aspergillus fumigatus* and N_2 for 10, 20 and 30 days at 30°C

	Time of incubation		
	10 days	20 days	30 days
Aspergillus fumigatus			
Talaromyces spectabilis			



N_1 N_2 N_3 N_4 N_5

Figure 1. The isolated fungal strains N_1, N_2, N_3, N_4 and N_5 from ancient parchment from Quran found in Al-Azhar Library, Egypt.

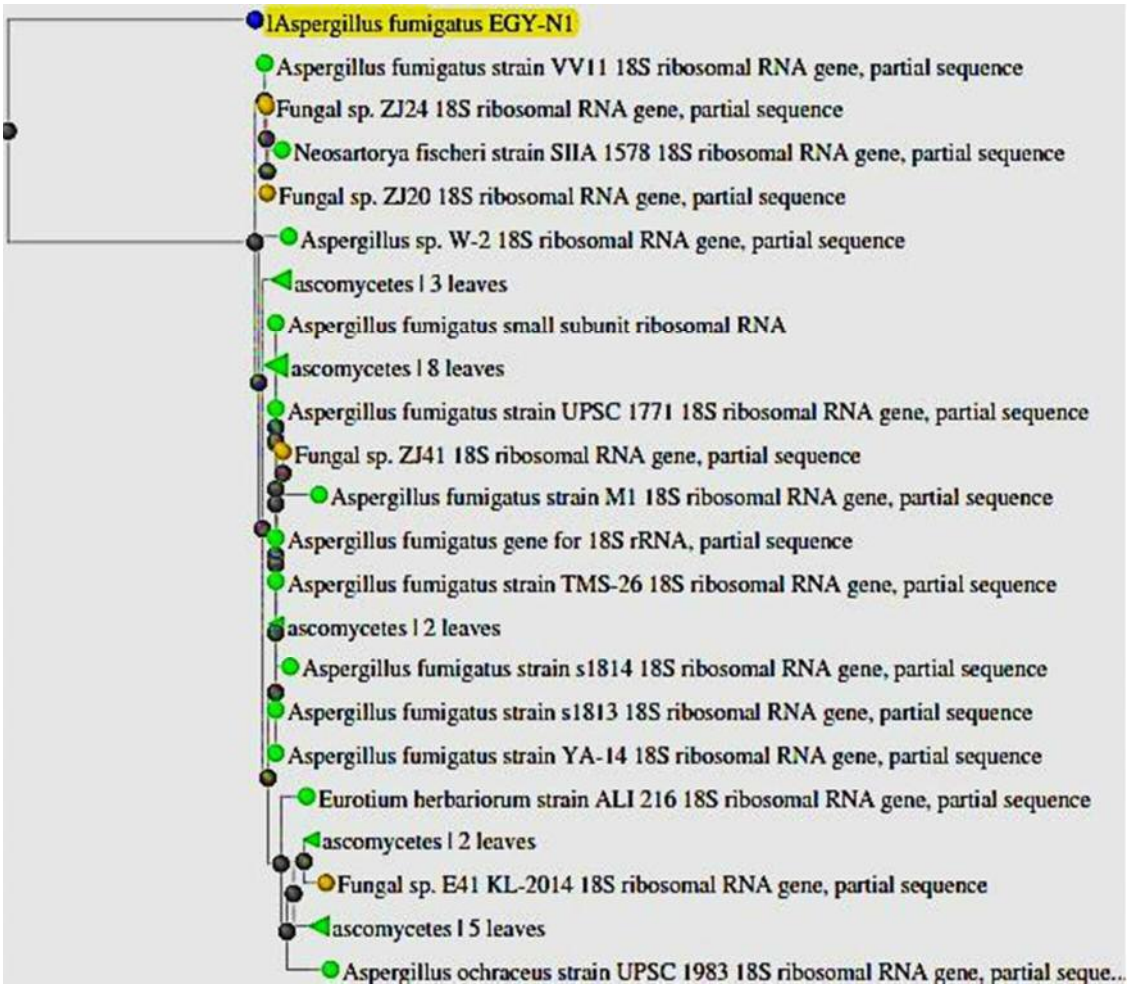


Figure 2. Phylogenetic trees showing relationship of strain N_1 with other related fungal species retrieved from GenBank based on their sequence homologies of 18srDNA

deterioration. A qualitative analysis based on genetic identification of bacteria and fungi has been done by Lech[21] for assessment of the bio-deterioration components of a parchment document from the 13th century. The isolated microbes with highest destructive potential were *Bacillus cereus* and *Acinetobacter lowffii* (bacteria) as well as *Penicillium chrysogenum, Chaetomium globosum* and *Trichoderma longibranchiatum* (fungi). In addition to *Alernaria, Aspergillus, Cryptococcus, Paenibacillus, Micrococcus, Massilia etc.* are lower destructive microbes.

3.2 Mechanical properties

Results represented in Table II revealed that the tensile strength of parchment samples treated with both fungal strains were decreased with increasing incubation periods. The reduction is clear for *T. spectabilis* after 30 days incubation (σ_B $N_2$3: 341.02kg/cm^2: σ_B $N_1$3: 578.61kg/cm^2) whereas the σ_B value for the control sample was 1168.57kg/cm^2.

The elongation percent (strain-to-break ε_B%) results displayed that the reduction is similar to the decrease in tensile strength. Also the reduction in elongation percent of samples inoculated with *T. spectabilis* is more than it is in the case of samples inoculated with *A. fumigatus* and this percent increased with increasing incubation period. ε_B% for the control sample was 25.86% and it reduced to 16.48% and 14.52% for samples N_1 and N_2 after 30 days of incubation. The decrease in mechanical properties may be attributed to the collagenolytic fungi that hydrolysed the collagen fibres.[2,3,22,23,24] *A. fumigatus* demonstrated proteolytic activity which represented a potential risk as it might hydrolyse different complex proteinaceous substrates containing collagen.[2] Certain species of fungi of the genera *Cladosporium, Fusarium, Ophiostoma, Scopulariopsis, Aspergillus, Penicillium, Trichodernia, etc.* produce non-specific proteolytic enzymes that can decompose denatured collagen in aerobic conditions.

3.3 Fourier Transform Infrared Spectroscopy (FTIR)

Results shown in Table III and Figures 4 and 5 present the Fourier Transform Infrared Attenuated Total Reflection (FTIR-ATR) of fungal-treated parchment as compared with the control. It has been found that there is a large negative shift in the amide I (39 and 28 wave number) and amide (41 and 25 wavenumber) for samples incubated for 30 days with *A. fumigatus and T. spectabilis* respectively.[25] These shifts have been occurring according to the separation of amide I and amide II ($\Delta\nu$) which means conversion of collagen triple helix into random coils found in gelatine.[26,27] These results were established with the occurrence of a shifted carbonyl vibration band around 1710 and

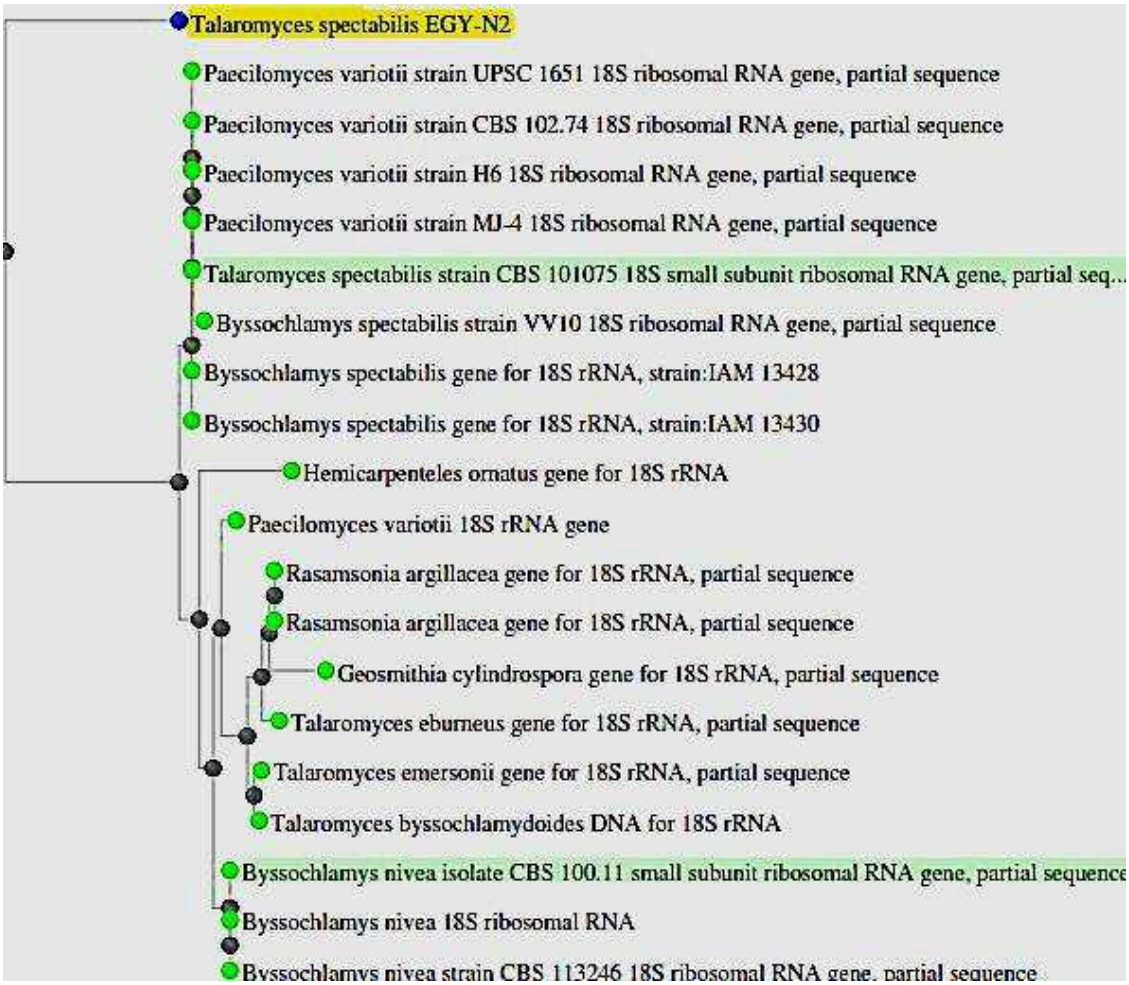


Figure 3. Phylogenetic trees showing relationship of strain N_2 with other related fungal species retrieved from GenBank based on their sequence homologies of 18srDNA

1720cm^{-1} which differ from the carbonyl of the amide group observed in 10, 20 and 30 days of incubated samples. This new carbonyl band is likely came from the formation of acidic species which may have different origins. This weak band between 1750 and 1700cm^{-1} may have been formed due to acidification, hydrolysis or oxidation.[28,29] In the case of gelatinization, the most likely mechanisms are acidification which result in protonation of the side chain on the carboxyl group of glutamic and aspartic acids, and the hydrolysis results in the realisation of COOH terminal and NH3^{+}. The positive shift of amide A (approximately 30 wavenumber) after 30 days of incubation confirms the denaturation of the collagen molecule. The amide A showed absorption at approximately 3300cm^{-1}, which is a practical indicator of the strength of the hydrogen bonds within the collagen structure. It shifts to a higher wavenumber with the decrease of hydrogen bonds fashioned between the triple helix of the collagen molecule, so indicating a partial denaturation of the collagen molecule.[25] The absorption bandings from the CH_3 and CH_2 functional groups can also be used to identify collagen conformational changes. Once collagen fibres are gathered into a triple helix motif the asymmetric and symmetric CH_2 are constrained and the asymmetric and symmetric CH_3 are predominant in the absorption spectra. Conversely, when collagen has denatured into the random structural motifs found in gelatine, there is less constraint of the asymmetric CH_2 and symmetric CH_3 producing an absorption banding with a significantly more intensity.[30] In our experimental study the band at 2960cm^{-1} and 2856cm^{-1} in the control sample, is associated with anti-symmetric and symmetric vibrations of CH_3 in the collagen triple helix, and, respectively, shifts to 2920cm^{-1} and 2845cm^{-1} are associated with asymmetric CH_2 and symmetric CH_2 in

TABLE II
Tensile strength and elongation of untreated and fungal deteriorated parchment samples

Sample ID	Tensile strength kg/cm^2	Elongation %
Blank	1168.57	25.86
$N_1$1	980.38	20.45
$N_1$2	903.30	18.48
$N_1$3	578.61	16.48
$N_2$1	976.61	18.83
$N_2$2	710.83	16.32
$N_2$3	341.02	14.52

Blank; Undeteriorated sample,
N_1 1, N_2 1; Samples incubated for 10 days,
N_1 2, N_2 2; Samples incubated for 20 days,
N_1 3, N_2 3; Samples incubated for 30 days
with *A. fumigatus* and *T. spectabilis*, respectively.

TABLE III

FTIR spectra of untreated and fungal deteriorated parchment samples

Sample ID	A_I		A_{II}		A_A		A_B		CH		OH		Gel, C=O	
	Wave-number	Intensity %	Wave-number	Intensity %	Wave-number	Intensity %	Wave-number	Intensity %	Wave-number	Intensity %	Wave-number	Intensity %	Wave-number	Intensity %
Blank	1680	27	1559	70	3300	77	3087	75	2960,2856	74	3445	79-68	–	–
$N_1$1	1670	29	1525	58	3315	50	3129	53	2920,2845	52	3460	44	1710	29
$N_1$2	1646	22	1530	44	3324	33	3143	35	2920,2845	34	3459	28	1720	24
$N_1$3	1641	26	1518	42	3330	26	3170	43	2921,2832	36-46	3448	28	1720	47
$N_2$1	1673	30	1543	62	3315	72	3098	77	2925,2840	70	3456	60	1721	37
$N_2$2	1670	43	1540	77	3324	58	3130	63	2925,2840	64	3443	48	1721	43
$N_2$3	1652	37	1534	76	3332	78	3167	78	2925,2840	78	3458	66	1721	37

Blank, Undeteriorated sample; $N_1$1, Samples incubated for 10 days; $N_1$2, Samples incubated for 20 days and $N_1$3, Samples incubated for 30 days with *A. fumigatus*. $N_2$1, Samples incubated for 10 days, $N_2$2, Samples incubated for 20 days and $N_2$3, Samples incubated for 30 days with *T. spectabilis*.

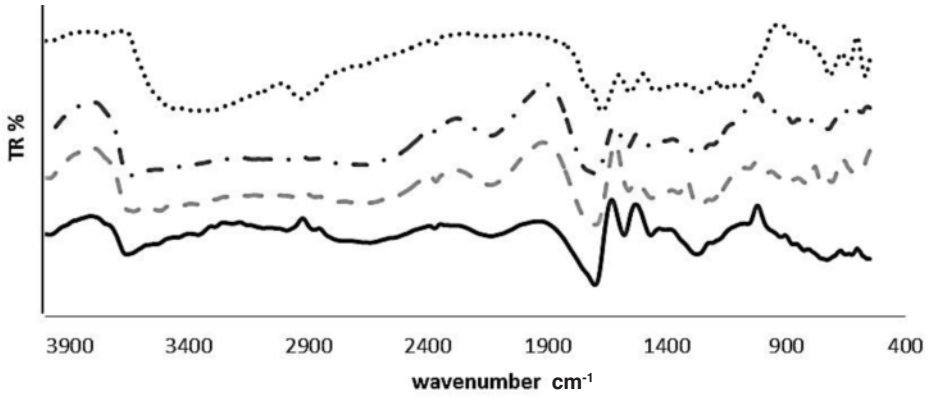


Figure 4. FTIR-ATR spectra of degraded parchment samples inoculated with *A. fumigatus*.
——— Undeteriorated sample ------ After 10 days of incubation
—·—·— After 20 days of incubation ····· After 30 days of incubation

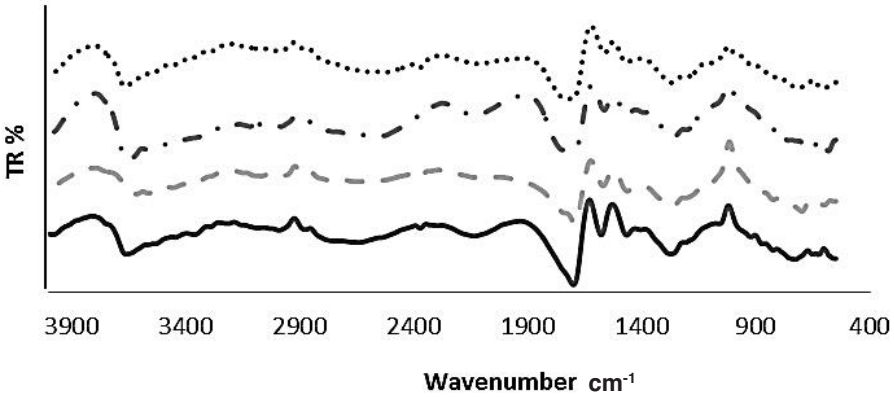


Figure 5. FTIR-ATR spectra of degraded parchment samples inoculated with *T. spectabilis*.
——— Undeteriorated sample ------ After 10 days of incubation
—·—·— After 20 days of incubation ····· After 30 days of incubation

unfolded collagen fibres. Intact collagen molecule exhibits anti-symmetric and symmetric vibrations of CH_3 at 2962 and 2872 cm^{-1}, respectively and CH_2 at 2922 and 2852cm^{-1} for asymmetric and symmetric vibrations, respectively and samples that have unfolded collagen molecules produce spectra with large, sharp peaks at 2952 and 2890cm^{-1}; assigned to a significant increase in the asymmetric CH_2 and symmetric CH_2, respectively.[25,31] The strong and broad band with indistinct shoulders centred at 3450cm^{-1}, assigned to the stretching of both OH and NH groups, variously hydrogen bonded assigned in all spectra of the control and incubated samples,[26,32] may be an indication of: (i) a dehydration process,[33] or (ii) conformational change of the collagen triple helix which allows a prevalence of polar regions that were otherwise embedded in the collagen fibril to bond with free OH^-.[25]

3.4 Investigation of the surface morphology by SEM

The intact untreated parchment showed a network of fundamental collagen fibres (Fig. 6a) whereas parchments inoculated with *A. fumigatus* and *T. spectabilis* incubated for 10, 20 and 30 days exhibited changes in their shapes; swollen and rounded fibres appeared (Fig. 6b). These findings indicate the deterioration occurred due to the fungal activity. With

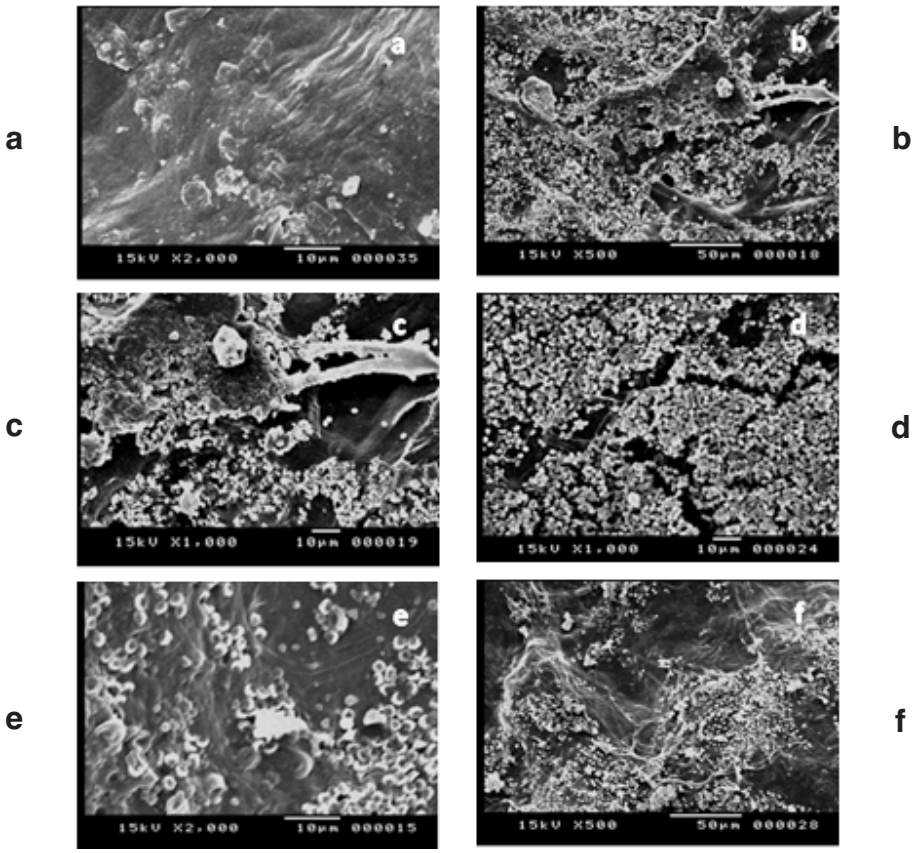


Figure 6. **a**; Fundamental collagen fibres, **b**; Swollen and rounded fibres, **c**; Increase of spacing between fibres, **d**; Loss of fibre network and presence of deep cracks, **e-f**; Melt-like zones and finally appearance of glassy surface.

TABLE IV
Change of colour parameters of new parchment samples before and after fungal deterioration

Sample ID	L	a	b	De
Blank	78.60	-2.04	8.90	0
$N_1$1	74.22	-0.75	16.28	8.68
$N_1$2	67.95	0.59	16.62	13.41
$N_1$3	63.09	1.27	16.67	17.66
$N_2$1	41.12	1.94	9.73	37.70
$N_2$2	39.97	3.67	12.57	39.22
$N_2$3	35.83	4.65	16.59	43.98

longer incubation periods a progressively deteriorating morphology is seen. (Fig. 6c). Moreover, fibre damage and the occurrence of deep cracks appeared (Fig. 6d). Furthermore, the creation of melt-like zones and finally a glassy surface appearance were noticed (Figs. 6e and f). These results are synonymous with the findings of Della Gatta *et al.,* 2005).[34] The fungal hyphae and spores covered the surface of all the degraded samples.

3.5 Change of colour

The results in Table IV represent the colour change of fungal-treated parchment samples in comparison the control parchment. It was found that the brightness (L-value) decreased with increasing incubation time. The reduction of the brightness for the samples inoculated with *T. spectabilis* was obviously greater than that inoculated with *A. fumigatus* at the same period. The reduction increased continuously during the whole period of incubation. Moreover, by increasing the incubation period of fungi with parchment samples, it has been noticed that a-values were also increased indicating that the treated samples were turned towards the red colour. The control sample (untreated parchment exhibited a-value of -2.04. The b-value (yellowness) was increased with the increasing incubation period and the yellowness was clearer with the parchment treated with *A. famugatus* than that treated with *T. spectabilis*. The colour changes could be due to the production of pigments and organic acids by fungi.[2,3,24,35] Moreover, the microbial attack also causes multi-coloured spots and white films.[22]

4 CONCLUSION

It has been found that *Aspergillus fumigatus* and *Talaromyces spectabilis* induce collagen denaturation as a result of their proteolytic activity. This leads to loss of mechanical properties. All parchment degraded samples showed a reduction in elongation and tensile strength in comparison with the control. FTIR analysis showed the unfolding of collagen fibres as a result of gelatinization. Moreover, SEM micrographs also presented variations between the control samples and the degraded ones due to the loss of the ordered fibre network as a result of fungal bio-deterioration. Colour change data proved that fungal deterioration induced reddening or yellowing that may be due to organic acids that the fungi produced as metabolites. It

becomes clear that parchment provides a good environment to fungi to grow if favourable conditions are found, and conservationists need to be aware of this.

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