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ORIGINAL ARTICLE

Cellulolytic potential and filter paper activity of fungi isolated from ancients manuscripts from the Medina of Fez

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Abstract The content of libraries in Fez is an important historical testimony and a treasure of knowledge of several civilizations. Unfortunately, this cultural heritage has suffered deterioration caused by many factors, including microbial deterioration specifically by cellulolytic fungi. The aim of our study was the identification and characterization of the microflora that damage historical manuscripts books from an old library of the Medina of Fez. A total of 31 filamentous fungi were isolated from deteriorated paper of ancient books. Nine of these isolates were screened positive for the ability to degrade carboxymethylcellulose (CMC). The ability of the nine isolates to produce filter paper activity (FPUase) in liquid media as well as the effect of temperature and pH were also studied. According to the molecular identification, the most frequent species were *Penicillium chrysogenum*, *Aspergillus niger*, *Aspergillus oryzae* and *Mucor racemosus*, and other less frequent, such as *Hypocrea lixii*, *Aspergillus melleus* and *Schizophyllum commune* were also present. Also, the effect of fungal contamination in paper artificially attacked for 18 months at 25 °C was examined. The fungal strains were able to degrade filter paper to varying degrees.

Keywords Fez · Filter Paper Activity · Biodeterioration · Cellulose · Fungi

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Introduction

Morocco, crossroads of several civilizations, has precious manuscripts. These documents play a major role in the field of scientific and historical research. One of the Moroccan cities most characterized by the wealth of manuscripts is the city of Fez. Historically, Fez has been known as a home to the oldest university in the world, "al qarawiyine university" (Saoudi 2008). However, much of this valuable cultural heritage that has been accumulated over time is today in an advanced state of deterioration due to poor storage conditions. Indeed, all of these manuscripts suffer from various destructive agents especially from microbial degradation that causes different kinds of damage depending on the organisms responsible for the attack. The biodeterioration of library materials surpasses 110 years (Zyska 1993), but the first research, which discuses microbial alteration of papers appears in 1917 (See 1919).

Fungi are considered as serious degrading agents of paper manuscripts, particularly cellulolytic fungi (Fabbri et al. 1997). Some filamentous fungi frequently colonize paper, and are able to degrade cellulose fibers through the action of cellulolytic enzymes (Reese and Downing 1951; Nyuksha 1983; Ciferri et al. 2000), or to alter aesthetic and visual appeal by releasing weak acids or pigments; a phenomenon referred as foxing (Arai 2000; Montemartini et al. 2003; Zotti et al. 2011).

Cellulose from native wood is used for paper production. In addition to cellulose fibers, paper can contain hemicelluloses (wood polyoses), lignin, and additives such as fillers and pigments (Fellers and Wegener 1991). Some filamentous fungi frequently associated with paper degradation are capable of dissolving cellulose fibres through the synergistic actions of endoglucanase (EC 3.2.1.4), exoglucanase or cellobiohydrolase (EC 3.2.1.91), and β-glucosidase (EC 3.2.1.21) (Lynd et al. 2002; Zhang and Lynd 2004), causing serious damage to paper materials of cultural and historical importance (Reese and Downing 1951; Nyuksha 1983; Ciferri et al. 2000). In this case,

Porck (2000) reported that the hydrolytic degradation of the cellulose molecules is the most frequent reaction, particularly when the presence of water in the storage environment plays an essential role. In fact, microbial activity depends largely on the water activity (aw) of the paper. It is a measure of free water which is available for the growth of the mould and is defined as the ratio of the vapour pressure of the water in the substrate and the vapour pressure of pure water at the same temperature and relative humidity (RH) (Arai 1987). According to standard reaction, the rate of the hydrolytic process is determined by the combined effects of aw, the temperature, the pH and the relative humidity. At a RH level higher than 65 %, and a temperature higher than 20 °C, moisture content of paper can reach 8 to 10 %, with consequent water activity (aw) higher than 0.65, microbial spores can germinate and develop by using the paper as a substrate of growth, consequently affecting the object of cultural value (Kowalik 1980). Furthermore, Prieto and Silva (2005) reported that the intensity of the microbial contamination is influenced by the climatic conditions of the atmosphere.

The fungal contamination of Moroccan manuscripts stored in archives is therefore problematic because of the imperfect knowledge of the deteriorative agents. Also, many efforts have focused on the preservation and maintenance of this written cultural heritage. For adequate conservation of these archive collections, it is important to identify and characterize the fungal species for their control.

This investigation was done to (1) assess the role of fungi in the degradation of manuscripts in librairies from the city of Fez, (2) identify fungi isolated from the decayed paper by molecular methods, and (3) evaluate their FPUase and cellulase activities in laboratory. Little is known about deterioration of historical manuscripts in old Fez city, and our results provide new information on the degradation caused by fungi, and therefore provide information that is crucial to conservators for the preservation of these manuscripts.

Materials and methods

Paper samples

Two types of historical books of an old library of the Medina of Fez at an advanced stage of biodeterioration were used in this study. The first one is the sacred book of Islam, Qur'an, dated from 1827. The second is an un-identified book of koranic interpretation from the 19th century. The samples books were selected and samples that present a visible alteration, mainly from the margins of degraded pages, were collected. Also, the powdery of the samples were observed with an Olympus optical microscope.

Isolation, culturing and incubation

For sampling, three methods were used. (1) The samples were swabbed from the historical books. (2) Other samples were scraped with a scalpel, then all of them were pre-incubated in lysogeny broth (LB) supplemented by chloramphenicol (500 mg/l) (Rojas et al. 2009). (3) The collected fragments of deteriorated paper were introduced directly in the same medium for 24 h at 25 °C. After, the supernatants from each sample were serially diluted in sterile distilled water up to 10–4 and cultivated using different media: MEA (4 % malt extract, 1.8 % agar), LB agar (1 % peptone, 1 % NaCl, 0.5 % malt extract, 1.8 % agar), and YPG antibiotics agar (1 % yeast extract, 2 % glucose, 2 % peptone, 1.8 % agar, ampicillin 60 μg/ml and kanamycine 30 μg/ml). The dishes were then incubated at 25 °C for 24 hours. The strains were isolated and purified after streaking several times.

Screening of hydrolytic activity

Pure isolated cultures of fungal mycelia were screened for their cellulase activity; for this, they were transformed individually on CMC agar plates containing ($NaNO_3$ - 3.0 g/l, K_2HPO_4-1.0 g/l, $MgSO_4{\cdot}7H_2O$-0.5 g/l, KCl-0.5 g/l, $FeSO_4{\cdot}7H_2O$-0.01 g/l, CMC-1.0 g/l, Agar- 20 g/l, pH 5.0) and then incubated at 25 °C for 72 hours. After incubation the plates were stained with 1 % Congo Red solution for 15 min, and afterwards the stain was neutralized with 1 M NaCl solution. The appearance of a discoloured zone around the mycelia of the isolates confirms the ability of cellulose utilization and cellulase activity of fungal culture. Additionally, a more quantitative assay method was used in order to determine cellulase activity in liquid medium for pre-selected fungi (Ariffin et al. 2006).

Molecular identification

For molecular identification, the genomic DNA was extracted using thermal shock. The rDNA ITS region 1, 5.8S, and ITS region 2 were amplified utilizing primers ITS1 (5′TCC GTA GGT GAA CCT TGC GG 3′) and ITS4 (5'TCC TCC GCT TAT TGA TAT GC 3') (White et al. 1990). The PCR reaction mix was performed in a total volume of 20 μl consisting of 1 x PCR reaction buffer, 1.5 mM $MgCl_2$, 0.2 mM deoxynucleotides, 1 μM of each primer, U *Taq* DNA polymerase (Promega, Madison, WI, USA). The thermocycling program was as follows: 94 °C for 5 min; 35 cycles of 94 °C for 1 min, 50 °C for 1 min, 72 °C for 1 min followed by a final extension step of 72 °C for 5 min. We sequenced both strands of all amplified fragments. Comparative sequence analyses were performed by comparing sequences with those available in

the online databases provided by the National Centre for Biotechnology Information (NCBI) using the BLAST search program (Altschul et al. 1997).

FPUase activity and stability as a function of PH and temperature

The enzymatic activity of cellulases was determined by the method of filter paper assay (FPA) (Ghose 1987). One unit of this activity, termed as FPU, was defined as the amount of enzyme that catalyzes the formation of 1 mM of reducing sugars that are liberated from the hydrolysis of filter paper, per minute of culture filtrate under assay conditions. It was assayed by measuring the release of reducing sugars in a reaction mixture containing Whatman filter paper no.1 (1.0× 6.0 cm =50 mg) as a substrate in 50 mM sodium phosphate buffer (pH 4.8) after a 30 min of incubation at 50 °C. Reducing sugars were assayed by the dinitrosalicylic acid (DNS) method. This procedure was repeated thrice and the average value of three absorbances was determined. The concentration of the sample was determined from the standard curve (prepared with glucose). To determine the optimum temperature, the cellulase was measured at a wide range of temperatures (20–50 °C) in 0.1 M sodium phosphate buffer (pH 4.0). To determine the optimum pH of the activity of cellulase, the enzymatic assays were carried out at pH values ranging from 3.0 to 6.0, using sodium phosphate buffer system (0.1 M).

Growth on paper

Pieces of filter paper (Whatman filter paper No 1, F 55 mm) were cleaned with acetone, then rinsed twice with distilled water and dried. The dried paper samples were put in Petri dishes, wetted with 2 ml of distilled water and autoclaved for 15 min at 120 °C. Each piece of sterilized filter paper was inoculated with 2 ml of single-strain suspension containing 10^6 spores/ml previously counted by optical microscopy in a Malassez Chamber. Others paper samples were not inoculated with fungi, to serve as a control. All the samples were incubated at 25 °C for 18 months. Additional 1 ml of sterile water was monthly added to samples. After various periods of incubation, the texture and color of pigments surface was well defined: brown, black, green, white and pink. Also, the mycelium of fungi growing on the filter paper was removed vigilantly and paper was dried and weighed. Then weight loss was determined by:

$$\text{Percentage Weight Loss} = \text{Initial weight} - \text{Final weight} \times 100/\text{Initial weight}.$$

Results and discussions

Macroscopic observations

In order to determine the levels of the degradation of these important manuscripts, we first observed our samples at a macroscopic level. All samples presented evidence of strong superficial alteration. Generally, the spots appear in the external pages of books; however, the internal pages were better preserved. Indeed, this visual inspection reveals a number of spots of different colors.

Furthermore, the results of the observation with the optical microscope of powdery samples showed the presence of fungal spores and pigmented cellular structures including some hyphae fragments and mycelia.

Fungi isolated, identified and screened

During this study, we isolated 31 fungal strains from the ancient documents; 16 of them presented different morphology and

Table 1 Fungi isolated from ancient documents: original document, accession numbers, genetic similarity with existing NCBI sequence and the total cellulase are presented.

ID	Orginal document	Identified fungi	Accession number	Similarity (%)	Class	Total cellulase (ICMC)
CPF1	Quoran	*Aspergillus oryzae*	EF661560.1	99	Eurotiomycetes	1.5±0.05
CPF4		*Aspergillus niger**	–	–	Ascomycetes	2.7±0.03
CPF6		*Hypocrea lixii*	FJ517550.1	99	Ascomycetes	1.9±0.02
CPF9		*Mucor racemosus*	HM641690.1	99	Zygomycetes	1.7±0.03
CPF11		*Penicillium commune**	–	–	Ascomycetes	2.5±0.02
CPF12		*Schizophyllum commune*	FJ478109.1	100	Ascomycetes	1.9±0.03
CPF13		*Penicillium chrysogenum*	GU985086.1	99	Ascomycetes	1.7±0.03
CPF14	Books	*Penicillium chrysogenum*	JF834167.1	98	Ascomycetes	0.9±0.01
CPF15		*Mucor racemosus*	HM641690.1	100	Zygomycetes	2.9±0.02
CPF16		*Aspergillus melleus*	FM986320.1	99	Ascomycetes	2,3±0.03

*Isolates identified using morphological examination

colour. Among these fungi, only nine were identified and screened for their ability to produce cellulase. Table 1 lists the molecular identification of fungal isolates from the deteriorated manuscripts. This report presents the first results of the microbial contamination study of the archives in Morocco.

The fungal contaminants detected in the paper samples were *Aspergillus niger* van Tieghem 1867, *Aspergillus oryzae* (Ahilburg) Cohn 1884, *Aspergillus melleus* Yukawa 1911, *Mucor racemosus* Fresenius 1850, *Hypocrea lixii* Patouillard 1891, *Schizophyllum commune* Fries 1821, *Penicillium commune* Thom 1910, *Penicillium chrysogenum* Thom 1910. Some of the mentioned genera occur more frequently, such as *Aspergillus* and *Penicillium*. Overall, the high frequency of those genera is in agreement with other works (Hyvarinen et al. 2002; Di Bonaventura et al. 2003; Lugauskas and Krikstaponis 2004; Silva et al. 2006; Michaelsen et al. 2006; Neves et al. 2009; Sohail et al. 2009). These are almost ubiquitous taxa, and can produce numerous mitospores and conidia that are easily dispersed by air. Moreover, they are usually found as contaminants or biodeterioration agents in many different habitats and materials, including those considered as representative of historical and cultural heritage (Das et al. 1997; Abrusci et al. 2005; Sohail et al. 2009). Shamsian et al. (2006) described a high frequency of the genera *Aspergillus*, *Penicillium*, *Mucor* and *Trichoderma* in the biodegraded books and manuscripts. Andersen et al. (2011) describe that *Penicillium chrysogenum* was evenly associated with wallpaper compared to other organic materials (wood, polywood, plaster, gypsum).

In fact, fungi are well known agents of decomposition of organic matter in general and degradation of cellulosic substrate in particular, as reported by Lynd et al. (2002). These microorganisms synthesized cellulases during their growth on cellulosic materials (Lee and Koo 2001) and as known, in time, paper suffers from deterioration processes because of the oxidation and hydrolysis of the various functional groups from the structure of cellulose. Several studies have reported that *Aspegillus niger* was isolated from paper materials (Silva et al. 2006; Michaelsen et al. 2006). In addition, it is well established that *Aspergillus niger* produces several of cellulolytic enzymes responsible for the degradation of cellulose from paper fibers (Deacon 1997). Commercial cellulase preparations, derived from culture filtrates of the fungus, have been fractionated by several workers (Van Whyk et al. 2000; Sukumaran et al. 2005; Khalid et al. 2006; Villena and Gutiérrez-Correa 2007; Sibtain et al. 2009; Eida et al. 2011). Besides, *Aspergillus melleus* is also known to produce cellulase (Ezekiel et al. 2010).

Similarly, *Mucor racemosus* and *Penicillium commune* are often present on biodeteriorated paper of historical documents (Gallo 1992; Florian and Manning 2000; Valentin et al. 2002; Abrusci et al. 2005; Zyani et al. 2009). Likewise, Andersen et al. (2011) found *Mucor racemosus* on wallpaper. These are

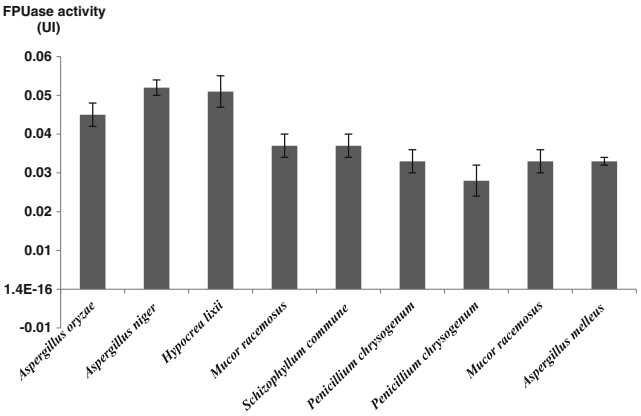

Fig. 1 FPUase activity of the fungal isolates grown on filter paper as the only source of carbon (pH = 7, T = 30 °C)

dangerous to books and documents due to their capacity to produce cellulolytic enzymes at high levels (Gopinath et al. 2005). Moreover, *Schizophyllum commune* was isolated from biodeteriorated paper and it has been found in a wide range of environments (soils, air, murals). In many cases the wood presents a specific substrate for this species (Cojocariu and Tanase 2010).

Finally, the fungal strains belong to genera already isolated from paper material, with the exception of *Hypocrea lixii*, which was never isolated on biodeteriorated paper until now. This microorganism is a teleomorph of *Trichoderma harzianum* (Chaverri and Samuels 2002; Eida et al. 2011) and produced a green pigment in conidial spores as reported by Hölker et al. (2002) and was recently reported to produce cellulase on several researches (Chandra et al. 2009; De Castro et al. 2010; Eida et al. 2011; Cabero et al. 2012).

All of these microorganisms are usually present in the air; their presence in these manuscripts may activate the start of a biodeterioration process if adequate environmental conditions are met—thus, the microbial contamination acts as a preliminary precursor for the alteration of the manuscripts.

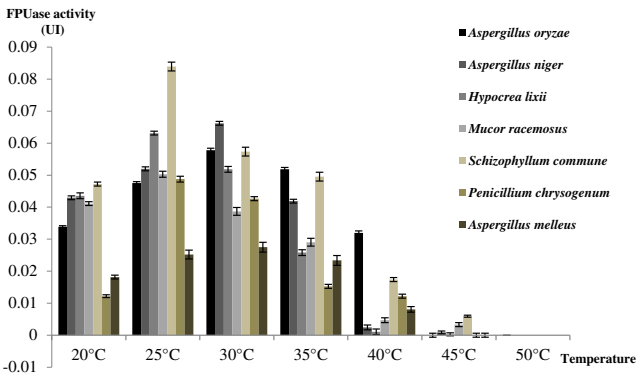

Fig. 2 Effect of the temperature on the FPUase activity of the fungal isolates grown on filter paper as the only source of carbon (pH = 5)

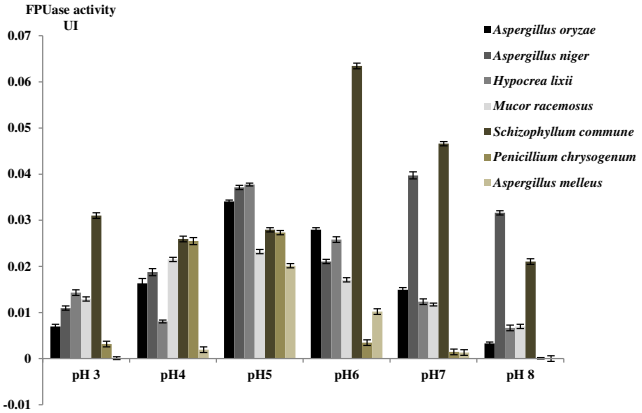

Fig. 3 Effect of the pH on the FPUase activity of the fungal isolates grown on filter paper as the only source of carbon (T = 25 °C)

Enzyme activity assay

Production of FPUase activity for the identified isolates is shown in Fig. 1. On the basis of the results, the production of this enzymatic activity was first detected after 48 h of cultivation and increased during growth and reached maximum level on the eighth day for all the isolates. Three of these isolates presented a high FPUase production, and the others presented low FPUase. It was observed that the levels of FPUase of *A. niger*, *A. oryzae* and *H. lixii* were higher than the level of the same enzymatic activity of *A. melleus*, *M. racemosus*, *S. commune* and *P. chrysogenum*.

Aspergillus niger and *Hypocrea lixii* enzyme activity was higher (more than 0.05U) on the eighth day. The first species is known to degrade filter paper (Villena and Gutiérrez-Correa 2007; Jahangeer et al. 2005), also, *Schizophyllum commune* is reported to produce a similar quantity of filter paper enzyme (Valencia et al. 2011). Furthermore, Ezekiel et al. (2010) found that *Aspergillus melleus* has a good FPUase activity.

This finding indicates that the cellulase produced by isolates (FPUase) to hydrolyze cellulose; the principal content of paper; to extract the nutrients required for fungal growth. For this, the filter paper activity would be an interesting subject to be assessed on degrading-paper microorganisms.

Effect of temperature and pH on the FPUase activity

The effect of temperature on the FPUase activity of the seven identified species was examined at various temperatures ranging from 20 to 50 °C as shown in Fig. 2. The FPUase activity of the isolates was greatly affected by temperature changes. Moreover, the enzyme showed a good activity at 25 °C and 30 °C, and inactive at 50 °C for all the strains.

The optimal temperature of the enzyme in *Aspergillus* was around 30 °C; however for *Hypocrea lixii*, *Mucor racemosus* and *Schizophyllum commune* and *P. chrysogenum* was of 25 °C. In contrast, most filamentous fungi are mesophilic requiring optimal temperatures between 25 and 35 °C (Reid 1998; Suresh et al. 1999). Philippidis (1994) report that some *Aspergillus* strains showed higher enzyme yield at 30 °C which is in agreement with the present study.

As a degradation factor, temperature acts indirectly by promoting fungal growth by accelerating the already initiated chemical reactions, as well as the biological degradation products. Keeping the thermo-hygrometric parameters constant is essential for a suitable, long-term preservation of the cellulosic materials. Moreover, Abrusci et al. (2005) noted that temperature is a very important factor affecting rate and extent of biodegradation. In the present work, there was a good correlation between the optimal temperature for enzyme activity and the temperature previously evaluated in the ancient libraries of the ancient Medina of Fez that not exceed 30 °C. This could reflect the adaptation acquired of these fungi to grow and to possess a maximum activity in the conditions of the novel environment they inhabit.

Similarly, the effect of pH on the FPUase activity was examined at pHs ranging from 3.0 to 8.0 as shown in Fig. 3. All isolates were able to produce FPUase activity at different pH, but at various degrees. The optimal pH for fungal cellulases varies between species, although in most cases the optimum pH ranges from 3.0 to 9.0 (Coral et al. 2002; Niranjane et al. 2007).

Enzymes from all isolates presented a major activity peak at pH 5.0, with the exception of *S. commune,* which showed higher activity at pH 6.0. Our findings are similar to those

Table 2 Paper decomposition by fungal strains and colours of pigmented stains

Fungal strains	*Aspergillus oryzae*	*Aspergillus niger*	*Hypocrea lixii*	*Mucor racemosus*	*Aspergillus melleus*
Visual colors of stains	Green yellow	Black	Green	Dark grey	Green yellow
Percentage weight loss					
0(months)	0	0	0	0	0
2	4,17	2,67	1,17	7,83	0,83
4	6,17	4,67	1,83	8,83	2,50
8	11,33	7,67	4,50	10,33	6,17
12	11,67	12,67	5,33	12,17	8,50
18	13,83	15,17	12,83	17,83	10,17

reported by several works with different period and temperatures of incubation. The present study showed that optimum pH for many *Aspergillus* cellulases is near pH 5.0 as reported by Vries and Visser (2001). Also, Sibtain et al. (2009) used pH of 5.5 for cellulases production from *H. lixii*. Similarly, Youssef (2011) previously described that maximum induction of FPUase activity of *A. oryzae* was achieved at pH 5. Furthermore, the pH requirement was determined at pH 5.0 for *A. melleus* as described by Ezekiel et al. (2010).

Artificial attack

During the examination of the artificially contaminated samples using pure fungal strains, unanticipated results were obtained. All the isolated fungal strains demonstrated a high capacity to grow on paper material after their reinoculation on sterile Whatman paper. In addition, the margins were extremely deteriorated, and in some areas, we observed the stains and spots of different colors like those naturally affected by biological agents. In fact, the brownish spots that appeared on paper are the typical marks of foxing resulting from the effect of biotic agents (Zotti et al. 2011), especially from the metabolic activity and pigments of the mycelia and spores. Table 2 shows that these cellulolytic strains were able to decompose filter paper cellulose at varying degrees.

Aspergillus niger shows black staining properties, characteristic of pigments (e.g., melanins) in the hypha of this fungus. Also, A. oryzae, *A. melleus*, *M. racemosus* and *H. lixii* produce respectively yellow, grey and green mycelia, could be responsible of intense stains. In most cases, the colours of the stains are often characteristic of particular genus or species of fungi. It is common for most fungal species to have aesthetical destructive effects by pigments and metabolic products. Such stains often badly disfigure the surface of paper, especially historical paper containing high levels of deterioration products like acidic components. Sterflinger et al. (1999) described that fungal growth on objects of cultural heritage often causes a serious aesthetical spoiling due to colony formation and fungal pigments.

Besides, all the fungal strains were able to degrade filter paper at varying degree. Table 2 shows that the maximum decomposition was observed with *M. racemosus* (17.8 %), this species was capable of decomposing the component of paper at two months at high levels, compared to the others. This can be explained by the fact that the test conditions coincide with the optimum conditions of the growth or/and of the cellulase production of this strains. However, the minimum was found with *A. melleus* (10.2 %). The loss of strength caused in the structure of paper is the result of enzymatic decomposition of the components of paper by fungi, and like final results, the paper become thinner and weaker.

Conclusion

In the present study, it could be concluded that the historical paper documents are colonized by variety of fungi including *Aspergillus niger*, *Aspergillus oryzae*, *Aspergillus melleus*, *Penicillium commune*, *Penicillium chrysogenum*, *Mucor racemosus*, *Hypocrea lixxi* and *Schizophyllum commune*. Most of these isolates were known as contaminants of paper, but some of them never were observed on paper materials of libraries. Moreover, all of these fungal strains were found to possess cellulose degrading ability when grown on carboxymethyl-cellulose or on filter paper as a sole source of carbon. In parallel, the capacity of the isolates to change aesthetic aspect and cause the mass losses of artificially contaminated paper was confirmed. The presence of these fungi within the paper materials, and their ability to have a cellulolytic potential and degrade cellulose in vitro, demonstrated that these fungi could be responsible of the degradation and the apparition of foxing in historical paper.

This report may increase the knowledge about the microbial communities colonizing paper of ancient library materials, as well as the relation between decomposition of paper and fungi cellulolytic enzyme activity. For that reason, it is useful to better study the other enzymatic activities of these fungi and others, like proteases and amylases that will enable them to use glues, starch and other additives of paper as a nutrient source for survival and maintenance.

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