THE EFFECT OF FUNGAL DECAY
ON FICUS SYCOMORUS WOOD

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Abstract

The deterioration of wood on account of microbiological agents is an acknowledged fact. Botryodiplodia theobromae - Trichoderma longibrachiatum - Aspergillus candidus - Aspergillus ustus and Aspergillus terreus were isolated from two wooden masks dating back to the Greek-Roman period in Egypt. The chemical composition of wood is easily affected after any attack and visible changes can be noticed clearly after some time, but the degree of deterioration of wood constituents cannot be estimated unless the wood is closely studied. Ficus sycomorus wood samples, which had been infected by the fungi isolated from the masks, were studied by using X Ray Diffraction (XRD) and Fourier Transform Infrared Spectroscopy with Attenuation Total Reflection (FTIR-ATR).

Keywords: *Ficus sycomorus; Botryodiplodia theobromae; Trichoderma longibrachiatum; Aspergillus terreus; Aspergillus ustus; Aspergillus candidus; XRD; FTIR.*

Introduction

Sycamore wood (*Ficus sycomorus*) was commonly used throughout the centuries in Egypt. Being a native wood, the timber was used for structural purposes and to make coffins and sarcophagi since at least the time of the Fifth Dynasty [1]. In one Eighteenth Dynasty document and in another dating from 251 B.C., sycamore wood is said to have been used for building a boat [2]. Statues and masks, commonly found in Egyptian museums, from storages and excavations, are often made of this wood.

Usually, objects made of wood suffer from numerous deterioration factors, depending on their surrounding environmental conditions. In a previous research, the fungal flora from Egyptian archaeological wood samples of different wood species, that were found in various locations in Egypt were isolated and identified. El-Sonbaty [3] identified twenty three species belonging to nine genera. Zidan *et. al.* [4] identified seven species belonging to five genera, after isolation from a wooden coffin. It is evident from previous researches that a large number of fungi isolated from wood, in Egypt, affect primarily cellulose on a higher scale, followed by the decay of lignin, on a slightly smaller scale [3, 5-7]. It is not clear, though, how fungi affected the different components of wood in the past.

Growth characteristics of fungi in wood and the type of degradation they cause, result in different decay patterns [8]. Depending on the type of decay, different physical, chemical and morphological changes occur in wood. These decay processes were well characterized and they

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provide useful insights into the deterioration of archaeological wood. Wood decay fungi are grouped into three major categories: brown rot, white rot and soft rot [9]. Fungi affect wood differently, but it was noticed that the oxidative enzymes that fungi excrete into the medium, mediate various biodegradation reactions, that involve the break of different carbon–carbon or ester bonds. In the case of hydrolysis, the break of aryl–alkyl bonds between the structural units of lignin occurs [10].

The present research aims to eliminate other deteriorating factors, in order to study the effect of early fungal decay on sycamore wood. We studied the early decay of sycamore wood, due to the infestation with five fungi, isolated from archaeological masks, with the aim that our results may help in the assessment of the deterioration of this commonly used wood throughout Egyptian history.

Materials and Methods

Samples

Biological samples were collected from different areas in the masks that either appeared to be associated with decay and discoloration, or showed frequent and typical microbial growths, particularly dense growths. The two badly deteriorated wooden masks were part of a collection at The Museum of the Faculty of Applied Arts at Helwan University, in Giza, Egypt. The masks (Fig. 1) date back, according to the records of the museum, to the Greek-Roman Period in Egypt, but their allocation is not known.

Fig. 1. One of the two deteriorated wooden masks in
the Museum of the Faculty of Applied Arts, Helwan University, Giza, Egypt

Sampling Method and Fungi Identification

Wood samples were sterilized on the surface by dipping them in Chlorox solution (10-15%) for 1 min., washed three times with sterile distilled water and dried, by using sterile filter paper, cut with a sterilized sharp knife and small wood pieces were removed aseptically, cultivated on Petri dishes containing a Potato Dextrose Agar (PDA) medium and incubated for 5-7 days at 25 – 30°C. During the incubation period, any emerged fungus was isolated on to PDA slants. The fungi were purified by using the hyphal tip and/or the single spore technique of Manandhar, *et al.* [11]. The identification of specific moulds was carried out on the basis of their macro and microscopical characteristic sporulation, according to the keys of Gilman [12], Nelson *et al.* [13] and Barnett & Hunter [14].

Fungal Inoculation

Spore suspensions of the previously isolated fungi from the deteriorated wooden masks were prepared separately by adding 10 mL of sterilized distilled water to each plated PDA

culture (15-days old) of each fungus and spores were freed by aid of a brush. Spore suspensions were strained individually through muslin and standardized to contain $1.2x10^6$ spores per ml, by using a haemocytometer slide.

Ten blocks (20x18x18mm) of sycamore wood for each fungus were dried at 105°C for 24 hrs, then autoclaved at 121°C for 20 min. After cooling, the wood blocks were sprayed until they were covered with spore suspension of the fungi and left for 8 weeks. Non-inoculated wood blocks were used as reference samples. The surface of the inoculated and the reference wood were investigated under a Quanta 200 ESEM FEG (FEI) Scanning Electron Microscope, which confirmed that the wood samples had been infested with the five fungi and the spores were obvious within the cell lumen. Samples from the infested surface were taken for XRD and FTIR analysis.

Cellulose Crystallinity

The band positions of crystalline and amorphous cellulose forms of the reference sample and of the inoculated samples were measured by using a Philips Analytical X-Ray B.V.; PC-APD diffraction software; diffractometer type: PW 1840, with a Cu tube anode; generator tension 40kV and generator current 25mA. The CuKα radiation consisted of Kα1 (0.154056nm) and Kα2 (0.154439nm) components. Scans were obtained from 5 to 30 degrees 2θ in 0.03 degree steps for 0.3 seconds per step. The crystallinity index (CI) was calculated according to the method of Segal et al. [15], i.e. $((I_{002} - I_{18})/I_{002})X100$, with the diffraction intensities, I_{002} at 002 peak position (2θ ≈ 22.5º) and I_{18} at 2θ = 18º (amorphous) [16,17]. The crystallinity index was also calculated by the FTIR method, according to Nelson and O'Connor [18], by measuring the ratio of band intensity at wave number 1425cm^{-1} to band intensity at wave number at 900cm^{-1}. The Crystallographic planes were labeled according to the cellulose I structure, as described by Popescu et al. [19].

FTIR Analysis

The structural changes occurring in the infected wood samples, compared with the non-inoculated ones, were monitored by a BRUKER'S VERTEX 70 – Fourier Transform Infra Red Spectroscope with Attenuation Total Reflection (FTIR-ATR), with a resolution of 4cm^{-1}.

Results

Isolation, Identification and Inoculation

Five species belonging to three genera were identified after their isolation from the masks. They were classified to: *Botryodiplodia theobromae - Trichoderma longibrachiatum - Aspergillus candidus - Aspergillus ustus* and *Aspergillus terreus* (Fig. 2). The sycamore wood blocks were then inoculated with the fungi that were isolated and monitored. To confirm that wood blocks became infested with the fungi, the samples were studied using SEM. (Figs. 3-5)
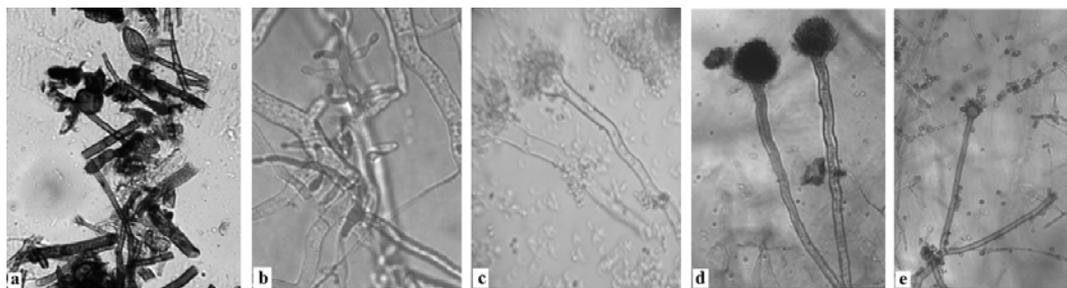

Fig. 2. The fungi isolated from the masks. a) *Botryodiplodia theobromae*, b) *Trichoderma longibrachiatum*, c) *Aspergillus terreus* , d) *Aspergillus candidus,* e) *Aspergillus ustus*
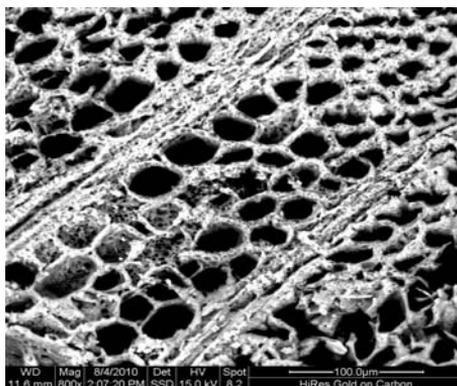
Fig. 3. SEM micrograph of control sample prior to inoculation,
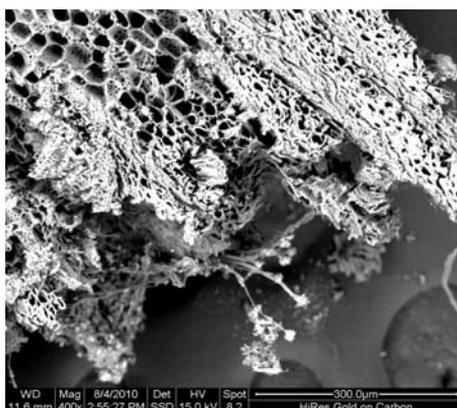showing no evidence of any type of the five inoculated fungi.

Fig. 4. SEM micrograph of the wood sample inoculated with *Aspergillus ustus* after 8 weeks.
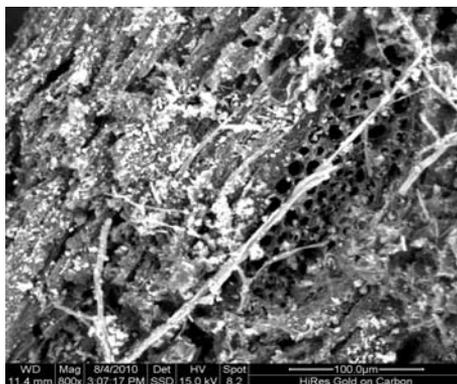The fungi started to grow on the outer surface of the sycamore wood.

Fig. 5. SEM micrograph of the wood sample inoculated with *Trichoderma longibrachiatum*
after 8 weeks. The fungi started to cover the outer surface and penetrate the cell walls.

Cellulose Crystallinty

Five bands observed in the XRD pattern of wood were assigned, according to Popescu *et al.* [19]. The results in Table 1 indicate a slight shift to a higher theta degree of the band positions in most fungal infected wood, in comparison to the non-inoculated wood sample. This

indicated that fungal degradation changes d-spacing of the crystalline area of cellulose. The different percentages of decrease in CI were calculated by both the XRD and the FTIR methods in all samples that were infected with fungi and were compared with the CI of the non-inoculated wood sample. Since the S2 layer of the secondary wall contains the largest amount of cellulose, fungal infection decreases the proportion of the crystalline material in this layer. It is obvious, though, that the CI varies significantly depending on the measurement method.

Table 1. The band positions of crystalline and amorphous cellulose forms
in the samples and the Crystallinity Index (CI) of the samples

Wood Samples	CI^a %	CI^b %	cr (101) 15°	cr (101$^-$) 16.8-17°	am 18.8°	cr (102) 20.6°	cr (002) 22.6°
Non-inoculated wood	85	81.25	15.1	16.9	18.8	20.5	22.5
Infected with Botryodiplodia theobromae	71	71.23	15.2	17.4	19.0	20.7	22.5
Infected with Trichoderma longibrachiatum	75	79.59	15.1	17.1	18.9	20.5	22.8
Infected with Aspergillus candidus	81	80.00	15.4	17.6	18.8	21.2	22.7
Infected with Aspergillus ustus	68	74.35	15.3	17.0	19.3	21.0	22.7
Infected with Aspergillus terreus	71	81.03	15.4	17.4	18.8	20.5	22.8

a: Crystallinity index calculated from XRD spectra; b: Crystallinity index calculated from FT-IR spectra
CR(101) = 2θ reflection around 15° assigned to (101) crystallographic plane
CR(101$^-$) = 2θ reflection around (16.8-17°) assigned to (101$^-$) crystallographic plane
AM = 2θ reflection around 18.8° assigned to amorphous phases
CR(102) = 2θ reflection around 20.6° assigned to (102) crystallographic plane
CR(002) = 2θ reflection around 22.6° assigned to (002) or (200) crystallographic plane of cellulose I.

FTIR Analysis

The results of our FTIR analysis of wood samples infected separately by *Botryodiplodia theobromae*, *Trichoderma longibrachiatum* and *Aspergillus terreus*, Figure 6 and Table 2, affected mainly wood carbohydrates and caused little lignin modification.

The results showed that the intensities of the C-O bands at 1030cm^{-1} and the O-H band at 3338-3288cm^{-1} increased as a result of hydrolysis of the hemiacetal bond between two glucopyranose rings C_1-O-C_4 and the formation of more C-O-H groups. The O-H bands were broadened and shifted to a lower wave number, due to an increasing hydrogen bonding effect. The terminal rings may open giving rise to the break of the C_1-O-C_5 bond in the same ring and to the formation of C_5-OH and C_1HO groups. The formation of these carbonyl groups were confirmed by an increase of conjugated C=O intensity at 1640cm^{-1} and a broadening of this band. A new small carbonyl band appeared at 1726cm^{-1} in the case of *Aspergillus terreus,* due to more oxidation effect, as the peroxidase enzyme was reported for *Aspergillus terreus* [20]. This enzyme can use H_2O_2 , which is produced by the action of the glucose oxidase and glyoxyl oxidase resulting during sugar utilization from polysaccharides of wood, as an oxidant [21, 22] and this is a prerequisite for the degradation of lignin [23].

Wood samples infected separately by *Aspergillus candidus* and *Aspergillus ustus* were affected in all the main wood components: lignin, cellulose and hemicellulose (Fig. 7; Table 3). Infection by these fungi caused a sharp decrease in the intensities of the C-O stretching band at 1026cm^{-1} and the disappearance of the bands at 1051, 1105 and 1157cm^{-1} , accompanied by a decrease in the O-H intensity at 3278cm^{-1}. These findings are attributed to the oxidation of functional groups of glucopyranose rings in cellulose and phenolic compounds in lignin to conjugated carbonyl groups, which appeared at 1640cm^{-1}, leading to a broadening of the band.

The results showed also that lignin bands at 1509cm^{-1} (aromatic >C=C< stretching) and 1263cm^{-1} (C-O-R stretching) decreased as a result of degradation.
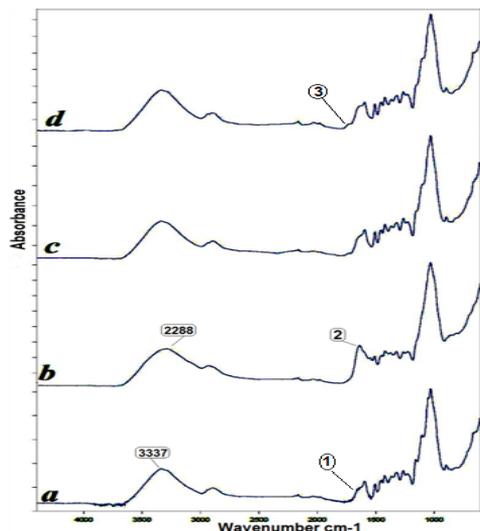

Fig. 6. FTIR spectrum of the reference sample (a), wood inoculated with *Botryodiplodia theobromae* (b), wood inoculated with *Trichoderma longibrachiatum* (c), and wood inoculated with *Aspergillus terreus* (d), indicating the following bands:
1 and 2 - conjugated C=O stretching bands at 1640 cm^{-1},
3 - new carbonyl band at 1726 cm^{-1}

Table 2. The effect of *Botryodiplodia theobromae, Trichoderma longibrachiatum and Aspergillus terreus* on the chemical bonds of the wood samples.

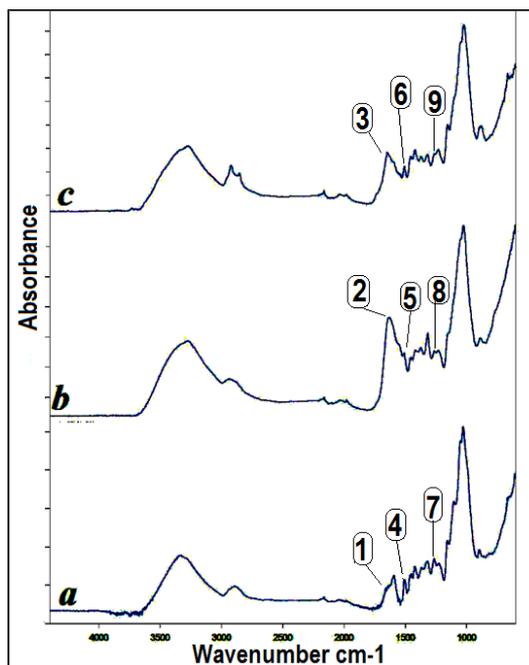| Wave cm^{-1}	Functional groups	Intensity				Comment
		Standard	*Botryodiplodia theobromae*	*Trichoderma longibrachiatum*	*Aspergillus terreus*	
3337	Bonded O-H stretching	0.036	0.055 appeared at lower wavenumber (3288 cm^{-1})	0.045	0.055	Increase of the intensities of these bands indicates formation more hydroxyl groups resulting from hydrolysis effect.
1157±5	C–O–C stretching in cellulose and hemicelluloses	0.043	---	---	---	The absence of these bands indicates advanced breaking of cellulose chains and shows that depolymerization has occurred.
1051±5	C-O stretching in cellulose and hemicelluloses	0.095	---	---	---	
1030±5	C-O stretching in cellulose and hemicelluloses + C–H deformation in the guaiacyl unit of lignin	0.103	0.167	0.131	0.147	The increase of this band is attributable to an increase in the guaiacyl lignin relative to carbohydrate components [31]
898±5	C–H deformation in cellulose.	0.064	---	0.049	0.058	The decrease in intensity is attributed to cellulose decay.
1593±5	C=C stretching vibration in aromatic ring in lignin	0.025	---	0.036	0.041	
1510±5		0.025	0.045	0.034	0.039	
1461±5	C–H deformation in carbohydrates and lignin	0.025	0.047	0.036	0.041	
1422±5		0.030	0.052	0.041	0.047	Increase of the intensities of these bands indicates that as decay progresses, extensive carbohydrate loss occurs and lignin concentrations increase in the residual wood [31], [39]
1368±5	O-H bending + CH deformation of cellulose and hemicelluloses	0.029	0.050	0.040	0.044	
1320±5	C–H vibrations in cellulose and a C–O vibration in syringyl (a main component of lignin) derivatives.	0.032	0.051	0.043	0.049	These fungi caused decrease in the number of stretching bonds C–OH and C–O–C and increase the bending and wagging of C–H, O–H bonds. This indicated the effect of these fungi eventually coming into the crystalline zone in the course of hydrolysis [40]
1265±5	This band is assigned to guaiacyl ring structure in lignin and C-O stretching in xylan and hemicellulose.	0.034	0.051	0.047	0.053	
1226±5	C-O stretching of guaiacyl ring of lignin	0.031	0.051	0.044	0.050	

Fig. 7. FTIR spectrum of the reference sample (a), wood inoculated with *Aspergillus candidus* (b), wood inoculated with *Aspergillus ustus* (c), indicating the following bands; 1, 2 and 3 - conjugated C=O bands at 1640 cm^{-1}, 4, 5 and 6 - aromatic >C=C< stretching bands at 1509cm^{-1}, 7, 8 and 9 - C-O-R stretching bands at 1263cm^{-1}

Table 3. The effect of *Aspergillus candidus* and *Aspergillus ustus* on the chemical bonds of the wood samples

Wave cm^{-1}	Functional groups	Intensity			Comment
		Standard	*Aspergillus candidus*	*Aspergillus ustus*	
1593±5	C=C stretching vibration in aromatic ring in lignin	0.025	---	---	The absence or decrease of these bands which are characteristics of lignin indicates decomposition of the aromatic skeleton of lignin.
1510±5		0.025	0.022	0.023	
1461±5		0.025	0.023	0.022	The decrease or absence of absorptions at these wave numbers showed that advanced decay has occurred in cellulose as well as lignin which indicate that a depolymerization process has occurred [41]
1422±5	C–H deformation in carbohydrates and lignin	0.030	0.026	0.029	
1368±5	C-H deformation of cellulose and hemicelluloses	0.029	0.027	0.027	The decrease in intensity is attributed to samples degradation.
1320±5	C–H vibrations in cellulose and a C–O vibration in syringyl (a main component of lignin) derivatives.	0.032	0.031	0.027	The decrease in intensity is attributed to lignin decay
1265±5	This band is assigned to syringyl ring and C-O stretching in xylan and hemicellulose.	0.034	0.025	0.028	The decrease in intensity of this band suggests a decrease of lignin and adjacent hemicelluloses in the ultrastructure of the wood [41]
1226±5	C-O stretching of guaiacyl ring of lignin	0.031	0.026	0.030	The decrease in intensity is attributed to lignin degradation
1157±5	C–O–C stretching in cellulose and hemicelluloses	0.043	---	0.040	The decreased intensity indicates advanced breaking of cellulose chains and shows that depolymerization has occurred.
1105±5	aromatic ring stretching	0.064	---	---	The decrease or absence in intensity in this region is attributed to structural degradation of lignin.
1051±5	C-O stretching in cellulose and hemicelluloses	0.095	---	---	
1030±5	C-O stretching in cellulose and hemicelluloses + C–H deformation in the guaiacyl unit of lignin	0.103	0.067	0.083	The intensity decrease of these bands is attributed to cellulose and lignin decay.
898±5	C–H deformation in cellulose.	0.064	0.030	0.039	The decrease in intensity is attributed to cellulose decay.

Discussions

The wood infested with brown rot, which attacks primarily cell wall carbohydrates, can be greatly weakened even before decay is visible. White rot fungi, which attack both cellulose and lignin, cause a loss in strength gradually, until the wood becomes spongy to the touch. Soft rot fungi cause a gradual and shallow softening, from the surface inward that resembles brown rot [9]. This is the usual appearance of decayed archaeological wood that has been either buried for centuries in different conditions that allow microorganisms to gradually affect wood, or exposed to different environments. In our samples that was, with certainty, not the case, because the time for inoculation was not enough to reach such advanced stages of disintegration, but the enzymatic effect of the fungi was clearly recorded in the FTIR analysis.

The crystalline structure of cellulose has been studied since its discovery in the 19th century. There is still a lot of controversy concerning the use of XRD in assessing the decay of wood, but in archaeological wood, where sampling is very difficult, the use of this technique is still a quick option that may indicate changes due to decay. All five inoculated samples showed different shifts, even if very slight, in the theta degree of the band position, which is an indication that every fungi affects wood components in a different manner.

The CI of cellulose was used to interpret the changes in cellulose structure after physicochemical and biological treatments. The determination of CI by using FTIR spectroscopy differed from that measured by using XRD. This can be explained as FTIR method gives only relative values and the spectrum always contains contributions from both crystalline and amorphous regions [24].

FTIR spectra of different types of wood have been studied before and peaks in the fingerprint region were well defined. Fungal decay of wood has been studied before by using FTIR [25-31].

In the three wood samples that were inoculated with *Botryodiplodia theobromae*, *Trichoderma longibrachiatum* and *Aspergillus terreus* the hydrolysis of the glycosidic bonds of cellulose and hemicellulose may have occured due to the capability of the fungi to secrete hydrolytic enzymes, as proved by other researchers. These fungi removed structural carbohydrate components, with reduction or absence of carbohydrate bands, at 1738, 1158, 1051 and 898cm^{-1}, leaving elevated levels of lignin bands at 1593, 1510, 1461, 1422, 1320, 1265 and 1226cm^1. This resulted in an increase in lignin: carbohydrate peak area ratio as decay proceeded. These fungi also caused a decrease in the number of stretching bonds C–OH and C–O–C and increased the bending and wagging of C–H, O–H bonds. This indicated the effect of these fungi eventually coming into the crystalline zone in the course of hydrolysis.

Umezurike [32] and Florence *et al*. [33] found that the wood staining fungus *Botryodiplodia theobromae* produced a series of cellulases enzymes involved in cellulose degradation. Royer and Nakas [34] examined the production of extracellular xylanase and cellulase enzymes by *Trichoderma longibrachiatum*. Biely and Tenkanen [35] and de Vries and Visser [36] studied the production of Xylanases enzymes by *Trichoderma* spp. and *Aspergillus* spp. The hydrolytic enzymes involved include: endo-1,4-β-glucanases; exo-1,4-β-glucanase, and 1,4-β-glucosidases for cellulose hydrolysis; endo-1,4-β-xylanases and β-xylosidases for hemicelluloses hydrolysis [35].

Oxidation of wood may be caused by the capability of *Aspergillus candidus* and *Aspergillus ustus* to secrete strong oxidizers, such as hydrogen peroxide, lignin peroxidase and laccase and/or a recently discovered oxidative enzyme of importance in cellulose degradation, which seems to be a cellobiose oxidase. An oxidoreductase, cellobiose, quinone oxidoreductase, is of importance both in cellulose and in lignin degradation. These results agreed with those of other researchers: Keppler, *et al*. [37] who proved that many *Aspergillus* strains showed alcohol dehydrogenase and Baeyer–Villiger monooxygenase activities. Westermark, *et al.* [38] noted that degradation of lignin results from breaking down of aryl-ether inter-unit linkages, so the amount of methoxyl groups in lignin diminishes. Kirk and Farrell [21] and Hatakka [22] mentioned that fungal attack is an oxidative and non-specific process, which decreases the methoxyl, phenolic and aliphatic content of lignin breaks aromatic rings and forms new carbonyl groups.

Conclusions

In the present study we monitored the decay of sycamore wood due to the infestation with fungi isolated from archaeological masks, in order to understand the chemical changes that may occur in archaeological wooden objects in Egypt. FTIR analysis of wood samples, inoculated separately by *Botryodiplodia theobromae*, *Trichoderma longibrachiatum* and *Aspergillus terreus* , indicated that mainly wood carbohydrates were affected and that little lignin modification occurred. Wood inoculated separately by *Aspergillus candidus* and *Aspergillus ustus* was affected in all the main wood components: lignin, cellulose and hemicellulose. Infection by these fungi caused a sharp decrease in the intensities of both carbohydrates and lignin bands. FTIR results were confirmed by the decrease in CI calculated by using both XRD and FTIR methods.

Our research clearly showed the changes that occur in wood samples due to fungal infestation, and that uncontrolled environmental conditions need to be changed, or adjusted to minimize microbiological damage emphasizing the need to establish standard conditions for the display of wooden archaeological objects.

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