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SPECTROSCOPIC AND MOLECULAR INVESTIGATION OF CHEOPS WOODEN BOAT FOR MICROBIAL DEGRADATION APPLYING PROPER MICROBICIDES AND METHODS

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ABSTRACT

The present research paper aims to determine the effect of microbial deterioration on wood housed in Cheops Boat and ways to control it. Khufu, the second king of the Fourth Dynasty 2650 BC, established Cheops Boat that was found in 1954 on the southern side of the pyramid. Wood is usually very sensitive to biological attacks, therefore, fungi, bacteria, and insects can easily attack and metabolize it, leading to significant physical, chemical, and morphological changes. Microbial infections were identified using light microscopy and PCR after their cultivation. The biological activities of the isolated microorganisms were investigated and the causative microorganisms from Cheops Boat were isolated and characterized as *A. niger, A. flavus, A. sulphureus, P. janthinellum, Cladosporium herbarum, Botryotrichum piluliferum, and Bacillus megaterium*. The historic wood was analyzed from different perspectives: Stereo microscopes, SEM with EDX, FTIR, as well as wood structure and chemical composition. The results illustrated that the best concentrations of a specific microbicide for the bio-treatment of the infected wood materials is *pentachlorophenol* at (900 ppm) as it is sufficient to inhibit all isolated microorganisms. It is followed by *Nano Chitosan* at (900 ppm) concentration, by plant extract of *Ceratophyllum demersum* at (1000 ppm) concentration and *p-chloro-m-cresol* at (2000 ppm) concentration.

KEYWORDS: Wood; Microbial; Antimicrobial agents; Enzymes; Biocides treatments

1. INTRODUCTION

All historical wooden objects are at risk from insect and fungal attacks (Abdelmoniem et al., 2020; Hunt., 2012) and biological degradation (Walsh et al.,2014). The Growth of fungi on wooden artifacts is accompanied as a rule by various physic-chemical processes making wood rigid, brittle and deformed (El-Gamal et al.,2018, Salem et al., 2016). Fungi playa tremendous role in the deterioration of cultural heritage due to their enormous enzymatic activity (Sterflinger, 2010; Osman,2014; Nilsson, 2008). Most of the studies focus on the bio deterioration of valuable wood objects damaged by fungi and bacteria. Furthermore, fungi are very often found in the display and storage conditions of museums (Abdel-Kareem, 2021). In museums and storage rooms, climate control, regular cleaning and microbiological monitoring are essential in order to prevent fungal contamination (Sterflinger, 2010). The natural durability or resistance of wood against decay or mold fungi is primarily dependent on the chemical composition of the wood (Mansour, 2015; Salem, 2015). Wood decay by fungi (Goffredo, 2017) is typically classified into three types: soft rot, brown rot, and white rot. The wood decayed by brown rot fungi is typically brown and crumbly. It is degraded by both non-enzymatic and enzymatic systems. White rot fungi are typically associated with hardwood decay, and their wood decay can take different patterns (Goodell, 2008). White rotted wood normally has a bleached appearance as a selective decay or a pocket rot. White rot fungi possess both cellulolytic and lignin degrading enzymes. Therefore, they have the potential to degrade the entirety of the wood structure under the correct environmental conditions (Krstulović, 2018). Soft rot fungi typically attack higher moisture, and lower lignin content wood and can create unique cavities in the wood cell wall (Srivastava et al., 2013; Hamed et al., 2018). Most of the literature published on the Cheops boat in the past refer either on data obtained from the Chief Restorer Hag Ahmed Youssof or a report published in the year 1960 (El Hadidi, 2005), while the Cheops boat have received much attention in the last years by several authors (Černý, 1955; Kadry, 1986; West, 2002; Lipke & Moustafa, 1984; Morabito et al., 2020)

The present study aims to further investigate for first time the selection of the best microbicide to stop the growth of all microbial isolates on the wood housed in Cheops Boat. We approach this question through spectroscopy and molecular sequencing methods.

2. MATERIALS, EXPERIMENTS AND METHODS

2.1 Sampling

Nine swabs were taken from the Cheops Boat (Fig.1). There were some symptoms of brown spots and spongy wood on the boat wood. Swabs were taken by scratching the surface of the infected materials by sterilized cotton swabs (Geweely et al., 2014) and transferred right onto two prepared agar media (cellulose agar for fungi and nutrient agar for bacteria) (Atlas, 2005; Difco, 1984). Plates were incubated at 28-30°C for 1- 21 days, depending on the microorganism.

2.2 Fungal isolates and bacteria

The identification of fungal isolates was carried out at the Laboratory of Microbiology, Grand Egyptian Museum, Ministry of Antiquities, whereas the identification of bacterial isolates was done by the sequencing of rRNA gene at Solgent Company, South Korea.

Identification of fungal isolates

Colonies grown on cellulose agar plates medium were purified on the same medium, and every single colony was picked for identification (Gilman, 1974; Domsch et al., 1980) by determining the morphological characteristics using the light microscope (Carl-Zies Microscope with an analysis unit and a digital camera). They were compared with the standard criteria in references. The identification process was performed in the Laboratory of Microbiology, Grand Egyptian Museum, Ministry of Antiquities.

Identification of bacteria

Bacterial identification was performed based on the molecular approach. Bacteria were grown on nutrient agar at 28° C for 4 days. A small amount of bacterial culture was scraped by sterile spatula suspended in 100µl sterile distilled water in 2ml sterile vials and boiled at 100° C for 15 minutes. Bacterial DNA was extracted and isolated using the SolGent purification bead. Before sequencing, the ribosomal rRNA gene was amplified using the polymerase chain reaction (PCR) technique in which two universal bacterial primers 27F (forward) and 1492R (reverse) were incorporated in the reaction mixture. Primers used for gene amplification have the following composition:

- 27F (5'AGAGTTTGATCMTGGCTCAG)
- 1492R (5'TACGGYTACCTTGTTACGACTT).

The purified PCR products (amplicons) were reconfirmed using a size nucleotide marker (100 base pairs) by electrophoreses on 1% agarose gel. Then,

these bands were eluted and sequenced with the incorporation of dideoxynucleotides (dd NTPs) in the reaction mixture. Each sample was sequenced in the sense and antisense directions using the same primers (Lane,1991). Sequences were further analyzed using the Basic Local Alignment Search Tool (BLAST) from the National Center of Biotechnology Information (NCBI) website. Phylogenetic analysis of sequences was done with the help of Meg Align (DNA Star) software version 5.05. moreover, the identification of bacterial isolates was done by sequencing the rRNA gene at Solgent Company, South Korea (Sakr et al., 2013, Konkol et al., 2009).

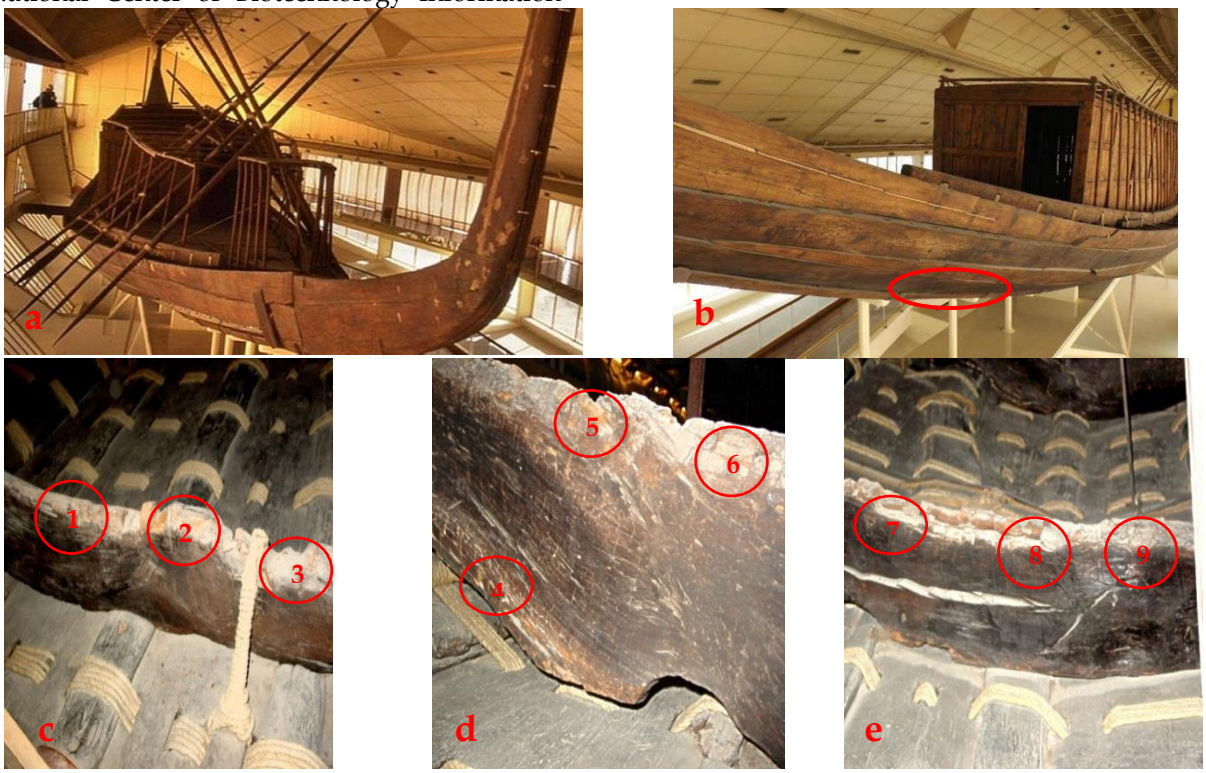

Figure 1. Microbial deterioration of Cheops Boat; a) Cheops Boat; b) the position of the samples from inside; c) basic bar tom; d) basic bar tom with high magnification showing spongy wood; f) another bar tom

2.3 Enzyme production and Assay

Enzyme production

Production was carried out in 250 ml conical flasks. Each contained 100 ml of the production medium (For the production of cellulose enzymes, the main source of carbon (sucrose) was replaced with 10g of cellulose. Flasks were sterilized at 121° C for 15min. After cooling, they were inoculated with 2ml of standard inoculums of each isolate. The inoculated flasks were incubated at 28 -30° C for adequate time. At the end of the incubation period, the liquid cultures were centrifuged at 3000 rpm for 15 minutes. The supernatant was taken for the determination of the cellulose enzyme activity, as described below.

Enzyme Assay

Cup plate Clearing Zone Technique (CCZ) was used for assaying the activities of cellulase enzymes. The procedure was carried out by pouring 20 ml aliquots of the detection medium (Ammar et al., 1998; Gamal et al., 2011) into a sterile Petri dish and was allowed to solidify. A sterile cork borer (15mm diameter) was used to make three cups in each plate, and 0.1 ml of the supernatant (cell-free enzyme) of each isolate was placed into the three cups. Plates were incubated at 30°C for 24h after which plates were flooded with Lugol's iodine solution to assay cellulase. Enzyme activities were compared based on the diameter (mm) of the clear zone (Osman et al., 2014). After that, the isolates showing the highest activity for the enzyme were used in the following experiments.

2.4 Minimal Inhibitory Concentration (MIC) of antimicrobial agents Versus the isolated microorganisms.

Four microcides were purchased from Aldrich Company (Germany) and used for testing their effect against the isolated microorganisms and determining their minimal inhibitory concentrations (Mahmoud et al., 2021). These microcides were plant extract of *Ceratophyllum demersum*, Pentachlorophenol, Nano Chitosan and p-chloro-m-cresol. The mode of action of

the antimicrobial agents on the microorganisms was to inhibit active transport by disrupting the cell membranes through solubilizing lipids and denaturing proteins. The cell is more permeable to protons, and ATP synthetase is destroyed (Geis, 2006).

The stock solution of each microcide was prepared by dissolving 1ml/ L ethyl alcohol at 95%. In the case of plant extract gradient, concentrations ranging from 800 to 2000 ppm were prepared by diluting the stock solution with alcohol 1g/L ethyl alcohol 95%. Gradient concentrations of other microcodes ranging from 800 to 2000 ppm were prepared by diluting the stock solution with alcohol. One ml of bacterial isolate suspension was spread onto a nutrient agar plate, and one ml of fungi spore suspension (described below) was spread on a cellulose agar plate. Plates were allowed to dry; then, a cork purer (diameter 15 mm) was used to make three pores in each plate. In one plate, 100µl of each concentration (from 800 to 2000 ppm) of the tested microcides were placed in each pore. Plates were incubated at 30^{o}C for 1-3 days compared with control plates (ethyl alcohol instead of microcides). The Minimal Inhibitory Concentration (MIC) was determined by measuring the inhibition zone (Brantner et al., 1993).

Infection of modern wood with isolated microbial strains

Modern pieces of pine wood specimen were used to determine the effect of isolated microorganisms on their deterioration. The pieces were inoculated with the spore suspension of each specific isolate by spraying 5 ml of a suspension of each microbial isolate, containing 0.5x106CFU. The infected modern pieces were incubated for 2 months at ambient temperature and 60-70% humidity. Physical and chemical properties of wood pieces were determined before and after inoculation, as described below.

Binocular stereomicroscope (in the Laboratory of Microbiology, Grand Egyptian Museum) was used at 8-36X magnification to examine wood specimens before and after infection.

2.5. Chemical properties

The chemical properties of modern wood before and after infection were determined by FTIR and ESEM.

A. Fourier Transform Infrared Spectroscopy (FTIR)

The FTIR of Shimadzu- Peristege 21 in the IR Lab at the Grand Egyptian Museum was used to monitor the changes in the main components of wood; cellulose, hemicellulose and lignin (Hamed & Abd El-Kader; 2019; Zidan et al ., 2016; Hamed et al., 2020) and to examine and determine functional groups of compounds according to wavelength and absorbance capacity (Kılıç and Kılıç, 2021; Abdelmoniem et al., 2020; Abdallah et al., 2020).

B. Environmental Scanning Electron Microscope (ESEM)

SEM use was of Model Quanta 250 FEG (Field Emission Gun) attached with EDX Unit (Energy Dispersive X-ray Analyses) with accelerating voltage 30 kV, magnification 14x up to 106. The analysis of specimen chemical elements were made at magnification power ranging 500 -10000X.

The alteration of surface morphology and the fungal deteriorated gilded painted cartonage samples were carried out by in the Central Laboratories Sector, the Egyptian Mineral Resources Authority, Ministry of Petroleum (Darwish et al., 2013; Hamed et al., 2020; Afifi et al., 2015; Helmi et al., 2021; Medhat et al., 2015).

3. RESULTS AND DISCUSSION

Nine swabs were taken from brown spots and spongy wood of Cheops Boat. Moreover, 15 colonies of fungi were isolated, and 5 colonies of bacteria were isolated. The detailed identification of the microbial isolates, the determination of the cellulase(s) enzymes production by the isolated microorganisms, as well as the determination of the MIC effect of microbial infection on the physical and chemical properties of modern wood, and the resulted infection of modern wood in relation to the physical, chemical and morphological properties is elaborated below.

3.1 Identification of the microbial isolates

The resulted microbial colonies were subjected to preliminary characterization depending on the type of organism. Fungal colonies, 15, were preliminarily characterized according to morphology (Ellis et al., 2007; Kidd et al., 2016). The following four genera were identified: *Aspergillus, Penicillium, Cladosporium, and Botrytrichum* (Table 1). From these results, it can be seen that the genus *Aspergillus spp.* was the dominant genus in the nine swabs, followed by *Penicillium spp*. and *Cladosporium spp*. Isolates of each genus were subjected to identification based on morphological characteristics. The results showed that the isolates of *Aspergillus spp.* were *A. niger, A. flavus,* and *A. sulphureus* (Fig. 3). The isolates of Penicillium were identified as *P. janthinellum*, and the isolates of other genera were identified as *Cladosporium herbarum* and *Botryotrichum piluliferum* (Table 1, Fig.3) (Sarah et al., 2006).

Table 1. Identification of fungal colonies up to species isolated from archaeological materials in Cheops Boat.

No. of swabs	*Cellulose agar medium*
1	*A. sulphureus, A. niger, and A. flavus.*
2	*P. janthinellum, Cladosporium herbarum*
3	*A.niger, A. flavus*
4	*P. janthinellum and Cladosporium herbarum*
5	*Botryotrichum piluliferum and A.niger*
6	*P. janthinellum*
7	*A. sulphureus*
8	*Botryotrichum piluliferum*
9	*Cladosporium herbarum*

Total bacterial counts are shown in (Table 2). Bacterial colonies were identified by sequencing the 16S rRNA sequencing analysis compared with closely related strains accessed from the Gen Bank (Table 3). The isolates were identified as Bacillus megaterium.

Phylogenetic tree based on 16S sequences of rDNA of the bacterial strains isolated in the present study (samples 3, 5, and 7) aligned closely with related sequences and accessed from the GenBank (Fig. 2).

Table 2. Identification of bacterial isolates according to the molecular approach

No. of swabs	*Nutrient agar medium*
1	*Bacillus megaterium*
2	*Bacillus megaterium*
3	---
4	*Bacillus megaterium*
5	---
6	*Bacillus megaterium*
7	---
8	*Bacillus megaterium*
9	---

Table 3. Identification of bacterial isolates obtained from the Cheops Boat

Isolate	**No. of nucleotides with primers**		**Closely related strains accessed from the Gen Bank**				**Identified Name**
	27-F	**1492-R**	**Strain No.**	**Source and Locality**	**Accession No.**	**Similarity (%)**	
Bacillus-3	1052	1077	RKJ 600	Soil, India	HM027880	99	*Bacillus megaterium*
			ML257	Honeydew, Italy	KC692200	99	
			SVC4	*Gossypiumhirsutum*-rhizosphere, India	JQ910928	99	
			KU13	Fly ash, Japan	JF895484	99	

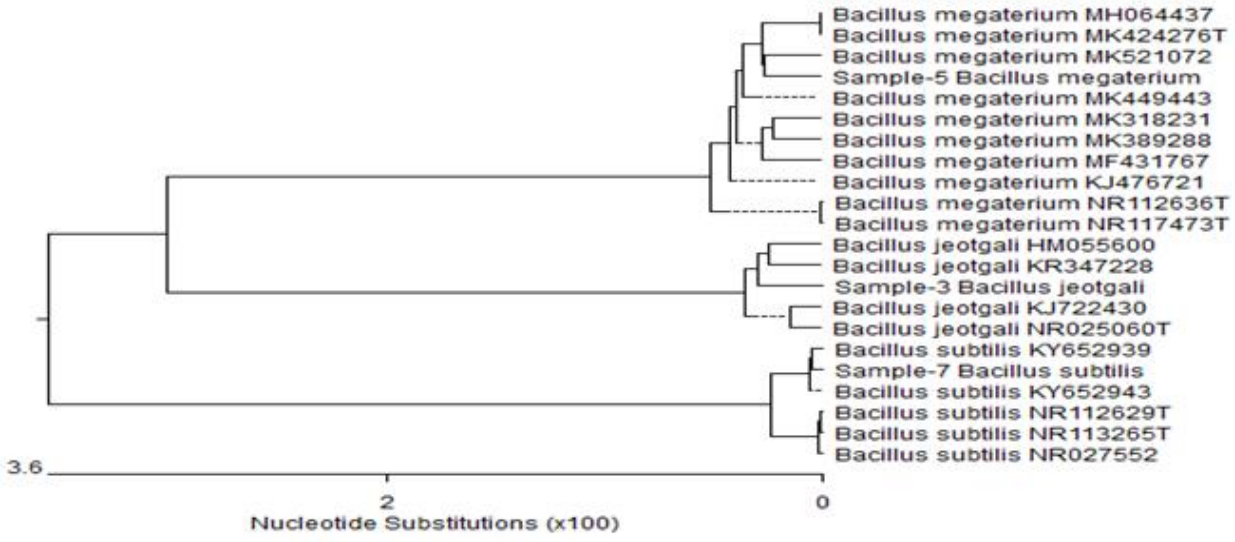

Figure 2. Phylogenetic tree based on 16S sequences of rDNA of the bacterial strains isolated in the present study

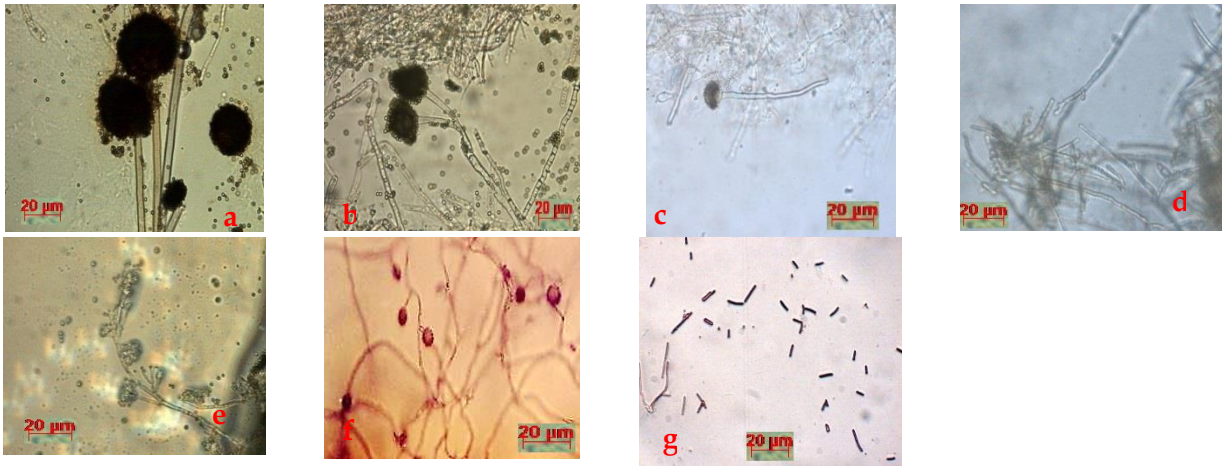

Figure 3. The fungi and bacteria isolated from wood housed in Cheops Boat a) A. niger; B) A. flavus; C) A. sulphureus; d) C. herbarum; e) P. janthinellum; f) B. piluliferum and g) Bacillus megaterium

3.2 Determination of the Cellulase(s) enzymes production by the isolated microorganisms based on the cup plate technique

Data recorded in (Table 4) and illustrated in Fig.4, indicate that the tested microorganisms varied in their abilities to produce the enzymes. Thus, they vary in the degree of decomposing cellulose. The tabulated data show that the highest cellulolytic activity was observed by *Aspergillus flavus*. It was found that genus *Penicillium* and *Aspergillus* exhibit a relevant cellulolytic activity (Pangallo et al., 2007) (Fig.4).

Table 4. Determination of cellulose(s) produced by the isolated microorganisms using the cup plate technique

Microbial isolates	Diameters of clearing zone (mm) Cellulase(s)
Aspergillus flavus	30
Aspergillus sulphureus	22
Aspergillus niger	25
Botryotrichum piluliferum	20
Cladosporium herbarum	23
Penicillium janthinellum	20
B. megaterium	22

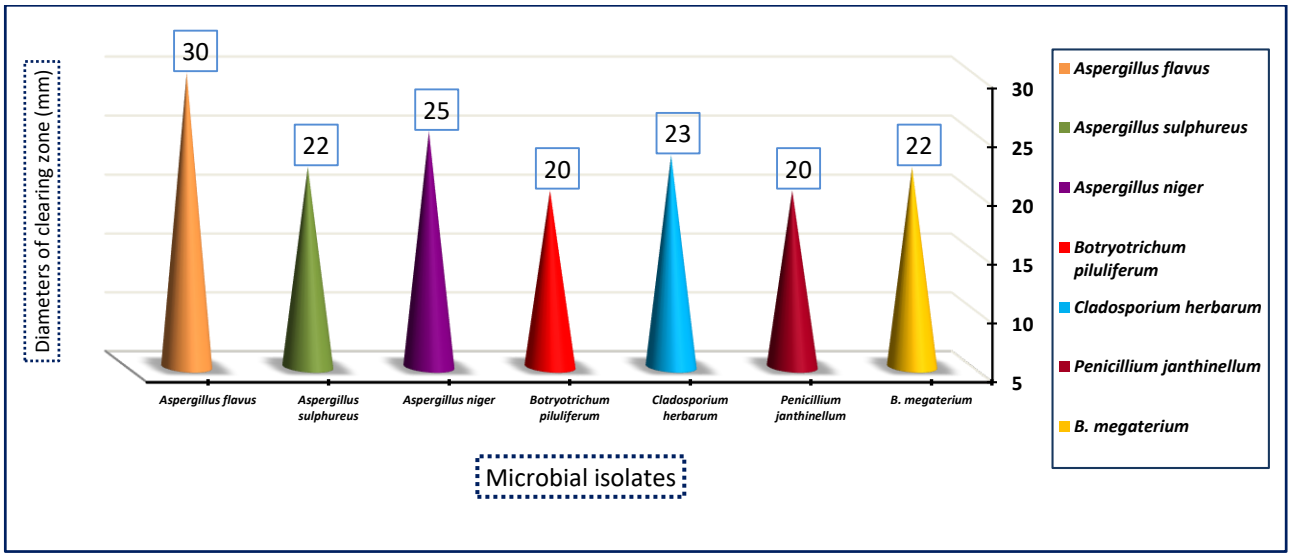

Figure 4. Determination of cellulose produced by the isolated microorganisms using the cup plate technique

3.3 Determination of the Minimal Inhibitory Concentration (MIC) of antimicrobial agents against the isolated microorganisms

To test the effect of antimicrobial agents on the isolated microorganisms, four microcides plant extract of *Ceratophyllum demersum*, *Pentachlorophenol, Nano Chitosan* and *p-chloro-m-crysol* at concentrations ranging from 800 to 2000 ppm were prepared and applied on the isolated microorganisms. Inhibition zones were measured to determine the MIC that inhibited each species.

Table 5. Determination of the inhibition zone (mm) of fungal and bacterial species grown on cellulose agar for fungi and nutrient for bacteria as affected four microcides.

Fungal isolate	Mean diameter of inhibition zone (mm) at different concentrations of															
	plant extract of Ceratophyllum demersum				Penta-chlorophenol				p-chloro-meta-cresol (ppm)				Nano Chitosan			
	800	900	1000	2000	800	900	1000	2000	800	900	1000	2000	800	900	1000	2000
Aspergillus flavus	0	0	22	30	0	23	31	43	0	0	0	20	0	20	22	26
Aspergillus sulphureus	0	0	21	29	24	32	43	50	0	0	0	17	0	23	24	27
Aspergillus niger	0	0	23	32	17	24	34	45	0	0	0	18	0	20	21	24
Botryotrichumpiluliferum	0	0	19	27	22	33	42	55	0	0	0	19	0	19	23	25
Cladosporium herbarum	0	0	25	33	24	30	44	57	0	0	0	19	0	19	20	22
Penicillium janthinellum	0	0	20	28	0	20	29	38	0	0	0	21	0	21	23	26
B. megaterium	0	0	23	34	30	42	50	60	0	0	0	18	0	23	24	28

The results of the plant extract of *Ceratophyllum demersum* (Table 5) show that 1000 ppm was the minimum concentration that inhibited all the isolated where the diameter of inhibition zone ranged (19-25 mm). Therefore, it was concluded that using plant extract of *Ceratophyllum demersum* at a concentration of 1000ppm that was the MIC that inhibited all the tested isolates. The results of Penta-cholorophenol showed that at a concentration of 900ppm were inhibited all isolates. Therefore, it can be concluded the MIC of *Penta-cholorophenol* that inhibited all the tested isolates was 900 ppm, and the mean diameter of the inhibition zone ranged 20-42 mm.

The results of p-chloro-m-cresol showed that up to 1000 ppm, no inhibition was detected. At 2000 ppm, all isolates were inhibited, where the diameter of the inhibition zone ranged (18-21 mm).

The results of Nano Chitosan showed that at a concentration of 900ppm were inhibited all isolates. It can be concluded the at a concentration of 900 ppm was the MIC of Nano Chitosan that inhibited all the tested isolates, the mean diameter of the inhibition zone ranged (19- 23mm).

From the above results, it can be concluded that pentachlorophenol at 900ppm was the best microcide to stop the growth of all microbial isolates using the lowest possible concentration. These results recommended spraying pentachlorophenol at 0.25% for protecting objects from fungal biodeterioration and preventing the possible fungal attack (Shash & Arya, 1999).

3.4 Effect of microbial infection on physical and chemical properties of modern wood

New pieces of pine wood were infected with isolated microorganisms (*A. niger, A. flavus, A. sulphureus, Botryotrichum piluliferum, P. frequentans and B. megaterium).* Infected pieces were incubated for 2 months at ambient temperature and under 60-70% humidity. The physical and chemical properties of the specimen were determined before and after the infection.

Physical properties of the infected modern wood

The results presented in (Table 6) illustrate changes in the physical properties of wood. They show that black and red spots occurred on the infected pieces of wood. Data also show that the pH of wood decreases as a result of acid production resulting from digesting organic matter by the microbial attack, thereby altering and weakening those materials.

Fungal biodeterioration changes the quality and/or value of material and makes it less functional in utilization terms. A pack of extracellular hydrolytic enzymes excreted by fungi is responsible for the formation of acidic products that cause chemical alteration of the material under attack (Naji et al., 2014).

Table 6. Changes in physical properties of wood as a function of microbial infection

Properties	wood Before microbial infection	wood after microbial infection
Appearance	Yellow	Black and Red spots
Shape	No change	No Change
Texture	Smooth	Roughness
Water absorption capacity (%)	58.3	60.5
pH	7	5

SEM and FTIR on the morphological and chemical properties of wood (Pinus pinea)

Morphological observations, obtained by Binocular dissecting stereomicroscopy, low magnification (8-36 X), enabled the examination of detailed morphological features, e.g., color, profile, and the density of microbial presence. Chemical properties were determined by SEM combined with EDAX unit and FTIR equipment. The changes in the properties of each material after infection were described below.

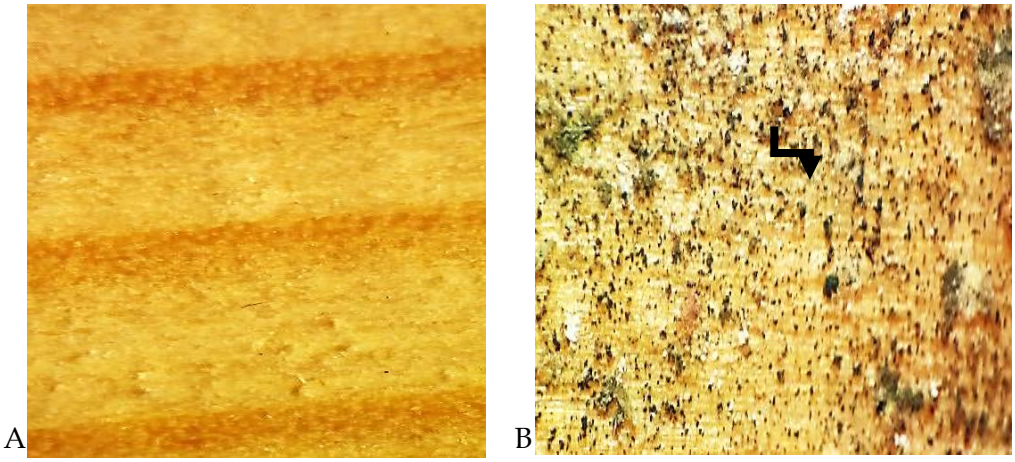

Figure 5. Optical photomicrographs showing the course morphology of the modern Pinus wood sample; A) wood sample before infection(26X); B) After infection(26X)

Morphological examination obtained by the stereomicroscope showed modern Pinus wood sample (Fig. 5a) before infection, and fungal infiltration and mat of filamentous fungal threads on Pinus wood sample (Fig. 5b) after infection for 60 days at room temperature. Also, it shows the presence of black and red spots, roughness, and weakness in wood cells.

No significant change was detected in carbon (Table 7), as well as a decrease in aluminium from 0.59 to 0.18%, and potassium from 0.82 to 0.18%. The following ions appeared after infection: Sodium (1.02%), Silicon (0.30%), Sulfur (0.28%), and Chloride (1.08%). Micrographs and major elements component of modern Pinus wood sample prior infection (Figs. 6a, b), and modern infected Pinus wooden sample (Fig. 6c) show fungal growth on the surface and elements component of modern Pinus wood sample after infection (Fig. 6c).

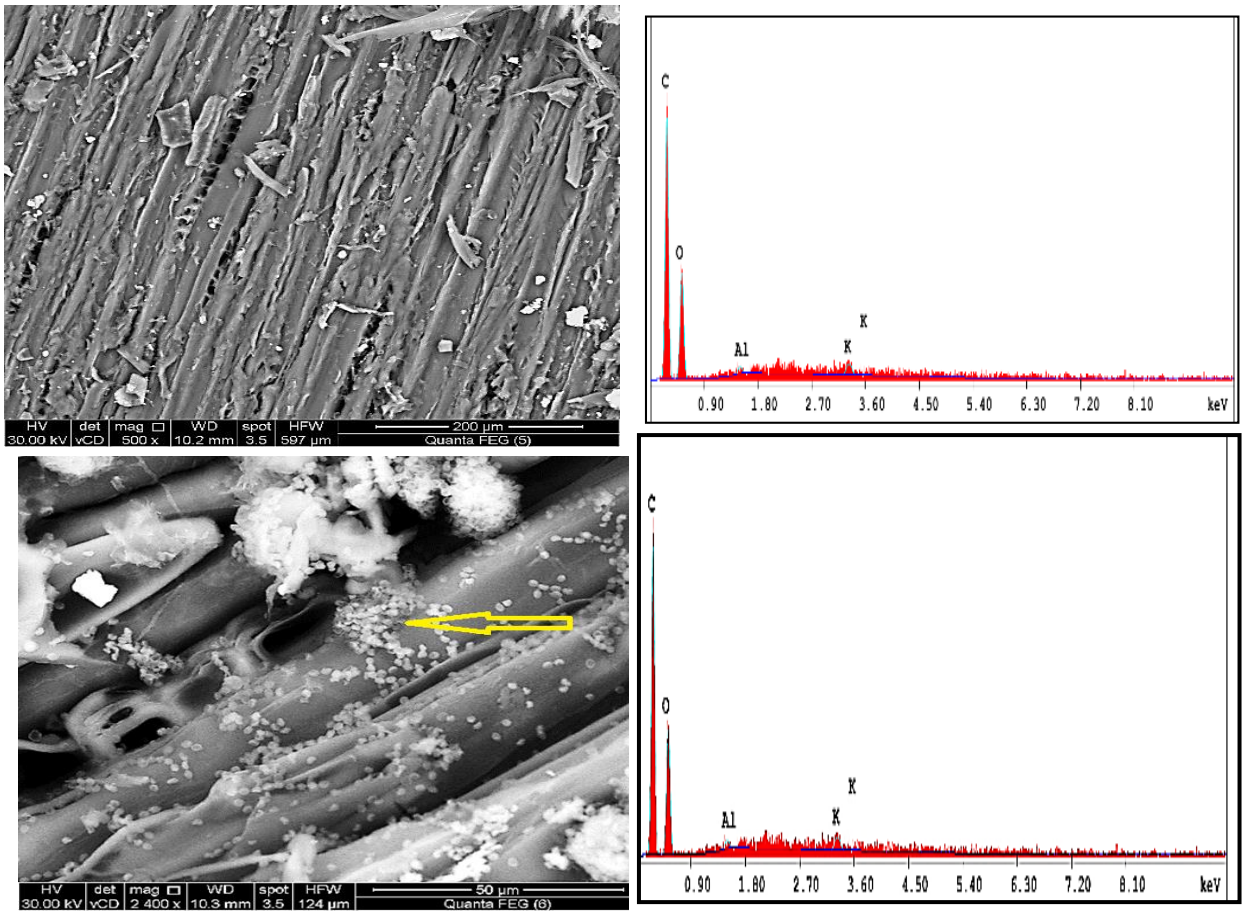

Figure 6. SEM-EDX spectrum of (Upper left): modern Pinus wood sample prior infection; Upper right: elements component of modern Pinus wood sample prior infection; Lower left: modern infected Pinus wood sample; Lower right: elements component of modern Pinus wood sample after infection.

Table 7. Chemical analysis of elements of modern Pinus wood sample before and after infection for 2 months

Elements	Wt. % before	Wt. % after
C	59.09	59.96
O	39.51	37.00
Na	-	1.02
Al	0.59	0.18
Si	-	0.30
S	-	0.28
Cl	-	1.08
K	0.82	0.18
Total	100.00	100.00

The chemical properties of wood were determined by FTIR (Fig. 7) indicate the occurrence of chemical changes in wood resulted from the degradation of large organic compounds into small compounds (which have other chemical functional groups) and the disappearance of other chemical groups. In considering the different functional groups in the uninfected sample (control) against the infected sample, the following bands were obtained:

1) Change in the shape of (C-H) asymmetric bending linkage movement at wave number area (1450-1422 cm^{-1}) assigned to amorphous and crystalline cellulose

2) Two strong absorption bands at around 1650 cm^{-1} (amide I, C=O stretching) and 1550 cm^{-1} (amide II, N–H bending) are also visible and typical protein bands assigned to the amide functions of the peptide groups. a significant change in typical bands of lignin (Rana et al., 2010)

3) Change in the shape of (C=C) bending linkage movement at a wave number area of (1636-1508 cm^{-1}).

4) The bound water bands at 3432 cm^{-1} and 1634 cm^{-1} showed changes due to various moisture levels in the samples (Hamed, et al., 2020; Funda, et al., 2020; Báder, et al., 2020).

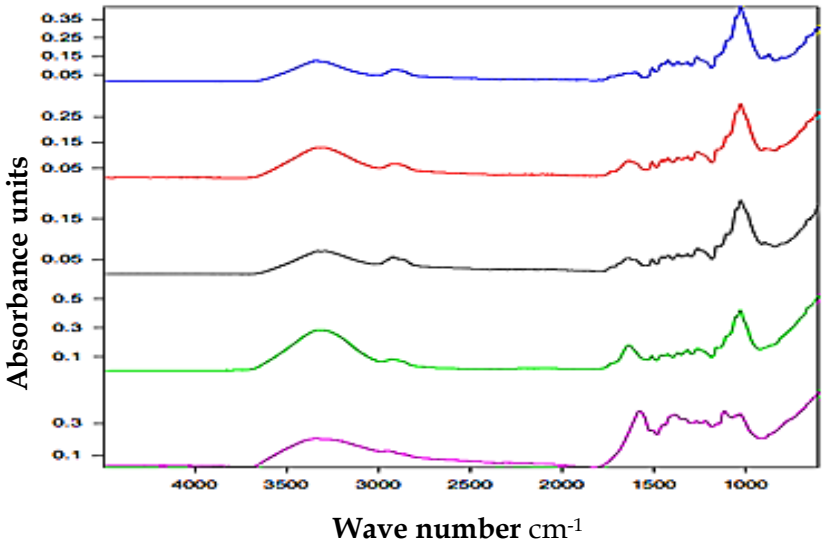

Figure.7. Pinus wood fragment analysis by FTIR before and after infection (2 months). Curves from up to down are: Control, Asp.niger, Cladosporium, Pen. Purpur and khopho wood.

4. CONCLUSION

In the present study, the effect of microbial deterioration on wood housed in Cheops Boat has been studied and the causative microorganisms that were isolated and characterized are: *A. niger, A. flavus, A. sulphureus, Botryotrichum piluliferum, P. frequentans*,and *B. megaterium*. The results show the side effect of causative microorganisms on the cellulose of the wood housed in Cheops Boat data whereby the highest cellulolytic activity was observed by *Aspergillus flavus*. Four microcides were used for testing their effect against the isolated microorganisms and determining their minimal inhibitory concentrations (MIC). These microcides were plant extract of *Ceratophyllum demersum,* Pentachlorophenol, Nano Chitosan and p-chloro-m-cresol. From the results, it can be concluded that pentachlorophenol at 900ppm was the best microcide to stop the growth of all microbial isolates using the lowest possible concentration followed by Nano Chitosan showed that at a concentration of 900ppm and plant extract of *Ceratophyllum demersum* at a concentration of 1000ppm.

The natural extracts were applied to modern Pinus wood sample and there was no negative effect on the properties of wood. The use of plant products will reduce the over-dependence on synthetic chemicals in controlling microbial pathogens and reduce the cost of the management and conservation of archaeological wood.

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