JOURNAL OF PURE AND APPLIED MICROBIOLOGY, October 2011. Vol. **5**(2), p. 501-509

Penicillium italicum, *Penicillium griseoroseum* and *Pseudomonas trivialis* are the Novel Species that Degrade Cedar Wood in Historical Moroccan Sites

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(Received: 12 February 2011; accepted: 20 March 2011)

Sbaiyine and Sehrij are two Medersas among the many historic buildings of the old medina of Fez. The wood in these foundations suffer from the advanced state of deterioration by microorganisms. This present work investigated the presence of fungi and bacteria in this cedar wood. Twenty fungal and eleven bacterial species were isolated and screened for their ability to produce cellulase and ligninase enzymes, responsible for wood degradation. Among the fungi, twelve species showed cellulase production, whereas only three species are ligninase positive. All the bacterial isolates produce cellulase. The fungi belong to species, *Aspergillus niger*, *Penicillium italicum*, *Penicillium griseoroseum* and *Lewia infectoria*, on the other hand, the bacteria belong to three species, *Bacillus sp*, *Bacillus subtilis*, *Pseudomonas trivialis*. In general, *A.niger* was found to be a high extracellular cellulase producer with 0.235U/ml when it was grown on CMC as the only sources of carbon. Additionally, *Penicillium italicum*, *Penicillium griseoroseum* and *Pseudomonas trivialis* are the novel species that degrade the wood. Artificial inoculation of cedar wood with the seven species of microorganisms was used to confirm the origin of the rot in Sehrij and Sbaiyine Medersas. The results showed respectively significant white and brown rot in vitro by the two species of *Penicillium*, *L.infecoria* and *A.niger*. However any rot was observed of the bacterial species.

Key words: Fungi, Bacteria, Cedar wood, Cellulase and ligninase.

The Medersas constitute a living testimony to the intellectual and spiritual past of the city of Fez in Morocco. Conceived by the sultans during the Merinid Dynasty, in 1321, where they reached their apogee in the Sehrij and Sbaiyine Medersa complexes. These splendid structures, built out partly of cedar wood, functioned as both an educational and cultural institute as well as a congregational mosque[1]. Over time, several factors have contributed to the deterioration of these structures, one of which is rot of cedar wood by microorganisms. Wood and other lignocellulosic materials are formed from three main polymeric constituents: cellulose, lignin, and hemicelluloses[2]. These organic polymers can serve as substrates for microorganisms and accelerate the deterioration process. Most research has identified the damage

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caused by fungi. These microorganisms are able to degrade all the components of wood using their enzymes[3,4] and the non-enzymatic systems[5]. On the other hand, the association of bacteria with wood has been recognized until the 1971s, although, little attention has been given to the causal bacteria compared with the extent of degradation caused by wood decay fungi[6,7,8,9]. Indeed, rot fungi are important and potent wood-destroying organisms that attack various components of the wood[10,11]. They can cause changes in appearance, structure, and/or chemical composition, which range from simple discoloration until the alterations that render the wood completely useless.

Based on wood decay patterns, it is possible to distinguish three main types of fungal infections: soft-rot fungi (SRF), brown-rot fungi (BRF) and white-rot fungi (WRF). The BRF preferentially depolymerizes and degrades the structural carbohydrates (cellulose and hemicelluloses) in the cell wall leaving behind the modified lignin. WRF can progressively utilize all the major cell wall components, including both carbohydrates and lignin[6]. In contrast to white and brown rot fungi, little is known about the degradation of lignocelluloses by the SRF fungi. Soft rot fungi typically attack in higher moisture and lower lignin content materials[12]. Bacteria are also able to colonize and affect wood under both aerobic and anaerobic conditions[13]. Shigo and Hillis[14] reported that during the initial invasion of wood, there is most likely a succession of bacteria before fungal wood inhabiters take their place in the overall succession of microorganisms. Moreover, some investigations have shown that bacteria, sometimes together with soft rot fungi, play a major role in the degradation of wood [15, 8]. Because soft rot fungi and bacteria are slow degraders as compared to white and brown rot fungi.

This study's goal was to identify the fungi and bacteria which degraded the wood in these monuments and examine the biodeterioration type that may have been caused by them. Knowledge of the fungal and bacterial species growing on the wood, and their deterioration potential, is crucial to the design of appropriate measures for their control.

MATERIAL AND METHODS

Sample collection

The wood samples were collected in February, 2008 from two historical sites located in the old Medina of Fez in Morocco: Sehrij and Sbaiyine Medersas. The reasons for choosing these medersas for this study were twofold: First, studies conducted to characterize the biodeterioration of historical monuments in Morocco have been very limited; and secondly, Sehrij and Sbaiyine complexes suffer from a serious degradation which required immediate intervention. We identified these complexes to be in an advanced stage of biodeterioration (Fig 1), especially the Sbaiyine Medersa which is in the course of restoration. Three samples were collected from each site and 1g of wood was dissolved in a 250 ml flask containing 40 ml of autoclaved sterilized Lysogeny broth (LB). The flasks were incubated for 6 h at 25°C. Samples' collection was stored at -20°C to reduce the microorganism activity.

Isolation and culture conditions of microorganisms

In order to isolate microorganisms, the supernatants from each sample were serially diluted in sterile distilled water up to 10^{-8} and cultivated using the following 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, ampicilline 60 µg/ml and kanamycine 30 µg/ml). The effect of growth at different temperatures was examined at 15, 20, 25, 30, 35, 37, 40 and 45°C. Cultivations were carried out for 7 days. The results were then analyzed to give the optimum growth temperature. The fungi isolated were compared with those isolated by Zyani *et al.,* [16] from an Old house of the Medina of Fez.

Screening of enzymatic activities on plates

The presence of extracellular cellulases was tested using agar dishes containing 1% (w/v) carboxymethylcellulose (CMC) (pH 7.0). The cultures were flooded with an aqueous solution of congo red (1% w/v) for 15 min to detect cellulase production[17]. The congo red solution was then poured off and the plates were destained with 1 M NaCl for 15 min. The formation of a clear zone of hydrolysis indicated cellulose degradation by the

microorganisms[18]. Similarly, as an initial screening method for detecting the ability of the fungal and bacterial strains to produce lignin-modifying enzymes, solid 2% (w/v) MEA plates containing 0.005 g/L rhemazol brilliant blue-R (RBB-R) were inoculated with 5 mm diameter agar plugs from 4 day old mycelia and bacterial colonies previously grown on 2% (w/v) MEA. Ligninase production was tested by formation of a halo in the medium resulting from RBB-R decolorization[19]. Duplicate cultures were incubated for 96 h at 25°C, and the diameter of the clear zone and the colony was measured in two dimensions at 90° to each other and the values averaged. An index of relative enzyme activity (RA) was calculated by dividing the total area of activity (the area of the clearing zone less the area of the colony) by the area of the colony. This index of relative enzyme activity (RA) provides a gross measurement of the enzymes production responsible for the degradation of a given substrate[20].

Enzyme and protein content assay

Activities of the enzymes in the culture supernatants were determined by the method of Ghose[21]. A reaction mixture for testing the CMCase activity contains 0.5 ml of 1% CMC (carboxymethylcellulase) in 0.1M sodium phosphate buffer (pH 7.0) and 0.5 ml of the crude supernatant. Activity of filter paper cellulase (FPase) 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 incubation at 50 °C. Reducing sugars were assayed by the dinitrosalicylic acid (DNS) method[21]. Units (IU) of CMCase and FPase were defined as the 1 imol of glucose liberated per minute of culture filtrate under assay conditions. The specific activity was computed and defined as IU per mg protein. This procedure was repeated thrice and the average value of three absorbances was determined. Using the average value of the absorbance, the concentration of the test sample was determined from the standard curve.

Soluble proteins were determined by Lowry's method by measuring optical density at 550nm[22]. The amount of protein was calculated using Bovine Serum Albumine (BSA) as standard curves.

Wood decay experiments in vitro

Cedar wood pieces measuring approximately 2.5 x 1.8 x 0.4 cm were used. Prior to biodegradation, pieces of wood were immersed in water for 12 h and then drained at room temperature and sterilized at 121°C for 60 min[23]. The pieces of wood were placed on dishes containing a sterilized water-agar medium (20% agar in distilled water), inoculated with fungus and bacterial isolated strains and incubated at 25°C for 10 weeks[16]. This test was done to determine the rate and extent of rot by the microbial agent.

Identification of microorganisms

Both fungal and bacterial cultures obtained from the isolates were transferred and maintained on MEA media and their genomic identification was undertaken using respectively the ITS, and the FD1and RS16 primers of rDNA of fungi[24] and bacteria[25]. The DNA was extracted by simple thermal shock and amplified by PCR (polymerase chain reaction). The PCR conditions for amplification in both fungi and bacteria were: 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 fragments. The obtained sequences from different fungal mycelia and bacteria were searched in the NCBI using the BLAST search function

RESULTS AND DISCUSSION

Isolation and identification of microorganisms

The advanced stage of deterioration observed in different historical buildings of the city of Fez. In fact, microbial degradation plays an important role in this degradation and causes different kind of damage depending on the organisms responsible for the attack. In our study, the microorganisms were isolated in the cedar wood, from the historical sites in the medina of Fez: Sehrij and Sbaiyine Medersa. The presence of microorganisms show that the cedar wood of Sehrij and Sbaiyine Medersas provide an environment for the development of a variety of fungi and bacteria, and represents an environment and metabolic substrates for these microorganisms.

Twenty fungal and eleven bacterial isolates were maintained, purified and identified. The first step of identification of the fungi is the

morphological examination. This identification was subsequently confirmed by the sequencing of 16S rRNA region of the fungi and bacteria. Twelve of twenty fungal isolates were found to belong to four different species: *Penicillium italicum*, *Penicillium griseoroseum*, *Lewia infectoria* and *Aspergillus niger*. Although, the bacterial species were: *Bacillus sp*, *Bacillus subtilis* and *Pseudomonas trivialis*. In general, several studies has been reported the attack of the cultural heritage by fungi[13,27,28] and demonstrated that the fungal decomposition of wood appears to predominate. However, archaeological investigations of the bacterial attack have only recently begun[8]. Our study showed the presence of the two species of *Penicillium* and *A. niger*, these species were reported on cedar wood in buildings in several parts of the world[29,30,16,31,32]. In the same case, *L. infectoria* was recently isolated from an old wood[33].

In our Knowledge, our results shows for the first time that *Pseudomonas trivialis*, *Penicillium italicum* and *Penicillium griseoroseum* are part of the microorganisms affecting wood. This result suggests that the bacterial and fungal diversity in the Moroccan cedar wood is not characterized thoroughly and the microbial diversity that alters the cedar wood in the old medina of Fez requires further characterization.

Culture conditions

Many investigations have demonstrated that the development of microbial degradation of historic woods was influenced by environmental conditions especially, temperature. This factor had important effect of fungal[34] and bacterial growth. In order to confirm that, the temperature in the Medina of fez was primarily evaluated; it was found vary between 16 and 30°C. Similarly, the optimum temperature of development of isolated strains was determined. Figure 2 shows the growth results for the seven species. It was found that growth occurred at 25 and 30ºC; less growth was noticed at 4ºC for five species. At 40 to 60°C, only three bacteria were grown: *B. subtilis*, *Bacillus sp* and *P. trivialis*, while no growth fungi was observed at these temperatures. Thus, this observation confirms the ability of fungi and bacteria to well grow range 25 and 30°C, which represent their optimal growth temperature (Fig 2). Therefore, these findings were in agreement with those reported by Zyani *et al.,*[16].

Screening of enzymatic activities and wood decay experiments

Subsequently, the characterization of enzymes produced by these microorganisms has been realized. For this, a screening of cellulolytic and ligninolytic fungi and bacteria was done. Among the twenty fungi isolated, sixteen were able to produce carboxymethylcellulase after 4-7 days of incubation, however all the bacterial strains were produced CMCase only after 2 days. According the literature, wood fungi may serve as good producers of extracellular enzymes capable of hydrolysis of polysaccharides[35,36].

Table 1. Origin of wood decay microorganisms in the present study and enzymatic activities in solid cultures for the isolates

	Historical site	Isolation medium	Identification	Total cellulase	Ligninase (I*CMC)	Rot (I*L)
	Sehrij M	LB	*Lewia infectoria*	1.64±0.04	0,15±0.04	Brown
Fungal isolates	Sbaiyine M	EM	*Penicillium italicum*	1.75±0.04	1,82±0.06	White
	-	EM	*Penicillium griseoroseum*	1.95±0.06	1,74±0.08	White
	-	EM	*Aspergillus niger*	2.25±0.14	0,26±0.12	Brown
	Sbaiyine M	EM	*Bacillus sp*	1.3±0.05	-	-
Bacterial isolates	-	EM	*Bacillus subtilis*	2.05±0.05	-	-
	-	LB	*Pseudomonas trivialis*	1.54±0.09	-	-
Control 1		LB	*Erwinia chrysanthemi*	1.6±0.34	-	-
Control 2		EM	Control 2	-	2,05±0.15	-

I*CMC: Index of Relative Enzyme activity determined on CMC medium
I*L : Index of Relative Enzyme activity determined on MEA medium containing RBB-R

Table1 shows the location where the decay wood microorganisms were isolated, the index of relative enzyme activity of both CMCase and ligninase (ICMC, IL), and type of rots that the four species of fungi cause in the cedar wood. On the basis of this screening, *Aspergillus niger* was qualified as the best producer of cellulose, with important value of clearing zone of hydrolysis of CMCase (ICMC = 2.25 ±0.14), however, this fungus shows the lowest of ligninolytic activity by the decolorization of Rhemazol (IL = 0.26 ±0.12). At the same time, the test of the rot showed the typical appearance of a brown-pitted mycelium on the surface of the wood during 60 days, this is the brown rot. Also, the results in Table 1 indicate that two *Penicillium* species (*P.italicum* and *P.griseoroseum*) are also good producers of the CMCase and ligninase activities. The Relative Index of Enzyme Activity is between 1.74 and 1.95 for both enzymes. These two species were able to

Fig. 1. Photographs of the historical monuments with signs of biodeterioration, the Sehrij (right) and Sbaiyine (left) Medersas

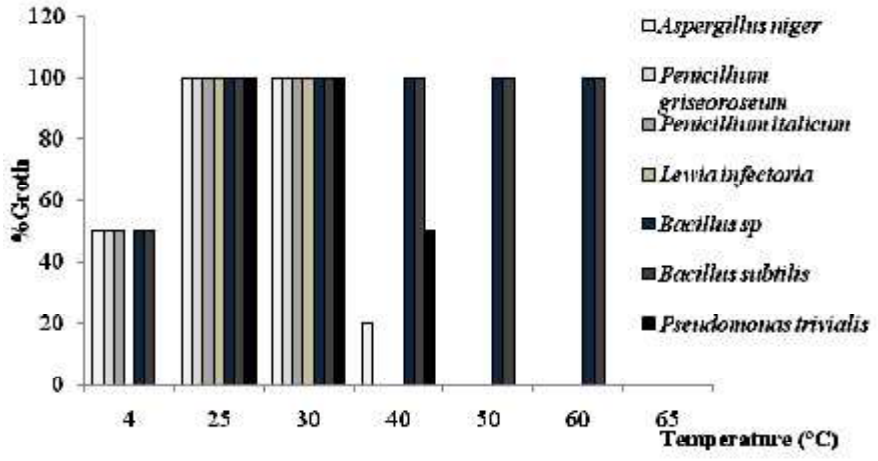

Fig. 2. Effect of temperature on the growth of the isolated strains on solid culture

attack wood in rot experiment and cause white rot. Finally, this study has allowed to isolate *Lewia infectoria*, a strain of fungus that can cause the wood decay. This species was identified recently and for the first time by Pfeffer *et al.,*[33]. *L. infectoria* showed the lowest endoglucanase activity (ICMC =1.64±0.04), and gave a very low relative ligninolytic activity index (IL =0.15±0.04). This fungus can degrade the wood in vitro by brown rot.

In the other hand, it was observed that the bacteria isolates were produced extracellular cellulolytic enzymes. The bacterial results obtained of ICMC ranging from 2.05 to 1.3. Activity ligninolytic and the bacterial rot were not observed among all of the bacterial species studied. In recent years various studies have indicated that bacterial wood attack cause considerable strength loss of waterlogged pile foundations in short periods of time.

Enzyme assay

The CMCase activity for all the microorganisms up to the sixth days is illustrated in Figure 3 (a and b). Early, the endoglucanase activity was detected at low levels after 24h in the culture fluid of the eight isolates. Though, the maximum production of the CMCase activity was observed at the 4th day of culture, which presented also the phase of active multiplication of all strains. Figure 3(a) shows also that the maximum CMCase activity was obtained with *A.niger* by 0.235 UI. Similar endoglucanase activity was observed in both *Penicillium*, *griseoroseum* and *italicum*. However, CMCase activity of both strains was significantly lower than that of *A.niger* and the reference strain during the whole time of incubation. The same findings as shown by several authors who demonstrated that genus fungal of *Aspergillus* has been extensively studied due to their ability to secrete cellulose-degrading enzymes and use wood as source of carbon and energy[37]. *Aspergillus niger* exhibited strong activity CMCase. Cellulase activity has been also reported in temperate isolates of many species of *Penicillium*[38]. *Penicillium griseoroseum* was found synthesizes cellulase enzyme at low quantity and appeared a moderate CMCase activity in agreement with observations made by Minussi *et al.,*[39]. Indeed, we noted that *Penicillium italicum* was able to produce CMCase activity. This fungus was also previously reported as a good producer of carboxymethylcellulase[40]. *Lewia infectoria* showed the lowest activity of the four fungi. Interestingly, our results also report that *Lewia infectoria* is a cellulolytic fungus showing a moderate CMCase activity. Indeed, it was found

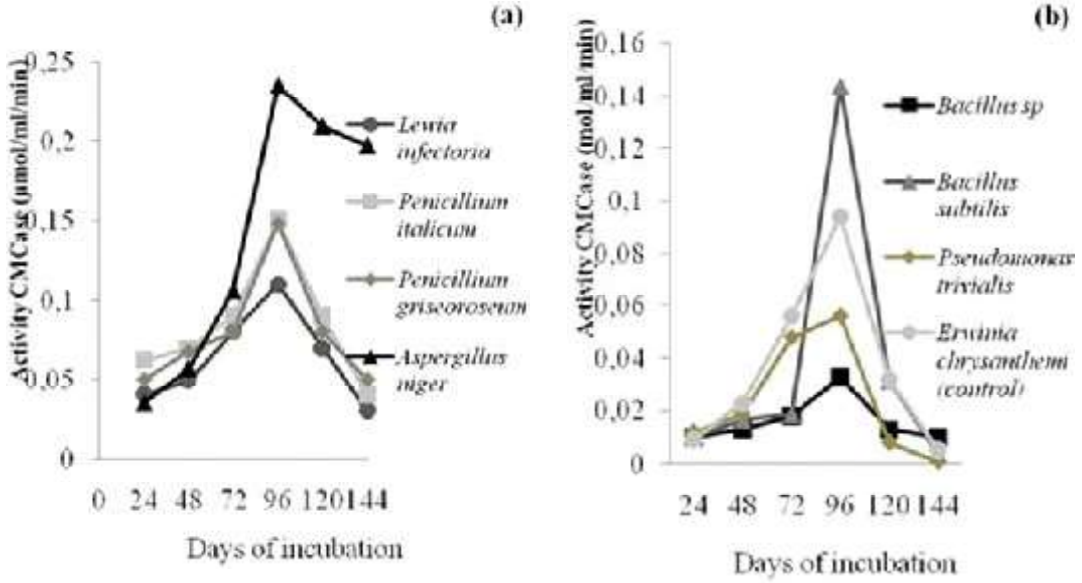

Fig. 3. Endoglucanase activity of the fungal (a) and bacterial (b) strains grown on CMC as the only source of carbon (pH = 7, T = 45°C)

that during the formation of brown rot, polysaccharides are degraded first and the lignin matrix remains nearly undigested but not unchanged[41].

In the case of the bacterial production of CMCase (Fig 3(b)), *B. subtilis* showed the highest rates of CMCase (0.144 UI) among the bacteria, however, *Bacillus sp* and *P. trivialis* showed a lower rate of CMCase. Furthermore, the activities of cellulolytic enzymes of the fungal strains were higher than those of the bacterial strains. Greaves[42] have demonstrated that the bacterial cellulases alter the permeability of wood, opening up the crystalline arrangement of the cellulose as a target for advanced diffusion of cellulolytic enzymes. Various *Bacillus* strains was a well-known cellulase-producing organism[43], and have an indirect influence on the decay process; it can work synergistically with other wood decay fungi to predispose wood to fungal attack[13]. Furthermore, several species of the genera of *Pseudomonas* are known by their degradation of wood by their cellulolytic system, as like as *Pseudomonas sp*[44]. However, any report has been described the degradation of cellulose or of wood by *Pseudomonas trivialis*.

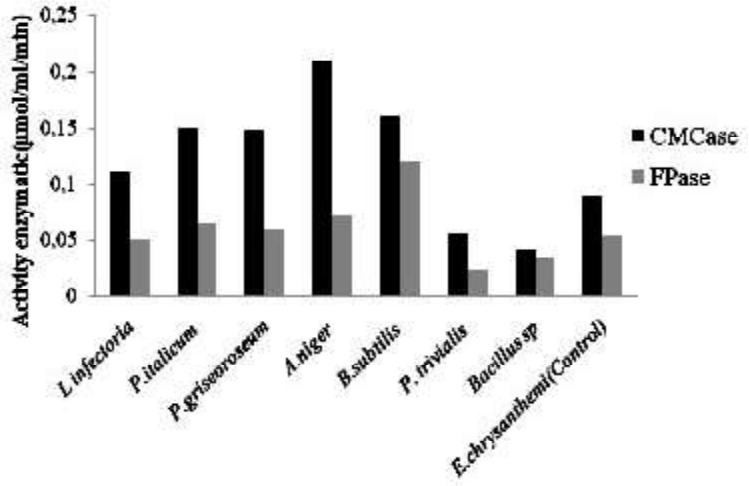

Fig. 4. CMCase and FPase activities of the microbial isolates respectively grown on CMC and Filter paper as the only sources of carbon (pH = 7, T = 45°C)

Finally, CMCase and FPase activities of isolated microorganisms were compared. Figure 4 shows the results when the CMC and Filter paper are the only sources of carbon. CMCase appeared in large quantities in the culture medium 4 days after inoculation; however the maximal of FPase appeared after 7 days of inoculation. As it is seen in figure, significantly production of FPase activity was observed with *A.niger* and *Bacillus subtilis*. In addition, it was observed that the FPase activity was higher than double of endoglucanase for all the isolates species studied. This is may be due the extent of polymerization and crystallinity in the paper substrata.

All isolates fungi and bacteria produce cellulase using filter paper us a source of carbon, but the amount produced was variable. This is because of the influence that carbon sources have on the growth of cellulolytic organisms[45]. Fungi and bacteria can alter their metabolism to make use of different substrates and can colonize materials with historical or economic value for humans[46], causing various levels of biodeterioration.

CONCLUSION

In agreement with our results, Zyani *et al.,*[16] previously reported the biodegradation of wood in vitro by the fungi that showed weak positive cellulolytic and ligninolytic reaction in solid and liquid media by enzymes that have a key role in this process, facilating microbial attack on wood and their components of these historic

buildings dating from the era of the Merinide.

Overall, knowledge of biology of the microorganisms of Merinid buildings and their role in wood decay are important to understanding the phenomenon of deterioration in these monuments. Studies of *P. trivialis, P.italicum and P.griseoroseum,* and their ability to degrade wood are also pertinent as part of long-term conservation plans to preserve the historical monuments of Fez and minimize conditions under which white and brown rot occurs.

ACKNOWLEDGMENTS

We express our sincere gratitude to Biomatec-US Manuscript Review Committee for helpful comments and valuable discussion.

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