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Psychrophilic Wood-inhabiting bacteria at the old Medina of Fez

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Abstract

In order to achieve a better understanding of the mechanism leading to the decay of wooden monuments in the old medina of Fez, we undertook a project aiming at identifying bacteria isolated from infected wood. We found that the principal wood-inhabiting bacteria are *Bacillus pumilis, Bacillus subtilis, Bacillus atrophaeus* and *Bacillus amyloliquefaciens*, all of witch present an important cellulotic activity with an Index of Relative Enzyme Activity greater than 1 (ICMC = 2.32, ICMC = 2.02, ICMC = 1.75 and ICMC = 3.22, respectively). Moreover, all four species showed the ability to grow at temperatures ranging from 4°C to 25°C. The highest FPUase activity was observed at 15°C for *Bacillus amyloliquefaciens* (0.34 UI) and at 25°C for *Bacillus pumilis, Bacillus atrophaeus* and *Bacillus Bacillus atrophaeus* (0.2 UI, 0.33 UI, 0.28UI respectively).

Key words: Cultural heritage, Wood-inhabiting bacteria, CMCase, FPUase

1. Introduction

The old Medina of Fez is a UNESCO World Heritage Site. It's believed to be the world's largest contiguous car-free urban area. The Medina of Fez is a walled city with madrasas(schools), fondouks (Hotels), mosques and palaces dating from Marinid rule in the 13th–14th centuries. Having been deserted since 1912 for the modern city, Medina's structural heritage and roadways did not present a problem until recently. Today it has become of an extreme urgency.

Wooden foundations are widely presents in the medina of Fez, as part of the historic buildings. The introduction of wood and other organic materials in the constructions of the medina provided nutrient sources for microorganisms that were indigenous and adapted to the harsh conditions. The wood used in the constructions is mainly the cedar. We have shown previously, (Zyani et al., 2009) that these wooden structures are commonly affected by fungal attack; they are principally *Penicillium commune, Penicillium granulatum,* Penicillium *chrysogenum, Penicillium expansum, Cladosporium cladosporioides* and *Thielavia hyalocarpa.* Normally wood is degraded under aerobic conditions where oxygen is available by fungi. Under special circumstances, become the main wood degraders, especially if sediment is filled with water and there is very little oxygen available.

The association of bacteria with wood has been recognized since the 1950s and 1960s. Little attention has been paid to the effects of bacteria on deterioration of wood compared with the extent of degradation caused by fungi. Lack of mobility restricts the bacterial mode of attack,

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prolonging the process of wood cell wall deterioration (Rossell et al., 1973). Notably, bacteria are the most numerous and ubiquitous of organisms. They are capable of colonizing wood under both aerobic and anaerobic conditions. As long as the wooden constructions remain under waterlogged conditions, bacteria remain the main wood degraders (Holt and Jones, 1983). They may be roughly classified into four groups for convenience and ease of comparison: 1- Bacteria which affect the permeability to liquids of wood but have no significant effect on strength properties 2- Bacteria which attack the wood structure 3- Bacteria which only function as integral members of the total microflora and are associated in the ultimate breakdown of the wood 4-The "passive" colonizers which have no effect upon the wood at all but have a marked influence on the remainder of the population by their antagonistic activities (Greaves et al., 1971).

Until the 1970s bacterial wood decay was considered to proceed very slowly, threatening only archaeological wood fragments such as shipwrecks, tools, and other wooden artefacts. In the 1980s, however, it was observed that in The Netherlands severe bacterial wood decay can cause considerable loss of strength in wooden foundations (Kretschmar et al., 2008).

Based on the decay patterns produced, three distinct wood-degrading bacterial types have been described in the literature, although the causal bacteria have not yet been identified (Blanchette et al., 1990; Singh and Butcher, 1990, 1991; Kim and Singh, 2000; Nilsson and Daniel, 1983; Daniel and Nilsson, 1986, 1997).). In an attempt to fill this gap, we designed the present study to isolate bacteria that affect wooden constructions in the old medina of Fez, identify isolated bacteria strains by molecular techniques, and evaluate their decay potential.

2. Materiels and methods

2.1 Prospecting and sampling.

Bacteria were isolated from three sites of a 450-years-old house located in the former Derb llamté in the Medina of Fez, Morocco. Samples were taken from visibly noticeable wooden, signs (grayed and softened) (Table 1).

2.2 Bacteria isolation

Ten grams of wood presenting decay signs of each wood sample were dissolved in 50 ml of sterile distilled water in 250 ml flack and shaken for 2 h. The supernatant was then recovered after settling the heavy sediment. Serial dilutions were plated on the solid media: LB agar (1% peptone, 1% NaCl, 0.5% malt extract, 1.8% agars). The plates were incubated at 4°C and 25°C.

2.3 Plate screening

Extracellular cellulases were tested using agar plate containing 1% (w/v) carboxymethylcellulose (CMC) (pH 7.0). The isolates were grown on Caboxymethylcellulose-agar (Fluka, Biochemika) medium containing (w/v): 1% carboxymethyl cellulose (CMC), 0.65% NaNO3, 0.65% K2HPO4, 0.03% yeast extract, 0.65% KCl, 0.3% MgSO4, 0.065% glucose, 1.7% agar. Plates were incubated at 25°C for two days. The pure culture on agar plates was flooded with aqueous solution of congo red (1% w/v) for 15 min to detect cellulase production. The congo red solution was then poured off and the plates were destained with 1 M NaClfor 15 min. The formation of a clear zone of

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hydrolysis indicated cellulose degradation by microorganisms (Ariffin et al., 2008).The Index of Relative Enzyme Activity (ICMC) was recorded as clear zone ratios = clear zone diameter / colony diameter (Bradner et al., 1999).

2.4 Enzyme assay

Total cellulase activity was determined by measuring the amount of reducing sugars released from filter paper. Endoglucanase (β-1,4-endoglucanase, EC 3.2.1.4) activity was assayed by measuring the amount of reducing sugar from CMC. Enzymatic activity was assayed according to the methods recommended by the International Union of Pure and Applied Chemistry (IUPAC) Commission on Biotechnology (Ghose, 1987). Endoglucanase (CMCase) activity was determined by incubating 0.5 ml of culture supernatant with 0.5 ml of 1% CMC in 0.05 M sodium citrate buffer (pH 4.8) at 50 °C for 30 min. Filter paper degrading activity (FPCase) was determined by incubating 1.0 ml of the supernatant with 1.0 ml 0.05 M of sodium citrate buffer (pH 4.8) containing Whatman filter paper strip (1.0 x 6.0 = 50 mg). After incubation at 50°C for 60 min, the reaction was terminated by adding 3 ml of 3,5-dinitrosalicylic acid (DNS) reagent to 1 ml of the reaction mixture. In these tests, reducing sugars were estimated by colorimeter with DNS according to the protocol described by Miller (1959) and Onsori *et al*. (2005), using glucose as standard. The enzymatic activities of total (FPUase and CMCase) are in International Units (U). One unit of enzymatic activity is defined as the amount of enzyme that releases 1 µmol of reducing sugars (measured as glucose) per ml per min.

2.5 Identification of bacterial strains based on rDNA sequences

To identify the bacterial strains, we used a molecular approach based on the amplification and sequencing of the 16S rRNA gene. This methodology is currently the most used for bacterial phylogeny (Woese et al., 1990). For PCR amplification, the following universal primers were used to amplify the 16S rDNA: D1 foroward (5'- AGAGTTTGATCCTGGCTCAG-3') and RS16 reverse (5'-TACGGCTACCTTGTTACGACTT- 3'). The amplification reaction was performed in a final volume of 50 µl containing 50 µmol of each primer, 200 µM each dNTP, 0.5 units Taq DNA polymerase and 3 µl of DNA sample in 1x Taq polymerase buffer. The mixture was first denatured at 94°C for 5 min. Then, 35 cycles of PCR were performed by denaturation at 94°C for 30 s, primers annealing at 55°C for 45 s, and primer extension at 72°C for 90 s. At the end of the last cycle, the mixture was incubated at 72°C for 10 min.

3 Results

Twenty bacteria were isolated from the 3 sites of an old house located in the Medina of Fez. The samples were taken from a wooden door, an outdoor window, and a decorative indoor board (Table 1). Of those twenty bacteria, twelve isolates were able to grow in a medium with cellulose only as carbon source. All of the twenty bacteria were screened for cellulotic activity by using the carboxymethyl cellulose (CMC) Congo red plate technique. Fifteen isolated bacteria demonstrated clearing of CMC with an Index of Relative Enzyme Activity (Bradner et al ., 1999) of 1 or greater (Table 2) . The lowest CMCase activity was seen in *Bacillus atrophaeus* and *Bacillus subtilis* (I_{CMC} = 1.75 and 2.02, respectively). *Bacillus*

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amyloliquefaciens exhibited the strongest CMCase activity (I_{CMC} = 3.22), slightly higher than the index value (I_{CMC} = 3.02) of *Penicillium chrysogenum* reported in our previous study (Zyani et al.,2009).
Of the twenty bacteria isolated from decaying wood, four isolates (*Bacillus atrophaeus*, *Bacillus subtilis, Bacillus amyloliquefacien* and *Bacillus pumilis*) were selected for further morphologic and molecular characterization (Table 3).The four bacteria were grown at different temperatures to determine their optimal growth conditions (Figure 2) and they all four species showed the ability to grow at 4°C, therefore qualifying as psychrophilic. *Bacillus subtilis* had higher growth rate, a larger bacterial biomass accumulation per hour in the log phase of growth, at 15°C. *Bacillus pumilis, Bacillus atrophaeus* and *Bacillus amyloliquefaciens* had higher growth rate at 25°C as compared 15°C and 4°C. All of the four species had a low rate of growth at 37°C. A previous study that addressed the influence of temperature on the growth of psychrophilic strains of *Bacillus* (Shetata et al., 1971) has shown that several strains of *Bacillus* had the growth characteristics similar to those reported for psychrophilic species *of Pseudomouas.* While the exact species of these bacteria remain unknown, we postulate that the environmental conditions, especially ambient temperature, may be crucial for the bacterial wood decay.

Then, all four strains screened as potential cellulose producers were investigated in the shake flask culture using CMC and the filter paper as a carbon source in the culture medium. After 72 hours of culture, enzymatic activity was measured by the production of sugar reducing end group, which is an indication of cleavage of cellulose molecules. Two standard substrates were used for the determination of cellulose activity in terms of overall (FPUase) and endoglucanase (CMCase) contents (Ghose, 1987). Filter paper was used as a standard substrate to measure the total cellulose activity (Wu et al., 2006). Different microorganisms vary in their optimal incubation temperature for production of hydrolytic enzymes (Habb et al., 1990).Therefore, the effect of temperature on the activity of crude CMCase and FPUase was determined at various temperatures ranging from 4°C to 25°C at pH 7.0 (Fig. 2 & 3). As shown in Fig. 2, three out of the four bacteria tested, three produced more endoglucanase activity (CMCase) at 25°C as compared to 15°C , however the remaning one (*Bacillus amyloliquefaciens*) produced more endoglucanase activity at 15°C but not at 25°C. *Bacillus pumilis* and *Bacillus subtilis* sowed low production of endoglucanase activity at 4°C. However, in the first study (Zyani et al.,2009) the fungal strains showed a good activity between 4 and 25 °C with a maximum activity at 25 °C.
The optimal FPUase activity was observed at 15°C for *Bacillus amyloliquefaciens* and at 25°C for *Bacillus pumilis, Bacillus atrophaeus* and *Bacillus Bacillus, respectively*. The higher value of FPUase activity (0.34 UI) was obtained by *Bacillus amyloliquefaciens* at 15°C (Figure 3). This activity was higher than the 0.02 UI reported earlier for *Cladosporium cladosporioides* was 0.02 UI (Zyani et al., 2009). So, the isolated bacteria are more producer of FPuase than fungi.

DISCUSSION

Finally, the isolation and identification of bacteria species participant in the degradation of the wood of the old Medina of Fez is a characterisation of favouring environmental conditions, so taking all this knowledge together can help to the development of preservation strategies and approaches.

Table 1 : Location of sample sites and type of sign of decay

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Location of Sample	Type of sign
Site 1 	Damaged wooden door (grayed)
Site 2 	Damaged windows (softned)
Site 3	damaged decorative wood (grayed)

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Table 2 : Identification of Bacteria, Index of relative enzyme activity determined on CMC medium and site of isolation

Identified Bacteria	(ICMC)*	Site of isolation
Bacillus pumilis	2.32	3
Bacillus subtilis	2.02	2
Bacillus atrophaeus	1.75	1
Bacillus amyloliquefaciens	3.22	3

* Relative index of enzymatic activity.

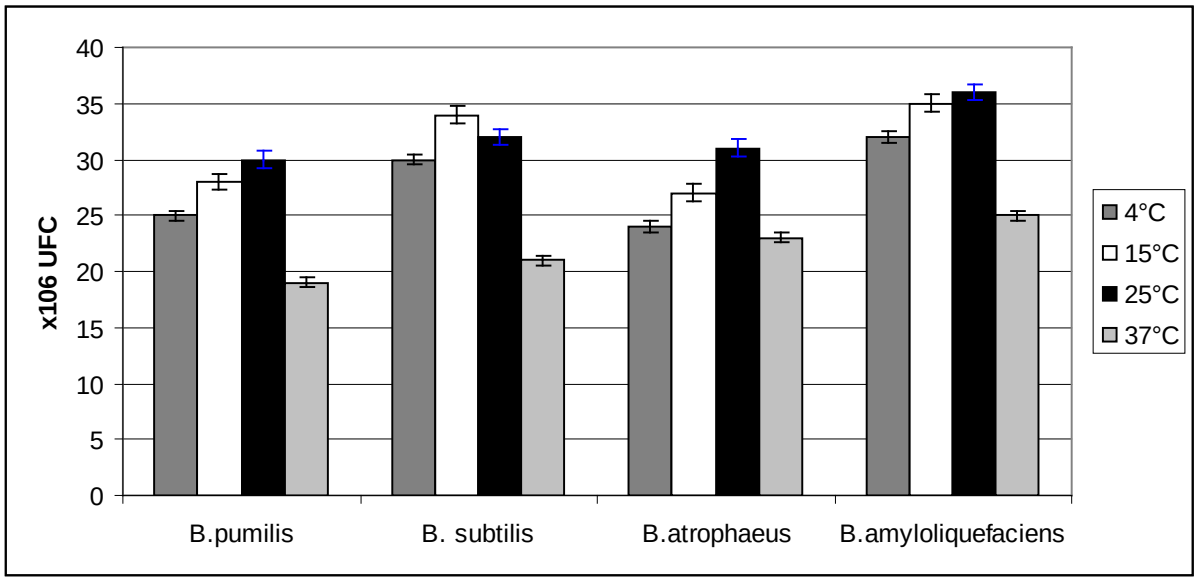

Fig 1 : Graph of growth rate of the four selected bacteria at 4°C, 15°C 25°C and 37°C.

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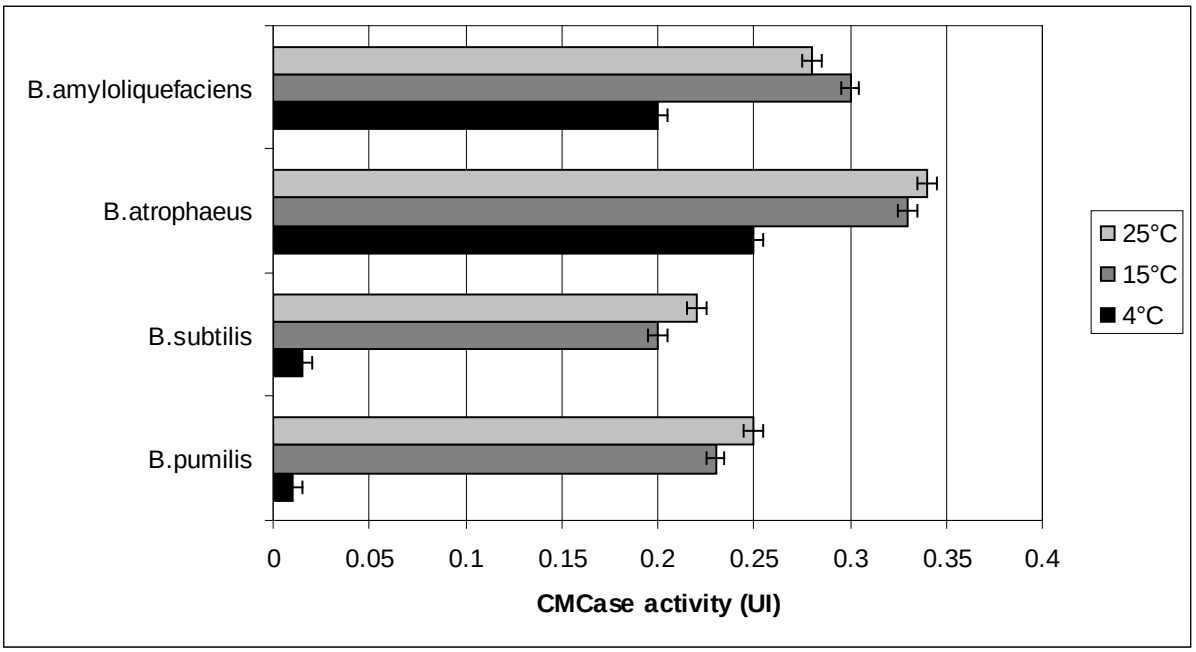

Fig.2 **:**Endoglucanase (CMCase) activity of bacteria after growth for 72 h in media with carboxymethyl cellulose as a sole carbon source at 4°C, 15°C and 25°C.

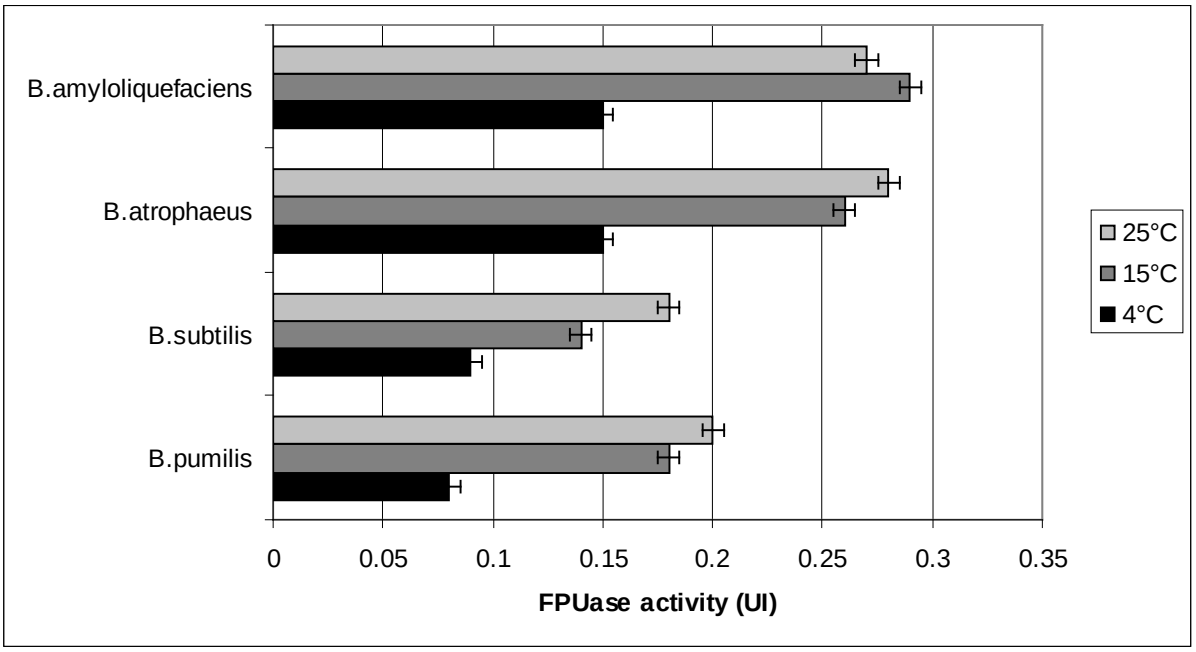

Fig.3 **:** Total filter paper cellulase (FPUase) activity of bacteria after growth for 72 h in media with filter paper as a sole carbon source at 4°C, 15°C and 25°C.

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