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Cellulolytic potential of fungi in wood degradation from an old house at the Medina of Fez

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Abstract - During the history of civilizations, advanced wood decay results from exposure to various agents for long periods of time. Bio-deterioration, under the influence of living organisms like fungi, can cause massive damage to historical monuments. In this work, we found that fungi participating in wood degradation share a single strategy for degrading wood polymers by secreting enzymes that break down the main constituents of wood such as cellulose, hemicellulose and lignin. While *Penicillium commune*, *Penicillium granulatum* and *Penicillium chrysogenum* showed the highest cellulase productivity and are therefore the most destructive for timber, other fungal species participate also in this biodegradation including *Penicillium crustosum, Penicillium expansum Cladosporium cladosporioides* and a cellulotic specie *Thielavia hyalocarpa* that we describe here for the first time.

Key words: wood, bio-deterioration, cellulose, cultural heritage, fungi.

INTRODUCTION

Fez is the oldest of Morocco's imperial cities. UNESCO has designated the entirety of the Fez Medina a World Monument. In this medina, the wood used in the constructions is mainly the cedar. Wood deterioration in temperate and tropical forest ecosystems and in wood products has been widely studied, and many studies on microbial decay and the mechanisms of wood degradation have been published (Eriksson *et al*., 1990; Zabel and Morrell, 1992; Eaton and Hale, 1993). Decay caused by many common white and brown rot fungi has been well characterized, but other types of decay, such as soft rot by fungi or bacterial degradation of wood, are not well understood (Blanchette *et al*., 2002).

Some microorganisms produce a complete set of enzymes capable of efficient degradation of native cellulose. Efficient cellulose systems are produced by white-rot and soft-rot fungi such as *Trichoderma, Fusarium, Humicola, Penicillium* and *Schizophyllum* (Wood and Garcia-Campayo, 1990; Nevalainen and Penttilä, 1995; Clarke *et al*., 1997; Mackenzie *et al*., 1997; Schülein, 1997). These cellulose degrading fungal systems usually contain various endo and exo-glucanases and at least one β-glucosidase. The number of enzymes produced depends on the fungus and on culture conditions.

Among the enzymes responsible for hydrolysis, the linear polymer of β-linked glucosyl units making cellulose, endo-1,4-β-glucanase (EC 3.2.1.4) is a cellulase catalysing the random hydrolysis of the β-(1-4)-glucosidic bonds (Duncan *et al*., 2006).

This investigation was done to (i) evaluate wood decay present at the medina of the Fez city, (ii) identify fungi isolated from the decayed wood by using internal transcribed spacer (ITS) sequences of ribosomal DNA (rDNA), and (iii) evaluate their decay potential in a laboratory. Little is known about the deterioration of wood in the medina of Fez city, and our results provide new information on the decay caused by fungi that are present in wood from the medina of Fez city, and elucidate the type and extent of degradation that has occurred over the past decades. In addition, these results will provide information that is crucial to conservators for the preservation of these important historic sites.

MATERIALS AND METHODS

Prospecting and sampling. Fungi were isolated from three sites of an old house built 450 years ago located in the former Derbllamté in the Medina of the Fez, Morocco (Fig. 1). This construction is made mainly by cedar wood, which includes an outdoor wall built with wooden planks showing damage from rain, wooden floors and untreated wooden walls indoors, wooden support poles, wooden painted outdoor windows, and decorative indoor boards. Samples were taken from visibly noticeable decay signs (grayed and softened) (Table 1).

Fungi isolation. Samples from each site were dissolved in 36 ml of sterile distilled water and shaken for 2 h. The supernatant

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TABLE 1 - Location of samples and the type of sign

Sample	Location (type of sign)
1	Outdoor damaged wood (grayed)
2	Indoor decorative wood (softned)
3	Indoor damaged wood (grayed)

was then recovered after settling the heavy sediment. Serial dilutions were plated on different solid media: EM agar (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).

Plate screening. CMC Congo red plate technique was used for screening of cellulase production. The isolates were grown on Caboxymethylcellulose-agar (Fluka, Biochemika) medium containing (w/v): 1% carboxymethyl cellulose (CMC), 0.65% $NaNO_3$, 0.65% K_2HPO_4, 0.03% yeast extract, 0.65% KCl, 0.3% $MgSO_4$, 0.065% glucose, 1.7% agar. Plates were incubated at 25 °C for two days then stained with 1% Congo Red dye for 0.5-1 h followed by distaining with 1 M NaCl for 20 min. The Index of Relative Enzyme Activity (I_{CMC}) was recorded as clear zone ratios = clear zone diameter / colony diameter (Bradner *et al*., 1999).

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 the 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 activity of total (FPCase) 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.

Protein determination. To determine the enzymes specific activities and therefore compare the enzyme productivities, total protein content in the culture supernatants was measured by the method of Lowry *et al*. (1951) using bovine serum albumin as standard. The cultures were incubated for 72 h. Cells were harvested by centrifugation at 6000 *x g* at 4 °C for 15 min. The cell free culture supernatant was used as source of crude extracellular enzyme.

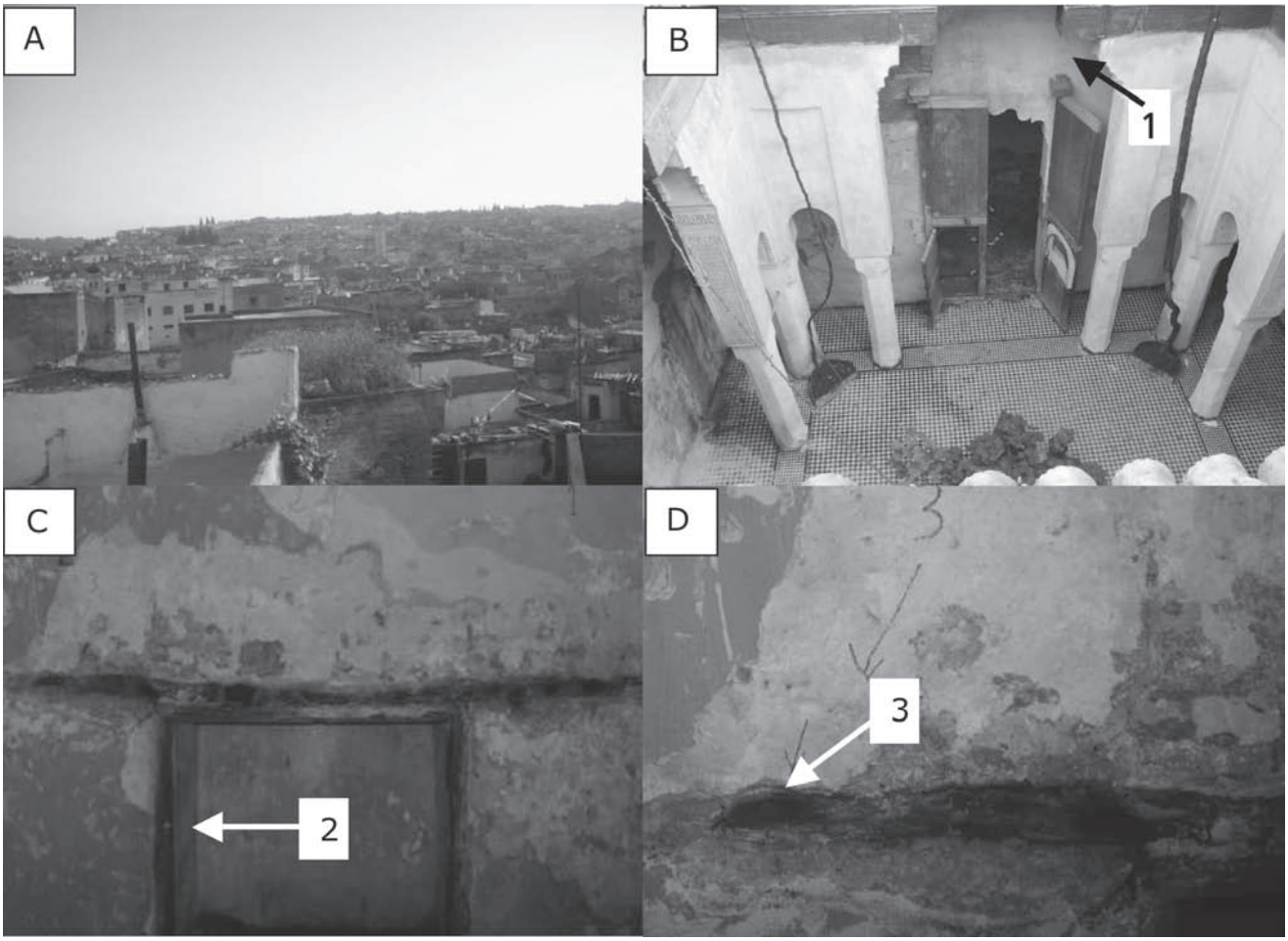

FIG. 1 - House in the old medina of Fez. A: Fez medina. B: A part of the house, where the study was done, shows in the middle the big room from which samples were taken. C and D: The spots from where degraded wood was taken are shown by arrows in photos B, C and D.

TABLE 2 - Identification of fungi, isolation media, Index of relative enzyme activity determined on CMC medium and type of rot

Identified fungi	Isolation medium	(I_{CMC})*	Type of rot
Thielavia hyalocarpa	LB	1.72	Rot white
Penicillium crustosum	EM	1.02	Rot white
Penicillium granulatum	EM	2.00	Rot white
Penicillium commune	YPG	1.87	Rot white
Penicillium chrysogenum	EM	3.05	ND
Penicillium expansum	EM	1.05	ND
Cladosporium cladosporioides	EM	1.62	Rot brown

* Relative index of enzymatic activity.
ND: Not determined.

Effect of temperature on the microbial growth. The effect of temperature on the microbial growth and activity CMCase in 1 M sodium acetate buffer (pH 4.8) was determined by incubating the plates at different temperatures ranging from 4 to 60 °C.

Test of the rot. The identification of the type of decay caused by various fungi isolated from our wood samples was made as follow: A piece of sterilized wood is placed in the centre of a plate containing a sterilised water-agar medium (20% agar in distilled water) and inoculated with a fragment of mycelium. Then the plate is incubated at 25 °C for 10 weeks.

Identification of fungi and bacterial strains based on rDNA sequences. Molecular characterizations, particularly DNA sequence analyses of the two internal transcribed spacer (ITS) regions of ribosomal DNA, ITS1 and ITS2, were used for species identification. Fungal material was scraped from pure cultures and DNA extracted using thermal shock. The rDNA ITS regions 1, 5.8S, and ITS region 2 were amplified using primers ITS1 and ITS4 (Gardes and Bruns, 1993). Polymerase chain reaction amplification was performed with the following protocol: 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. DNA sequencing was performed using ABI 3130; Applied Biosystems according to the manufacturer instructions. The GenBank BLASTN tools were used for sequence analysis.

RESULTS AND DISCUSSION

Thirty-six fungal isolates were obtained from the 3 sites of an old house located in the Medina of Fez. All of them were screened for cellulolytic activity using the CMC Congo red plate technique. I_{CMC} determined on CMC medium represented the preliminary endoglucanase activity characteristics of the fungal isolates belonging to different species. Of the 36 fungal isolates obtained from decaying wood, twenty isolates demonstrated clearing of CMC. An index value equal to or greater than one was noticed in fungi from seven species including *Thielavia hyalocarpa* (Table 2). Fungal strains belonging to the two species *Penicillium chrysogenum* and *Penicillium granulatum* showed the highest CMCase activity (I_{CMC} = 3.05 and 2.00, respectively). The lowest CMCase activity was seen in fungal strains belonging to *Penicillium crustosum* and *Penicillium expansum* (I_{CMC} = 1.02 and 1.05, respectively). Fast-growing fungi could not be classified as hypercellulase-producing fungi from the analysis of the relative activity index. The clearing of the cellulose medium was detected only in a close contact or in an area close to the mycelium.

The seven cellulotytyc strains were also tested for their ability to cause wood rot: three strains *Penicillium crustosum*, *Penicillium commune* and *Thielavia hyalocarpa* caused white rots, *Cladosporium cladosporioides* gave a soft rot with a brown colour and a wood surface degradation (Table 2). These results show that the isolated fungi can cause rots on fragments of wood, which confirm their participation in the wood degradation in the historic city of Fez.

CMCase activity, has also been described for *Penicillium chrysogenum* and *Penicillium granulatum* (Krystyna, 2007), *Penicillium crustosum* (Phil Haisley, 2002), *Penicillium expansum* (Duncan, 2006) and *Cladosporium cladosporioides* (Duncan *et al*., 2006; Pečiulytė, 2007). We report here, for the first time, that *Thielavia hyalocarpa* is a cellulolytic fungus showing a moderate CMCase activity (1.72 U). There has been an industrial interest in the thermophilic fungus *Thielavia terrestris* for the development of new enzyme preparations to boost the degradation of lignocellulose (Rosgaard, 2006). Our new cellulolytic specie *Thielavia hyalocarpa* should provide another interesting source for such valuable enzyme preparations.

The seven cellulolytic fungal isolates were further analysed as potential cellulase producers. They were investigated in shake flask cultures using CMC or cellulose powder as a carbon source. After 3 days of culture, the enzymatic activity was measured by the production of reducing sugars, which was taken as an indicator for the cleavage of cellulose molecules.

Two standard substrates were used for the determination of cellulase activity in terms of overall (FPCase) and CMCase contents (Ghose, 1987). Filter paper was used as a standard substrate to measure the total cellulase activity (Wu *et al*., 2006). The filter paper activity expressed summations of a simultaneous synergistic action of endoglucanases, cellobiohydrolases and β-glucosidase in a cellulose preparation. Soluble cellulose (carboxymethyl cellulose) was used as a substrate to determine the endoglucanase activity. Significant differences in activity were detected for the two enzymes CMCase and FPCase (Fig. 2). The overall cellulose activity levels were lower than the CMCase activity level for all fungal isolates.

The determination of cellulase activity is a complicated process because the hydrolysis of insoluble cellulose (filer paper in our case) is not always directly proportional to the amount of enzyme and/or reaction time. *Penicillium chrysogenum* is known as a very good producer of cellulases, perhaps due to its adaptability to anthropogenic substrates and resistance to different factors affecting fungal populations during the recycling procedures.

Although seven isolates showed a good filter paper disintegration and CMC utilization within 72 h, not all of them can be recommended as potential hypercellulase producers. We are most interested in the isolates that showed a high FPCase activity. These findings are comparable to results obtained by other research groups (Updegraff, 2004; Kluczek *et al*., 2006; Pečiulytė, 2007).

The selected seven fungal isolates were grown at different temperatures to determine their optimal growth conditions. Figure 3 shows the growth rates measured at five temperatures. All seven species showed the ability to grow at 4 and 25 °C, and had higher growth rates at 25 °C than at 4 °C and should be classified as cold-tolerant mesophiles. Another *Penicillium crustosum* isolate showed the ability to grow at 45 °C and should be classified as thermotolerant. No growht was observed for all the seven selected fungi

The production of hydrolytic enzymes varies between different microorganisms depending on their optimal growth temperature and the time of incubation (Kawai *et al*., 1988).

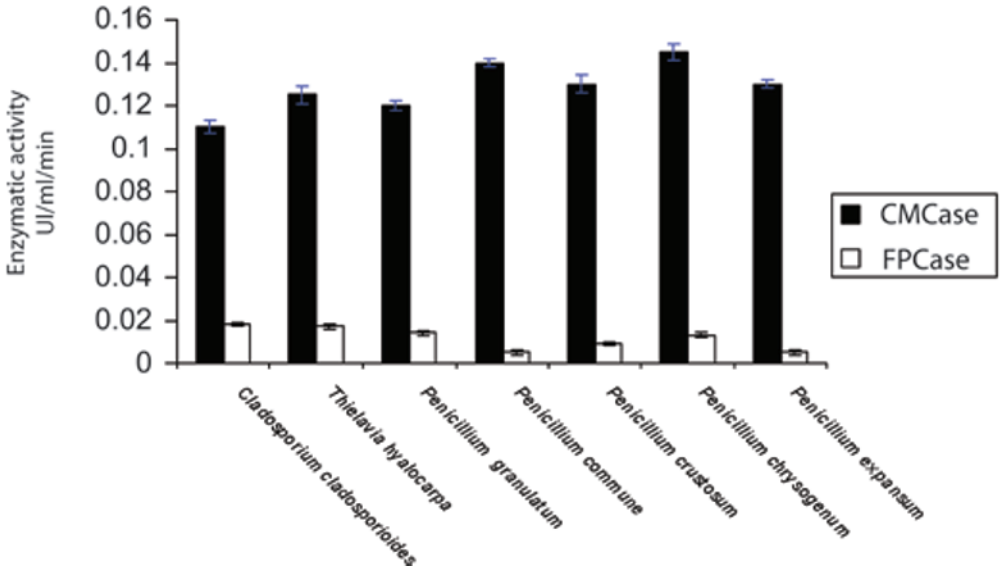

FIG. 2 - Endoglucanase (CMCase) and total filter paper cellulase (FPCase) activity of fungi after growth for 72 h in media with carboxymethyl cellulose or filter paper as a sole carbon source. Each bar represents enzymatic activity (U/ml/min)

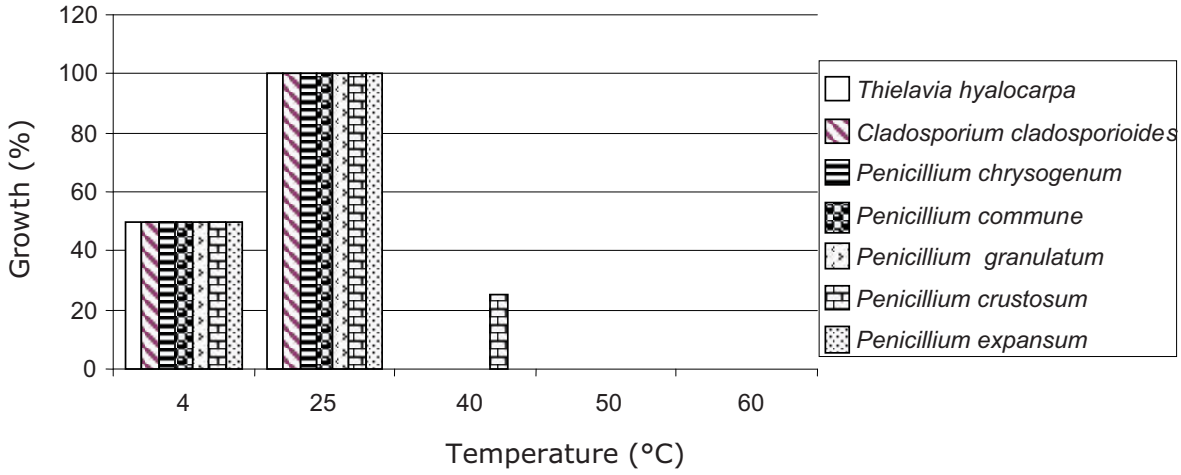

FIG. 3 - Growth rate of the seven selected fungi at different temperatures.

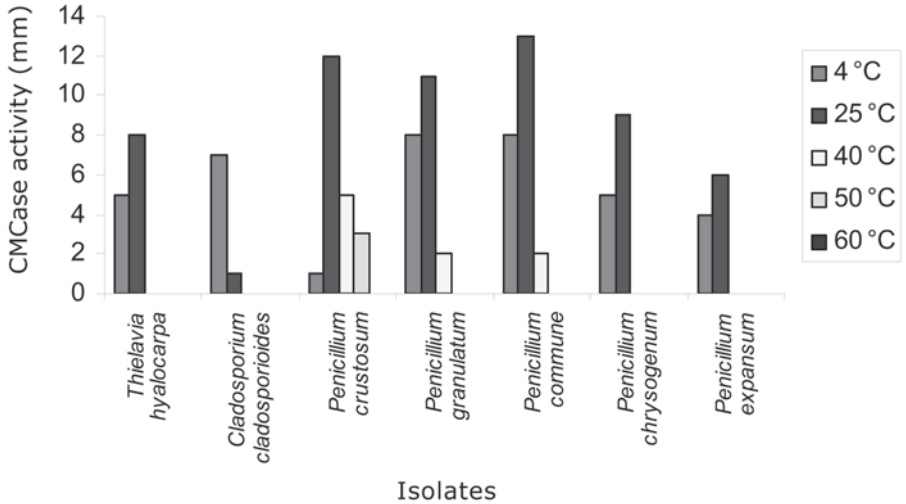

FIG. 4 - Comparison of CMCase activity for the seven isolates at 4, 25, 40, 50 and 60 °C. CMCase activity was calculated by measuring the diameter of the zones on the CMC agar plates indicated as clear orange halos after staining with 1% Congo red solution.

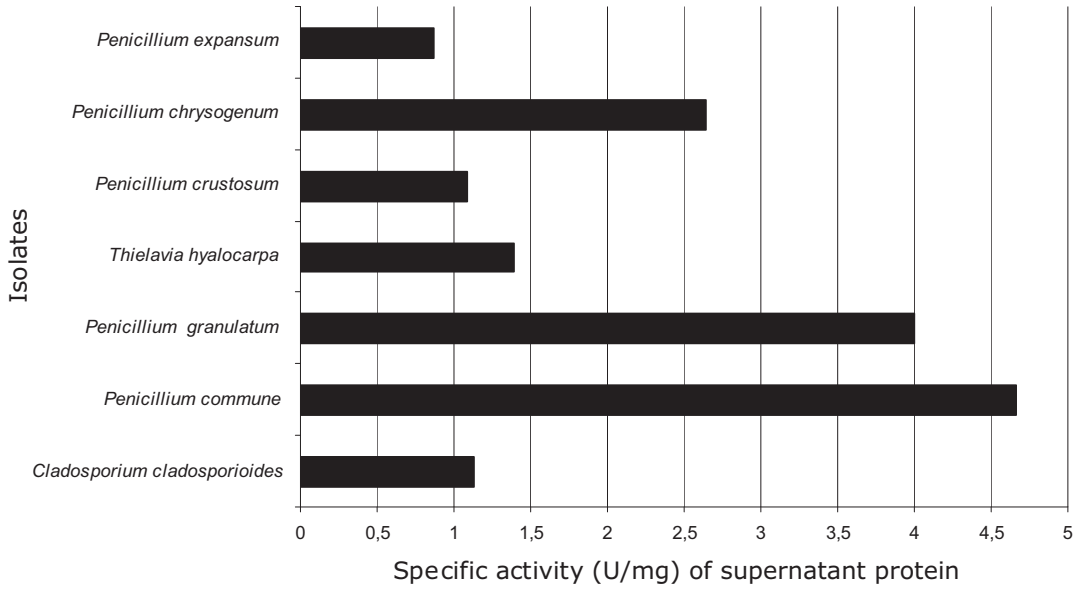

FIG. 5 - Specific activity on CMC agar medium of the seven selected fungi after incubation at 25 °C for 72 h.

The effect of temperature on the activity of crude cellulases was determined at various temperatures ranging from 4 to 60 °C (Fig. 4). The activity of enzymes decreases with increasing temperature because of enzyme inactivation at temperatures above a specific range probably due to the adaptation of these strains to an environment where the temperature does not exceed 30 ° C. The enzyme showed a good activity between 4 and 25 °C with a maximum activity at 25 °C. CMCase activity in the strain *Penicillium crustusum* remained constant at 40 °C. Inactivation of CMCase activity was observed at temperatures higher than 40 °C.

Penicillium commune, *Penicillium granulatum* and *Penicillium chrysogenum* showed the highest cellulase productivity as confirmed by CMCase values. This allowed us to infer that these three strains hydrolysed cellulose the most and therefore they are most destructive for timber. Results are shown in Fig. 5.

Cellulose is a mixture of crystalline cellulose, which is more resistant to microbial degradation, and amorphous cellulose, which is readily broken down to glucose (Eriksson *et al*., 1990). All fungi grew on the two cellulose carbon sources indicating that can use many different types of cellulose as nutrient sources. The presence of these fungi within the medina of Fez city, and their ability to degrade cellulose demonstrates that the conditions of Medina provide an environment conducive for the development of fungi. Future study will investigate searching for such species in different zones of the medina for the purpose of proposing solutions for the preservation of the cultural heritage of Fez historic monuments.

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