IN VITRO BIODEGRADATION OF KERATINIZED SUBSTRATES BY KERATINOPHILIC FUNGI

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Abstract

Keratinophylic fungi are present in the environment with a variable distribution, being influenced by human and animal presence and playing an important role in the biodegradation of keratinized substrates (skin, hair shaft, nails, claws, horns, wool and feathers). These fungi are geophilic, zoophilic and anthropofilic. Keratinophylic fungi have the ability to degrade keratinized materials, using the keratin as the sole source of carbon and nitrogen. This behaviour is based on the activity of keratinases, enzymes belonging to the group of proteases that can specifically degrade keratin. The aim of the present study was to evaluate the biodegradative ability of some keratinophylic fungal strains, clinical and geophilic isolates. The tested keratinized substrates were represented by animal hair strands. The rate of keratinized substrates biodegradation was expressed as weight loss over three weeks of incubation in minimal liquid medium in an orbital incubator. The morphological changes of hair samples were observed by light microscopy and scanning electron microscopy (SEM).

Key words: Keratinophylic fungi, biodegradation, keratinases.

INTRODUCTION

Keratinophilic fungi are present in the environment with a variable distribution, being influenced by human and animal presence and playing an important role in the biodegradation of keratinized residues and bioremediation of environment (Geetanjali et al., 2014; Khan et al., 2015; Mini et al., 2012; Sharma et al., 2010; Sharma and Choudhary, 2014). These fungi are geophilic, zoophilic and anthropofilic from ecological poit of view (Maruthi et al, 2012). The keratinophilic fungi have the ability to use keratin from keratinized materials (superficial layers of the skin, hair shaft and nails in humans and claws, horns, wool in animals) as the unique source of carbon and nitrogen (Ganaie et al., 2010; Narula et al, 2011). Keratin is found predominantly in feathers, hair, nails, horns, hooves, furs, claws, bird beaks, skin and consists of two type of keratin: α (alpha) and β (beta)-keratin; α keratin (soft) is usually found in hair, wool,

horns, nails, claws and hooves, whereas β keratin (harder) is found in bird feathers, beaks, and claws (Gopinath et. al., 2015). Keratins are also basic or acidic (Bragulla and Homberger, 2009).

The keratinophilic fungi can cause superficial mycosis both in humans and animals (Sarkar, 2014). They include a variety of taxonomic groups of filamentous fungi, one of them being the dermatophytes fungi (Gopinath, 2015; Jain and Sharma, 2012; Maruthi, 2012).

The keratinophilic fungi can produce a specific enzyme named keratinase that is responsible for keratin degradation. Keratinases can be serine proteases or metalloproteases (Kumar and Kushwaha, 2014).

The aim of the present study was to evaluate the biodegradative ability of some keratinophylic fungal strains from clinical and geophilic isolates.

MATERIALS AND METHODS

Microbial strains

The tests were carried out with the following keratinophilic fungal strains: *Trichophyton mentagrophytes*, *Fusarium* sp., *Cladosporium sp.* and *Trichoderma* sp. The tested strains were grown and maintained on potato dextrose agar (PDA) slants at 4°C.

Keratin substrate

Horse hair strands were washed thoroughly under running water, dried and sterilized with 3% ethanol. The hair strands were cut into pieces of about 2 cm in length, divided into portions of 50 mg weight and autoclaved at 121°C for 20 min.

Conditions of fungal cultivation

The minimal liquid culture medium had the following composition (g/L): 0.1, KH_2PO_4 ; 0.1, $CaCl_2$; 0.1, $FeSO_4 \cdot 7H_2O$; 0.005, $ZnSO_4 \cdot 7H_2O$; pH 7.0. In each Erlenmayer flask 50 ml of liquid medium and 50 mg of horse hair strands (cut into 2 cm fragments length) were added. The flasks were inoculated with a piece of mycelium from each strain. The flasks were incubated in an orbital incubator Heidolph Unimax 1010 at 27°C and 80 rpm, for three weeks (Figure 1).



Figure 1. Flasks in an orbital incubator Heidolph Unimax 1010

The tests include the following control flasks: microbiological control with the pure microbial culture on nutrient medium, flasks with only the nutrient medium, and the nutrient medium with the keratin substrates (horse hair strands) without the microorganism. The experiments were performed in triplicate. The broth culture was filtered through Whatman filter paper, washed carefully with distillated water. The fungal mycelium was removed gently from the horse hair strands, dried at 75°C for 48 hours and weighted. The biodegradation rate of keratinized substrates was expressed as weight loss over periods of incubation in experimental conditions. All tests were performed in the ICECHIM laboratories.

Morphological aspects of the horse hair strands

The fungal growth was observed by light microscopy with Olympus BX51 and by SEM on a FEI-QUANTA 200 instrument. All investigations were carried out in the ICECHIM laboratories.

RESULTS AND DISCUSSIONS

The test results are presented in Figures 2, 3 and Table 1.

The morphological examination of hair strands at different resolutions, with light microscopy as well as with SEM offer important data on the real capacity of a fungal strain to degrade keratin substrates. According to certain scientific reports, a model of the morphological expression of keratinolysis has identified two types of fungal attack, surface erosion and radial penetration (Marchisio et al., 1994; Marchisio, 2000). The authors considered that surface erosion is the hair keratin destruction from the exterior along the length of hair or in certain zones producing extensive "pockets". The radial penetration is a random attack by socalled "boring hyphae" acting at right angles with respect to the hair surface. The principles of this model are used in the present paper to discuss the images obtained from light microscopy and SEM.

As it can be observed in Figure 2a, the hair strand aspect was not changed by incubation in liquid culture medium in the absence of microorganism. The cuticle and the inner medullar channel of the control hair strand are preserved.

Trichophyton mentagrophytes develops "boring hyphae" specialized as perforating organs (Marchisio et al., 1994; Marchisio, 2000). (Figures 2b and 2c). Also, the lifting of the cuticle (Figure 2d) and modifications in the medullar channel (Figure 2e) can be observed. The images from optical microscopy obtained

for *Fusarium* sp. (Figure 2f), *Trichoderma* sp. (Figure 2h) and *Cladosporium* sp. (Figure 2j) show a normal network of hyphae grown

around hair strand. Further growing of fungal network may be responsible for the surface erosion of hair strand.



a) Control-horse hair strand (20x)



b) *Trichophyton mentagrophytes* (20x; pointed arrow - boring hyphae; 1 week)



d) *Trichophyton mentagrophytes* (20x; arrow - lifting of cuticle; lactophenol cotton blue; 3 weeks)



 f) Fusarium sp. (20x; hyphae around hair strand; interrupted medullar channel; lactophenol cotton blue; 3 weeks)



c) *Trichophyton mentagrophytes* (20x; arrow - perforating organ; 1 week)



e) *Trichophyton mentagrophytes*(20x; arrow - modification in medullar channel; lactophenol cotton blue; 3 weeks)



g) *Fusarium* sp. (40x; interrupted and narrowed medullar channel; lactophenol cotton blue; 3 weeks)



h) *Trichoderma* sp.(20x; hyphae and spores around hair strand; lactophenol cotton blue; 3 weeks)



j) *Cladosporium* sp. (40x; hyphae surrounding the hair strand; 3 weeks)



i) *Trichoderma* sp.(20x; arrow - slight boring hyphae in medullar channel; lactophenol cotton blue; 3 weeks)



 k) Cladosporium sp. (20x; dotted arrow – interrupted medullar channel; arrow - slight boring hyphae in medullar channel; lactophenol cotton blue; 3 weeks)

Figure 2. Light microscopy of entire fungus-hair units





a) control - horse hair strand (500x (left); 1000x (right) magnification)



b) Trichophyton mentagrophytes (1000x; arrow - fungal hyphae attached to hair strand)



c) Trichophyton mentagrophytes (1000x; dotted arrow – lifting of cuticle; arrow – fungal hyphae attached to hair strand)



d) *Fusarium* sp. (1000x; dotted arrow – "pocket" as surface erosion of hair strand; arrow - fungal hyphae attached to hair strand)



f) Cladosporium sp. (2000x; arrow – network fungal hyphae attached to hair strand)



e) *Fusarium* sp. (1000x; arrow - fungal hyphae attached to hair strand)



g) Cladosporium sp.
(500x; arrow – network fungal hyphae attached to hair strand)

Figure 3. SEM images of fungus-hair units after 3 weeks of incubation

The SEM micrographs offer more detailed information about the morphological modifications due to microbial contact (Figure 3).

In the case of *Trichophyton mentagrophytes*, hyphae network on the surface (Figure 3b, c) and cuticle lifting (Figure 3c) can be seen. The strain of *Fusarium* sp. produces surface erosion in an "extensive pocket" after 21 days of incubation (Figure 3d).

Attachment of fungal hyphae to the hair surface is observable also for *Fusarium* sp. (Figure 3e) and for *Cladosporium* sp. (Figure 3f and 3g). For *Trichoderma* sp. no significant changes in hair morphology were revealed by SEM images (data not shown).

The keratin substrates presented different rates of degradation according to the specific activity of the fungi under investigation (Table 1).

Table 1. Weight loss of keratin substrates after incubation with fungal strains

Funcol	Weight loss (%; w/w)		
species	1st week	2nd week	3rd week
Trichophyton mentagrophytes	15	49	75
Fusarium sp.	13	22	62
Trichoderma sp.	12	22	54
Cladosporium sp.	15	27	56

After the first week of incubation, there were minor differences between the weight loss values for the tested strains. The longer period of incubation facilitated the weight loss of keratinized substrates.

A significant difference between the weight loss values was observed after 2 weeks of

incubation, the highest value (49%) being obtained for *Trichophyton mentagrophytes*.

As regarding the others strains, *Cladosporium* sp. reached 27% weight loss, followed by *Fusarium* sp. and *Trichoderma* sp. with 22% weight loss each. The trend of the values was similar after 3 weeks of incubation. The weight loss values decreased in the following order: 75% for *Trichophyton mentagrophytes*> 62% for *Fusarium* sp. > 56% for *Cladosporium* sp. > 54% for *Trichoderma* sp.

CONCLUSIONS

The keratinophilic fungi play an important ecological role in the biodegradation of keratin substrates and can help in the environment provides useful protection. Our study information related to microbial degradation of According to our results, keratin. the Trichophyton mentagrophytes strain showed o good biodegradative activity. After 3 weeks of fungal contact, the hair strand lost 75% of initial weight and morphological changes were observed by microscopical analysis. The strain was selected for further studies dedicated to keratin biodegradation and isolation of keratinases.

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