Note

Detection of extracellular protease in *Mucor* species

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Summary The fungi are characterized by their abilities to produce and secrete enzymes to the external environment. The species of genus *Mucor* are a group of fungal microbes with important biotechnological potential, which are responsible for production of industrial enzymes. This work evaluated the ability of protease production in twelve species of genus *Mucor*. The strains were kept for 120 h under the incubation temperature of 28 ºC on a shaker at 120 rpm. The detection of proteolytic activity was evaluated in all species, the higher activity was detected in *Mucor racemosus* Fres. f. *chibinensis* (Neophytova) Schipper.

Key words *Mucor*, Enzyme activity, Protease activity

Detección de proteasas extracelulares en especies de *Mucor*

Resumen Los hongos se caracterizan por su capacidad de producción y secreción de enzimas al medio externo. Las especies del género *Mucor* forman un grupo de microorganismos importantes por su potencial biotecnológico, siendo responsables de la producción de enzimas industriales. Este trabajo investigó la producción de enzimas en 12 especies del género *Mucor*. Los experimentos fueron realizados durante 120 h de fermentación a 28 ºC, en un incubador orbital a 120 rpm. La actividad proteásica fue detectada en todas las especies, aunque la mayor actividad fue observada en *Mucor racemosus* Fres. f. *chibinensis* (Neophytova) Schipper.

Palabras clave *Mucor*, Actividad enzimática, Actividad proteasa

The order Mucorales comprises an omnipresent group of fungi, known to act as adversaries and/or allies of mankind. As allies, these moulds are being commonly used in different industries for synthesizing organic acids and alcohols. They are also employed in dairy industry for cheese production as well as other sources of protein for human consumption [14].

A great diversity of enzymes has been isolated from animals, plants and microorganisms. Among the enzymes produced by microorganisms, proteases have been extensively studied [5,7,15]. Although bacterial proteases have been used in detergents, the main drawback in their use is that they require cost-intensive filtration methodologies to obtain a microbe-free enzyme preparation. On the other hand, the proteases of fungal origin offer an advantage because the mycelium can be easily removed by filtration [13].

Enzyme production by fungi is usually assayed by grinding mycelium in a buffer and testing an extract for enzymatic activity, although enzyme assays on cultural filtrates have been also made [9].

The fungi of the order Mucorales, members of the genus *Mucor* occur typically as saprophytes in soil, organic matter and herbivorous dung. In general they are considered as plant pathogens of minor importance [16]. *Mucor miehei*, *M. pusillus and M. bacilliformis* have been much utilized in food industry and fermented beverage [10,12,14]. They are known for producing proteases for clotting of milk in cheese manufacturing [8]. These species have generally been preferred as a substitute for true calf rennet because of its specificity in splitting similar peptide bond in kappa-casein, high ratios of milk-clotting activity to proteolytic activity, similar calcium requirements, and good cheese quality [7].

This work report on the ability of 13 samples of *Mucor* species isolated from herbivorous dung to release extracellular protease into cultural medium.

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Materials and methods

Microorganisms. The *Mucor* species studied in this work were identified by Alves et al. [2] and were incorporated in the culture collections of the following entities: Section of Micologia and Liquenologia (SML), of Instituto of Botanica (SPC) of Secretaria of Meio Ambiente of State of São Paulo and the Universidade of Recife, Micologia (URM) of Federal University of Pernambuco (UFPE), they are preserved by the Castelani method [6] (Table 1).

Table 1. Strains of *Mucor* spp used in the present experiments.

Taxa	Culture collection no	
	SPC	URM
Mucor circinelloides van Tieghem f. *circinelloides* Schipper	1768	4136
M. circinelloides van Tieghem f. *griseo-cyanus* Schipper	1769	4183
M. circinelloides van Tieghem f. *janssenii* (Lendner) Schipper	1770	4141
M. circinelloides van Tieghem f. *lusitanicus* (Bruderlein) Schipper	1771	4137
M. genevensis Lendner	1772	4188
M. hiemalis Wehmer f. *hiemalis* Schipper	1773	4193
M. hiemalis Wehmer f. *luteus* Schipper	1774	4186
M. piriformis Fischer	1775	4145
M. piriformis Fischer f. *nanus* Alves; Trufem & Milanez forma nov.	1776	4189
M. racemosus Fres. f. *chibinensis* (Neophytova) Schipper	1777	4149
M. subtilissimus Oudem.	1778	4133
M. variosporus Schipper	1779	4219
M. carbonaceus Alves; Trufem.& Milanez, sp. nov	1780	4146

SPC: Instituto of Botânica; URM: Universidade of Recife, Micologia.

Microbiological assays. The spores of species studied were obtained from monosporic cultures growth in potato dextrose agar (PDA) plus chloramphenicol (PDAc) incubated at 28 ± 2 °C for six days.

The spores were removed by washing with sterile distilled water. The spore suspensions were assayed with a Neubauer camera to obtain a 10^7 spores/ml suspension. Shake flasks (250 ml) containing 50 ml of YPD [3] medium were inoculated with the spore suspension volume of 2.5 ml. The shake flask fermentation was carried out for 120 h at 28 °C using a shaking incubator (Bio-shaker BR-300L). Five shake flasks were inoculated for each sample and assayed daily.

Growth curve. Cellular mass was obtained daily by filtration and washed in membrane of 6-25 µm. The supernatant was transferred to a penicillin tube and the pH and enzymatic activity determined.

The mycelia mass was lyophilized for 24 h and maintained under vacuum until constant weight. The dry biomass was then analyzed by using neperian logarithm.

Proteolytic activity. The enzyme assay was based on the procedure described by Leighton et al. [11]. Azocasein (Sigma) was diluted at a 1% (w/v) solution of 0.2 M Tris-HCl, 1.0 mM $CaCl_2$, pH 7.2. The solution was stored at –20 °C. A 0.4 ml reaction mixture contained 250 µl of azocasein solution, and 150 µl supernatant fraction. The reaction was terminated by the addition of 1.2 ml of 10% (w/v) trichloroacetic acid. The reaction tubes were cooled at 0 °C for 15 min and centrifuged to remove the precipitate protein. A 0.8 ml amount of the supernatant was mixed with 1.4 ml of 1 N NaOH and the absorbance read at 440 nm.

Units of proteolytic activity are expressed as mg of azocasein hydrolyzed per hour. Specific activity was calculated by the ratio between total protease activity and total protein concentration in 1 ml of sample (U/mg). The experiments were done in duplicate.

Protein was measured according to Bradford [4] using bovine serum albumin (Sigma) as a standard.

Results and discussion

The ability of the members of genus *Mucor* to produce extracellular protease enzyme on liquid medium is shown in table 2. As seen in data all of the *Mucor* species tested presented protease activity. The enzyme production curve of *Mucor genevensis* and *M. variosporus* is shown in figure 1E-L, respectively. Cell growth increased rapidly in the early stages. Enzyme synthesis started in the first 24 h when nutrient consumption was high. There was reduction in the enzyme activity after reaching a maximum (for this particular experiment at 120 h). For all experiments it was found that the activity was associated to the pH for values higher than 7.8 and 3.8 (figure1A-1L). This behavior was also observed by Escobar & Barnett [7] for an acid protease of the specie *M. miehei*. They also demonstrated that

Table 2. Protease activity in different taxa of *Mucor* spp between 24 h and 120 h.

Taxa	Protease activity (U/mg)				
	Time (h)				
	24	48	72	96	120
Mucor circinelloides f. *circinelloides*	4.56	4.79	6.45	16.53	19.11
Mucor circinelloides f. *griseo-cyanus*	13.52	6.83	6.14	18.14	18.13
Mucor circinelloides f *janssenii*	6.77	4.20	5.60	5.53	16.58
Mucor circinelloides f *lusitanicus*	10.90	11.76	3.9	9.01	13.72
Mucor genevensis	19.42	11.22	3.41	4.4	5.67
Mucor hiemalis f. *hiemalis*	13.13	9.9	3.87	5.72	14.04
Mucor hiemalis f. *luteus*	13.57	8.42	7.50	8.58	14.78
Mucor piriformis f. *piriformis*	5.12	4.01	5.58	8.24	8.65
Mucor piriformis f. *nanus*	3.69	1.03	2.37	1.84	4.56
Mucor racemosus f. *chibinensis*	8.36	6.72	24.5	15.2	10.84
Mucor subtilissimus	5.88	7.84	4.21	7.59	8.55
Mucor variosporus	13.07	8.71	4.66	9.49	8.39
Mucor carbonaceus	12.61	8.17	4.31	14.54	7.12

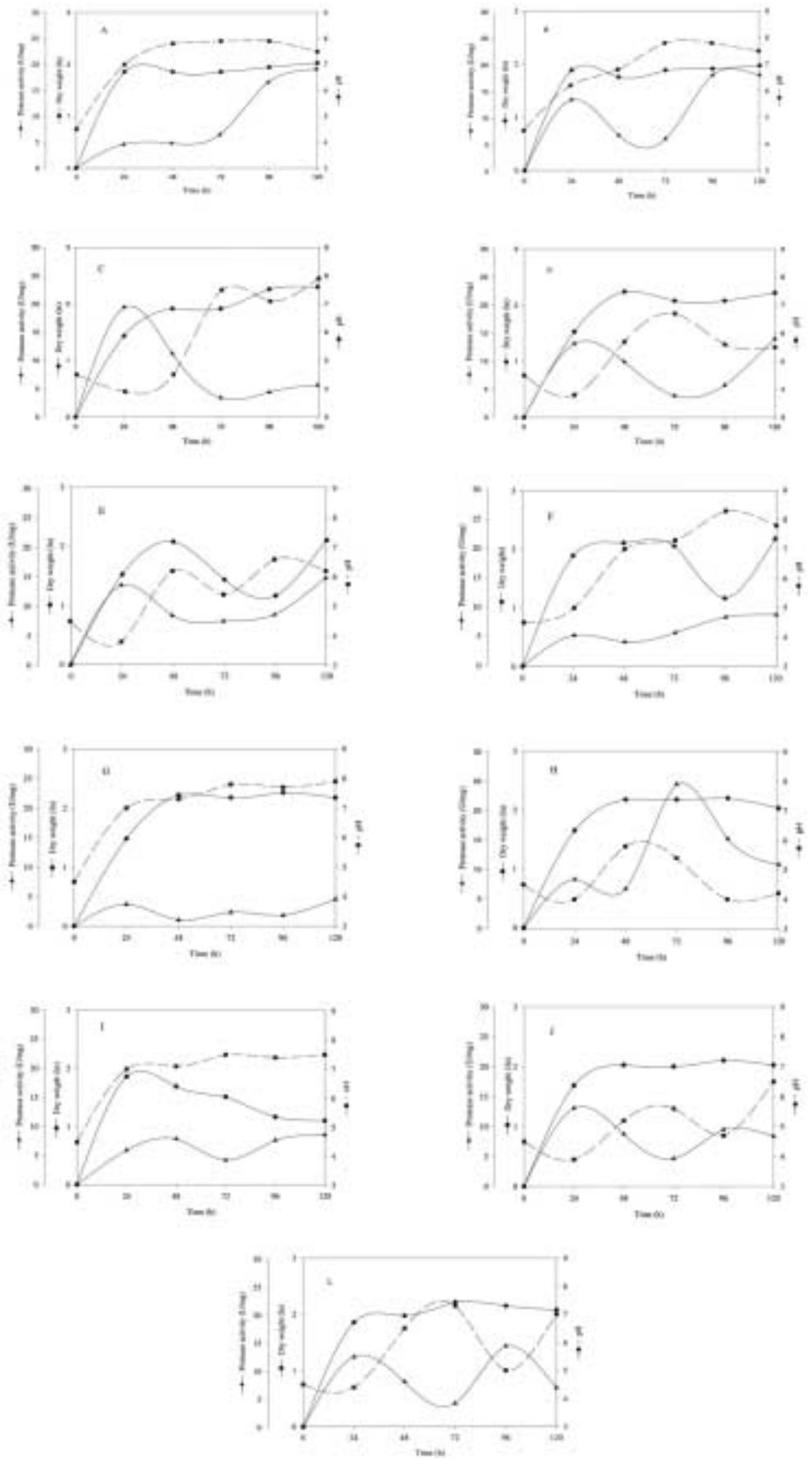

Figure 1. Protease activity in *Mucor* spp: 1A: *Mucor circinelloides* f. *circinelloides*; 1B: *Mucor circinelloides* f. *griseo-cyanus*; 1C: *Mucor genevensis*; 1D: *Mucor hiemalis* f. *hiemalis*; 1E: *Mucor hiemalis* f. *luteus*; 1F: *Mucor piriformis* f. *piriformis*; 1G: *Mucor piriformis* f. *nanus*; 1H: *Mucor racemous* f. *chibinensis*; 1I: *Mucor subtilissimus*; 1J: *Mucor variosporus*; 1L: *Mucor carbonaceus*.

there was a total loss of enzyme activity at pH 8.0 after 72 h. They also cited that is important to maintain pH below 6.5 to minimize losses in the enzyme activity.

The production of protease activity was high in: *Mucor genevensis*, *M. variosporus*, *M. carbonaceus*, *M. racemosus* f. *chibinensis* and *M. hiemalis* f. *hiemalis* in acid pH (3.9-6.2); *M. hiemalis* f. *luteus*, *M. circinelloides* f. *lusitanicus*, *M. circinelloides* f. *circinelloides*, *M. circinelloides* f. *janssenii* and *M. subtilissimus* in neutral pH (6.8-7.5); *M. circinelloides* f. *griseo-cyanus*, *M. piriformis* f. *piriformis* and *M. piriformis* f. *nanus* in basic pH (7.8-7.9).

The new taxa showed the same behavior when compared to the taxa previously identified. However, *M. carbonaceus* than *M. piriformis* f. *nanus*, once showed production in the stationary phase (48 h) while the last showed production only the end of stationary phase (120 h).

The Figures 1A.-L compares all of the taxa biomass production (ln), enzyme concentration (U/mg) and pH along the fermentation and can be that it protease activity increased with the biomass. On the other hand *M. piriformis* f. *nanus*, *M. subtilissimus* showed an increase of protease activity when the biomass decreased, while for *M. genevensis* and *M. variosporus* protease activity decreased when the biomass increased. The more expressive activity was showed for *M. racemosus* f. *chibinensis* (24.5 U/mg) in pH 5.4. The previous results indicate that pH influences protease activity.

Once, the protease activity of *M. circinelloides* f. *janssenii* and *M. circinelloides* f. *lusitanicus* was similar to *M. circinelloides* f. *circinelloides* and *M. circinelloides* f. *griseo-cyanus* respectively, the figures related to these species were not shown in the test.

In relation to the *M. circinelloides* f. *circinelloides* and *M. circinelloides* f. *griseo-cyanus* (Figures 1A-B) it could be observed that, even being of the same specie, the taxa showed a different protease activity profile along the fermentation. The same behavior was observed for *M. hiemalis* f. *hiemalis* and *M. hiemalis* f. *luteus* (Figures 1D-E) and *M. piriformis* f. *piriformis* and *M. piriformis* f. *nanus* (Figures 1F-G). Confirming so which really are variations of species.

Previously research for the qualitative test of the protease activity realized in solid media with same taxon utilized in this work, did not revealed enzymatic activity to *M. circinelloides* f. *lusitanicus*, *M. piriformis* f. *piriformis* and *M. subtilissimus* with 96 hours of experiment [1]. In fact, the present of enzymatic activity in liquid media for the genus *Mucor* related only the best detected with 120 h of fermentation (see Table 1).

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