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# Hydrolytic enzymes (proteases and lipases) released by biofilm-forming cells of *Scedosporium/Lomentospora* species

## Abstract

**Introduction and objective:** *Scedosporium/Lomentospora* species are human opportunistic filamentous fungi able to form biofilm in both biotic and abiotic surfaces.However, little is known about the ability of these fungi to release molecules into the biofilm environment. In this context, the present study aimed to detect hydrolytic enzymes, proteases and lipases, in the supernatants obtained from biofilm-forming cells of *S. apiospermum*, *S. minutisporum*, *S. aurantiacum* and *L. prolificans* (formerly *S. prolificans*).

**Materials and methods:** Biofilms were formed through incubation of *Scedosporium* and *Lomentospora* conidial cells for 72 hours over a polystyrene surface and validated by classical biomarkers (biomass, extracellular matrix and viability). In parallel, the cell-free biofilm supernatants were harvested in order to measure the activity of secreted hydrolytic enzymes, proteases and lipases. Azoalbumin and azocasein were used as proteinaceous substrates, while 4-methylumbelliferyl butyrate, 4-methylumbelliferyl heptanoate and 4-methylumbelliferyl oleate were used as lipid substrates.

**Results:** As previously reported, mature biofilms formed by *Scedosporium/Lomentospora* species were confirmed by the presence of a dense mycelial mass covering the polystyrene surface. Protease and lipase activities were detected in the biofilm-derived supernatants of all fungi studied. Regarding the protease, similar cleavages were observed for both azo-substrates, conjugated to albumin and casein, with an overall predilection to neutral-alkaline pH range. Concerning the lipase, substrates containing lipids with small, medium and large carbonic chains were degraded, with different predilection according to the fungal species.

**The inferences:** Collectively, the results suggest the presence of distinct proteases and lipases secreted by biofilm-growing cells of *S. apiospermum*, *S. minutisporum*, *S. aurantiacum* and *L. prolificans*.

**Keywords:** *Scedosporium*, *Lomentospora*, biofilm, proteases, lipases, secretion

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## Introduction

In the recent times, the frequency of fungal infections has increased in immunocompromised and immunocompetent individuals.[1] In this context, species from *Scedosporium* and *Lomentospora* genera emerged as human pathogens.[2] *Scedosporium* and *Lomentospora* species are ubiquitous, multidrug-resistant opportunistic filamentous fungi, with the ability to form biofilm on several biotic (e.g., lung epithelial cells) and abiotic (e.g., polystyrene, glass and different kinds of catheters) surfaces.[2–4] *Scedosporium/Lomentospora* mycelial cells are able to extracellularly release hydrolytic enzymes, including aspartic-, metallo- and serine-type proteases.[2] Secreted hydrolytic enzymes play vital role in different aspects of the fungal-host interplay, contributing to the development of a successfully infectious process. Fungal proteases and lipases, for example, cleavage host barriers, like biological membranes, allowing the fungal cells to disseminate inside the host environment as well as to escape from the immunological defenses.[2-4] Corroborating these findings, *S. apiospermum* mycelial cells were able to secrete metalloproteases capable of cleaving human serum albumin, casein, hemoglobin and immunoglobulin G.[3] However, absolutely nothing is known about the secretion of hydrolytic enzymes by the biofilm-growing cells in these opportunistic fungal pathogens.

### Study objective

In the present study, we aimed to detect the presence of protease and lipase activities secreted by biofilm-growing cells of *S. apiospermum*, *S. minutisporum*, *S. aurantiacum* and *L. prolificans*.

## Materials and methods

### Fungi

*Scedosporium apiospermum* (RKI07_0416) was provided by Dr. BodoWanke (Hospital Evandro Chagas, Instituto Oswaldo Cruz, Rio de Janeiro, Brazil), *S. minutisporum* (FMR4072), *S. aurantiacum* (FMR8630) and *L. prolificans* (FMR3569) were kindly given by Dr. Josep Guarro (Facultad de Medicina y Ciencias de la Salud, Reus, Spain). Fungi were maintained on Sabouraud liquid medium at room temperature with orbital shaking.[3] To obtain the conidial cells, each fungus was grown on potato dextrose agar (PDA; Difco Laboratories, USA) at room temperature. Conidia were obtained by washing the plate surface with phosphate-buffered saline (PBS; 10 mM $NaH_2PO_4$,10 mM $Na_2HPO_4$, 150 mM NaCl, pH 7.2) and filtering them through a 40-µm nylon cell strainer (BD Falcon, Franklin Lakes, USA) in order to remove the hyphal fragments.[5] The conidial cells were counted in a Neubauer chamber.

 

### Biofilm assay

For biofilm formation, conidial suspensions in Sabouraud medium (200 µl containing $10^6$ cells) were placed on flat-bottom 96-well polystyrene microtiter plates and then incubated without agitation at 37ºC with 5% of $CO_2$ for 72 hours. Medium-only blanks were also set up in parallel.[3]Subsequently, the supernatants were harvested and filtered through a 0.22-µm membrane (Millipore, Brazil). Protein concentration of the cell-free biofilm supernatants was determined by the method using the Folin phenol reagent and bovine serum albumin (BSA) as the protein standard.[4] In parallel, to confirm the ability of these fungi to form biofilm over polystyrene surface, the biomass was evidenced by crystal violet dye in methanol-fixed biofilm as well as the extracellular matrix and the metabolic activity were quantified in non-fixed biofilm by safranin incorporation and XTT metabolization, respectively.[5,6]

### Protease activity

Protease activity was assayed using two azo-containing proteinaceous substrates, azocasein and azoalbumin (Sigma-Aldrich, USA), at different pH's (4.0, 5.0, 7.0 and 9.0). Briefly, the cell-free supernatants (100 µg of proteins) were incubated with azocasein or azoalbumin (1.6 mg/ml) in 50 mM sodium acetate buffer (pH 4.0), 50 mM sodium phosphate buffer (pH 5.0), PBS (pH 7.0) or 50 mM glycine-NaOH (pH 9.0), for 1 hour at 37ºC. The reaction was terminated by adding 5% trichloroacetic acid. After centrifugation at 4000 g for 5 minutes, the supernatants were collected and the absorbance of the released azo-dye read at 366 nm in a microplate reader (SpectraMax M3; Molecular Devices, USA). The specific peptidase activity was expressed in arbitrary units (U) per milligram of protein, where 1 unit of enzymatic activity is equivalent to the variation in optical density of 0.001 nm per minuteat 366 nm.[4]

### Lipase activity

Lipase activity was assayed using three distinct lipid substrates, 4-methylumbelliferyl butyrate, 4-methylumbelliferyl heptanoate and 4-methylumbelliferyl oleate (Sigma-Aldrich, Brazil). To perform the reaction mixtures, 10 µM of each substrate was added with cell-free supernatants (100 µg of proteins) in 10 mM Tris-HCl buffer (pH 8.0), as suggested by the manufacture protocol. Cleavage of substrates was monitored for 1 hour at 37ºC in a fluorescence spectrometer (SpectraMax Gemini XPS; Molecular Devices, USA) using an excitation wavelength of 327 nm and an emission wavelength of 449 nm. The lipase activity was expressed as the fluorescence arbitrary units (FAU) obtained after 60 minutes of reaction.

## Results

The *Scedosporium/Lomentospora* mature biofilms were evidenced by classical methodologies, including the observation of biomass by crystal violet staining, the presence of extracellular polymeric substance by safranin dye and the metabolic activity of biofilm-forming cells by XTT metabolization (Figure 1). In addition, light microscopy analyzes clearly revealed a dense fungal mass formed with intertwined mycelial cells (Figure 1). Subsequently, the supernatants of biofilm-growing cells of *Scedosporium/Lomentospora* were harvested in order to measure the hydrolytic activity of two classes of enzymes, proteases and lipases.

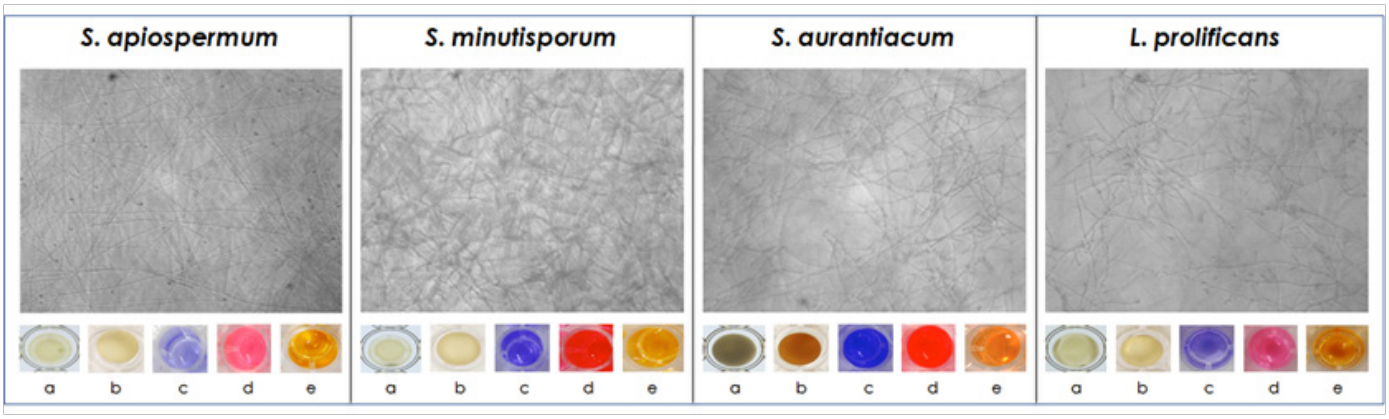


**Figure 1** Light microscopies of biofilms formed by *S. apiospermum*, *S. minutisporum*, *S. aurantiacum* and *L. prolificans* after 72 hours of interaction with polystyrene surface. In addition, images of the mature biofilms formed on 96-well microtiter polystyrene plate: (a) top and (b) bottom view of the wells, (c) biofilm biomass evidenced by crystal violet, (d) biofilm extracellular matrix evidenced by safranin incorporation and (e) biofilm metabolic activity evidenced by XTT metabolization.

Overall, protease activities secreted by *S. apiospermum*, *S. minutisporum*, *S. aurantiacum* and *L. prolificans* were preferentially evidenced at neutral-alkaline pH range regarding the two proteinaceous substrates tested, albumin and casein (Figure 2). However, considerable differences on preferential degradation of both proteins were evidenced. For instance, in the extreme acidic pH (4.0), higher protease activities were detected over azocasein than azoalbumin in all fungi. In pH 5.0, *S. apiospermum* and *S. minutisporum* hydrolyzed better azocasein than azoalbumin and an opposite cleavage pattern was detected for *S. aurantiacum* and *L. prolificans* (Figure 2).

Lipase activities able to cleave different lipid chain sizes were detected in all fungal species studied herein (Figure 3). For *S. apiospermum* and *L. prolificans*, the higher lipase activity was detected over the substrate containing a medium lipid chain size (4-methylumbelliferyl heptanoate), while *S. minutisporum* preferentially degrades the small lipid size chain (4-methylumbelliferyl butyrate) and *S. aurantiacum* the larger one (4-methylumbelliferyl oleate) (Figure 3).

**Citation:** Mello TP, Barcellos IC, Branquinha MH, et al. Hydrolytic enzymes (proteases and lipases) released by biofilm-forming cells of *Scedosporium/ Lomentospora* species. *J Microbiol Exp.* 2019;7(6):313–316. DOI: 10.15406/jmen.2019.07.00277

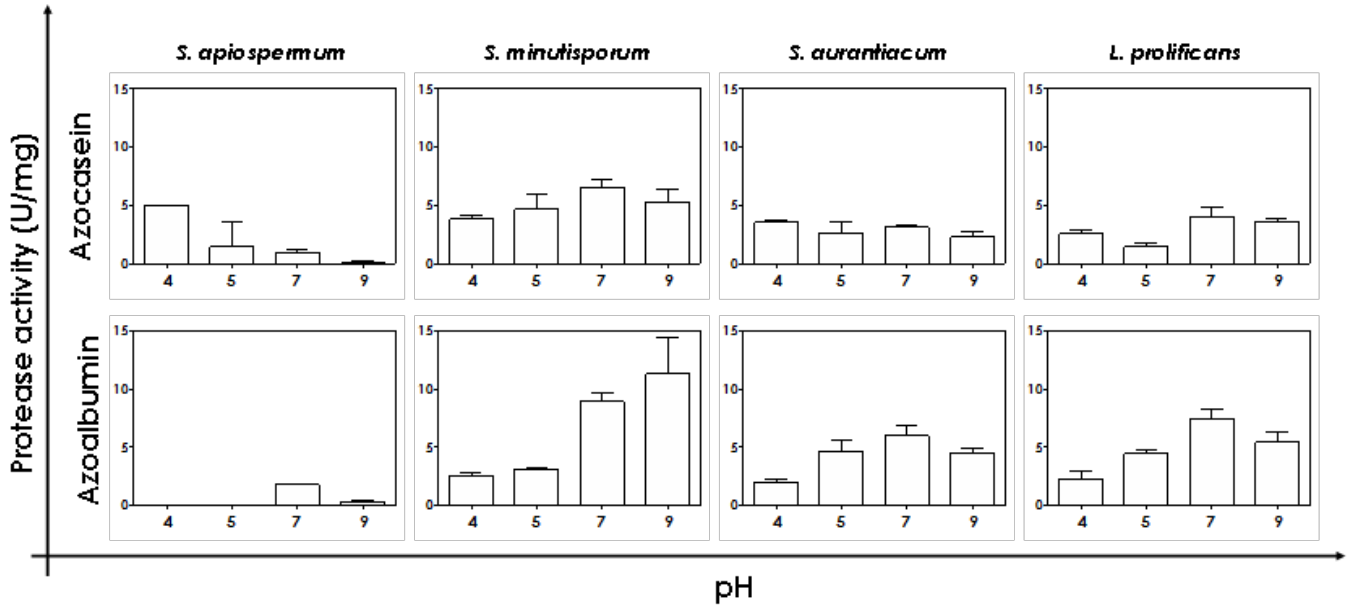


**Figure 2** Protease activity measured in the 72 hours-biofilm-derived supernatants from *S. apiospermum*, *S. minutisporum*, *S. aurantiacum* and *L. prolificans*. The enzymatic activities were measured after 1 hour at 37°C using different pH values and two distinct proteinaceous substrates, azocasein and azoalbumin. The protease activity was expressed as arbitrary units (U) per milligram of protein, where one unit of activity is equivalent to the variation of 0.001 nm in the optical density per min at 366 nm. The bars represent the means ± standard deviations from at least three independent experiments.

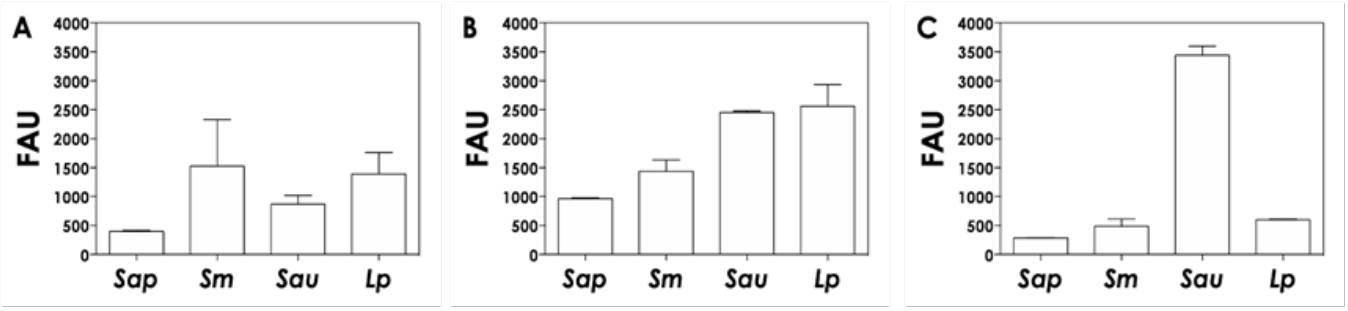


**Figure 3** Lipase activity measured in the 72 hours-biofilm-derived supernatants from *S. apiospermum* (Sap), *S. minutisporum* (Sm), *S. aurantiacum* (Sau) and *L. prolificans* (Lp). The enzymatic activities were measured after 1 hour at 37°C at, pH 8.0, using three distinct lipid substrates: (A) 4-methylumbelliferyl butyrate, (B) 4-methylumbelliferyl heptanoate and (C) 4-methylumbelliferyl oleate. The lipase activity was expressed as fluorescence arbitrary units (FAU). The bars represent the means ± standard deviations from at least three independent experiments.

## Discussion

As previously reported by our research group, *Scedosporium/ Lomentospora* species form mature biofilms after 72 hours of incubation on several abiotic and biotic surfaces.[5,6] In the present work, we described that those biofilm-forming cells were able to release both lipases and proteases. The role of secreted hydrolytic enzymes in mature biofilms has been linked to crucial biological events, such as nutrient acquisition and cell dispersion, in several fungal species, including *Candida albicans* and *Trichosporon inkin*.[7–9] The dispersion of cells confined in a mature biofilm structure is a relevant event on microbial transmission from environmental reservoirs to human hosts as well as in dissemination of focal infection within the host.[9] The secreted hydrolytic enzymes cleave the biofilm extrapolymeric matrix, which acts like a glue holding the biomass together, releasing microbial cells into the environment that permit the colonization of other environments. The expression of secreted aspartic proteases (*SAPs*), phospholipase B (*PLB*) and lipase (*LIP*) genes in *C. albicans* is directly associated with biofilm growth on mucosal surfaces as well in abiotic surfaces.[10] In addition, sessile *C. albicans* cells secrete more aspartic proteases than planktonic cells,[7] demonstrating the importance of secretion of hydrolytic enzymes on biofilm formation and maturation.

## Conclusion

Biofilm-growing cells of *Scedosporium* and *Lomentospora* species extracellularly released hydrolytic enzymes, including proteases (with the ability to cleave distinct proteinaceous substrates at wide range of pH) and lipases (capable of cleaving molecules with different lipid chain sizes), which can contribute with the physiological homeostasis of the biofilm structure.

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## Conflicts of interest

The authors declare that there is no conflict of interest.

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