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COMPARISON OF LIPOLYTIC ACTIVITY OF *Sporothrix schenckii* STRAINS UTILIZING OLIVE OIL-RHODAMINE B AND TWEEN 80

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ABSTRACT

The aim of this work was to identify the lipase activity of *Sporothrix schenckii* strains using Rhodamine B as an indicator of triglyceride hydrolysis (triolein from olive oil) and to compare these results with the use of Tween 80 as substrate. Free fatty acids released from lipase activity were detected on agar plate complexed with Rhodamine B, when exposed to UVA light, through orange fluorescence and by precipitation haloes on Tween 80 agar. There were differences in lipolytic activity among strains and between the media utilized. The highest lipase activity (orange fluorescence) was observed on the biomass of 10 strains, with no fluorescent haloes around the colonies, whereas only six strains showed large precipitation haloes. These plate assays are suitable for the detection of lipase-producing strains of *S. schenckii* in the yeast phase, but it is still unknown whether different enzymes act upon the substrates or whether there are variations of the same enzyme with different specificities.

Key-words: Rhodamine B, lipase, Tween 80, *Sporothrix schenckii*.

1 Introduction

Sporotrichosis, a widely distributed fungal infection caused by *Sporothrix schenckii*, causes chronic granulomatous and ulcerative lesions. *S. schenckii* is a dimorphic fungus, but the yeast form is dominant in affected tissues and is believed to be more virulent. However, factors related to the invasion of the parasite have not yet been clarified [1,2]. The release of enzymes to the extracellular environment might be an important adaptive mechanism during the life cycle of a variety of microorganisms, including fungi [3,4], and some extracellular enzymes are related to the virulence and pathogenicity of mycotic infections, such as proteases, lipases, and phospholipases [5-9]. Although *S. schenckii* commonly causes a chronic deep fungal infection, its pathogenicity has not been biochemically elucidated.

Lipases (triacylglycerol hydrolases, EC 3.1.1.3) are a group of enzymes that hydrolyze triacylglycerols to diacylglycerols and monoacylglycerols, with free fatty acids (FFA) as coproducts [10]. The activity of these enzymes can be assayed by monitoring the release of either fatty acids or glycerol from triacylglycerols or synthetic fatty acid esters [11]. Lipases are generally water soluble, and catalytic reactions take place after the adsorption of enzymes onto an oil-water interface [10,12]. There are ubiquitous natural sources of lipases, including animals [13,14], plants [15-17], insects [18], fungi, and bacteria [19]. Screening of lipase producers on agar plates is frequently done by using tributyrin [20] or Tween 80 [21] as a substrate. Fungal lipases can be visualized after ultra-thin layer isoelectric focusing gel when they are overlaid by an agar gel containing trioleoylglycerol and the fluorescent dye Rhodamine B, and adapted by Kouker and Jaeger [22] to a plate assay. Rhodamine B is a fluorochrome utilized to stain fungal cells directly or in enzyme assays [23,24]. The association between this dye and fatty acids [24,25] has encouraged its use in research about microorganisms with lipolytic activity [22]. The aim of this work was to identify lipase activity of *Sporothrix schenckii* strains using Rhodamine B as an indicator of triglyceride hydrolysis (triolein from olive oil) and to compare these results with the use of Tween 80 as substrate.

2 Materials and Methods

Fungal strains: *Sporothrix schenckii* strains 237, 329, 339, 424, 440, 441, 444, 450, 478, 576, 579, 611, 794, 805, 810, 853, and 864 were used. These strains were obtained from the mycological collection of the School of Medicine of Universidade de São Paulo.

Olive oil assay: The *S. schenckii* strains were incubated for 14 days in bilayer media for lipase induction (basal media: peptone 5 g; beef extract 3 g; sodium chloride 5 g; agar 15 g; distilled water 1 L; overlay media: olive oil 10 mL; gum arabic 20 g; agar 15 g; distilled water 1 L). 5-mm plug inocula were transferred to Petri dishes containing the same bilayer media, whose overlay media were added from a solution of 1 $mg.mL^{-1}$ Rhodamine B in distilled water, with a final concentration of 0.0015%. One strain of *Serratia marcescens* was used as positive

control. The experiment was made in triplicate and repeated twice. After seven days of incubation at 25 ºC, the plates were revealed in 365-nm light and the activity was determined by visual evaluation of fluorescence intensity.

Tween 80 assay [26]: strains from 7-day cultures were inoculated in triplicate on agar plates containing Tween 80 as substrate (bactopepton 10g; sodium chloride 5g; calcium chloride 0.1g; agar 20g; Tween 80 20% 50mL; water 950mL) and incubated for 7 days at 25ºC. Precipitation haloes and colony sizes were measured with a caliper. Degradation intensity was gauged by the ratio between colony diameter and precipitation halo (Pz). Pz coefficients were grouped into four classes: Pz between 0.9 and 1 (+), very low Pz group; 0.89–0.80 (+ +) low Pz group; 0.79–0.70 (+ + +) high Pz group; and Pz less than 0.69 (+ + + +) very high Pz group[27]. According to this system, a lower Pz ratio corresponds to a higher enzyme activity.

3 Results

The presence of fluorescence in the yeast form of the cell growth biomass of the colonies was detected at earlier growth stages (7 days), on the Rhodamine B based test (Figure 1). Precipitation haloes from the Tween 80 test could be observed after 24 hours of incubation, with measurement on the fifth day (Figure 2).

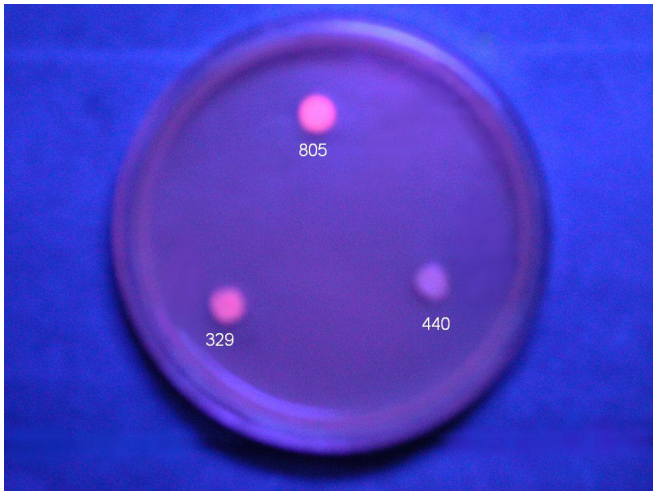

Figure 1. Plate assay showing lipase production on *S. schenckii* biomass, detected by Rhodamine B under UVA light (365nm) after seven days of incubation at 30ºC. On the top and lower left, two good lipase producers, strains 805 and 329, respectively. On the right, a weak lipase producer, strain 440.

Differences in the lytic capacity of the strains for substrate degradation were observed, as shown in Table 1.

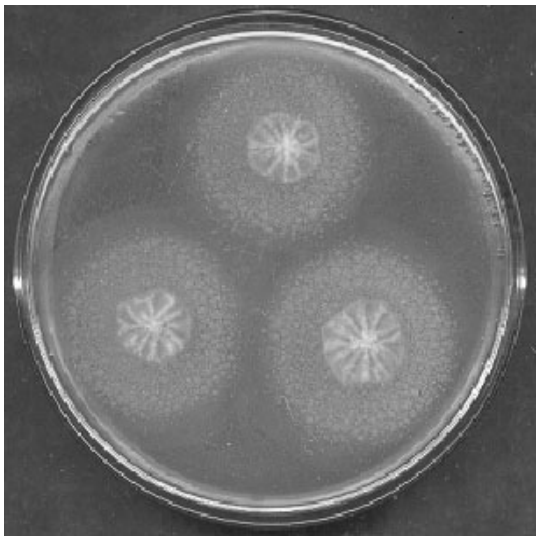

Figure 2. Plate with precipitation haloes characteristic of lipase production by *S. schenckii* 450 in the presence of Tween 80.

Table 1. Lipase activity of sporotrichosis agents cultured on olive oil and Rhodamine B-based agar and Tween 80-based agar.

Strain	Rhodamine B*	Tween 80 (Pz)†
237	+++	+++
329	+++	+
339	++	++++
424	+++	+
440	+	++
441	+++	++
444	+	+
450	+++	++++
478	+++	++
576	+	++++
579	+	++++
611	+++	+
794	+++	++
805	+++	++
810	++	++
853	+	++++
864	+++	++

* fluorescence intensity: (+) low intensity; (++) medium intensity; (+++) high intensity
†Pz values: (+) Pz = 0.9-1; (++) Pz = 0.89–0.80; (+++) Pz = 0.79–0.70; (++++) Pz < 0.69.

4 Discussion

The present data indicate that different isolates of *S. schenckii* are capable of producing lipases. However, the activity of these enzymes varied among the strains and between the substrates utilized.

Lipolysis can be determined through many different methods, such as titrimetry, colorimetry, spectrophotometry or fluorimetry, radioactive fatty acids, gas-liquid chromatography and immunological methods [28]. Visual assessment of colorimetric and/or fluorimetric assays can be used in experiments where quantitation is not necessary, such as investigation into microbial lipases on agar plate media. Lipolysis determination, however, varies and is specific to each microorganism tested.

Tweens have been used because some microorganisms which do not hydrolyze tributyrin or other butyric acid esters can do this to true fats, but Tweens are not suitable as substrates because they are heterogeneous [28], do not resemble triglycerides, and are hydrolyzed by esterases (carboxylic ester hydrolase, EC 3.1.1.1) [7,29], whose main substrates are phospholipids and sterols. When used as emulsifiers, Tweens may be hydrolyzed by lipases, resulting in competition with the intended substrate [11]. Olive oil is an easily obtainable and easy-to-use substrate, bearing strong similarity to triolein, but an emulsion is necessary to increase the proportion of surface area, facilitating lipase activity [30]. Emulsification requires surfactants, such as detergents, other lipids, proteins, or bile acids. However, emulsifying agents can affect lipase activity. Detergents with structures such as those of fatty acids (e.g.: Triton X-100) can inhibit lipases, or be toxic (bile salts) [31]. Strong detergents may also denature the enzyme, resulting in loss of activity[11]. Tiss et al. [32] found that gum arabic enhanced the lipase activity of olive oil in up to 3%, with or without sodium taurodeoxycholate (a lipase-stimulating agent), without interfering in enzyme kinetics. Association between an emulsifier and a bilayer agar, where olive oil was dispersed and maintained in droplet form until agar solidification, may be involved in the fast production of lipases by this fungus.

The detection of lipase activity using Rhodamine B is based on the acidic change to its cationic form and formation of complexes with uranyl-fatty acid ion, proportionally inverse to the fatty acid chain length [33] with orange fluorescence excitation at 350nm [22]. As Rhodamine B reveals reactive FFA, interference of phospholipids may be avoided [33]. Victoria Blue B is another indicator of lipolysis, but its action as acid indicator may cause false positives through the production of acids from carbohydrates [31].

Some of the most detailed studies reported are those dealing with the lipases produced by *Rhizopus delemar* [34-36], *Mucor lipolyticus* [37,38], *M. hiemalis* [39], *M. javanicus* [40], *Humicola lanuginose* [41], *Geotrichum candidum* [42-45], *Penicillium cyclopium* [46], *Acinetobacter calcoaceticus* [47], *Fusarium oxysporum* [48], *Rhizopus oryzae* [49], *Aspergillus japonicus* [50], *A. oryzae* [51] and *A. niger* [50,52]. However, there are still many gaps in the knowledge about the production of lipolytic enzymes by various species of fungi. Lipase activity had not been demonstrated or identified in this fungus up until this work. In this study, the highest lipase activity was observed on the biomass of fungi grown on olive-oil-Rhodamine B agar. In *Malassezia furfur* yeasts lipase activity is located within the glucan-rich wall and/or in membrane systems [5] and plays a role in cell growth and hyphal formation [7]. As morphological transition of the yeast to the hyphal phase is associated with disease, lipases may be involved in adhesion and interactions with the immune system.

In vitro lipase production has been observed in dermatophytes such as *Trichophyton rubrum*, *T. mentagrophytes*, *Epidermophyton flocosum* and *Microsporum canis,* and these findings are useful for the differentiation of biotypes and in indicating the role in the virulence of certain strains [53]. In addition to dermatophytes, opportunistic fungi such as *Candida* species are known to secrete extracellular lipases [7], which can be an important virulence factor during different types of *C. albicans* infections [54]. These findings allow us to suggest that wall/secreted lipases produced by other fungi may be associated with increased adherence of the yeast to host cells and with virulence, mainly on those which cause subcutaneous mycosis, such as *S. schenckii*. These plate assays detected lipase-producing strains, but it is still unknown whether different enzymes act upon the substrates or whether there are variations of the same enzyme with different specificities.

For this purpose, our future studies will be focused on the importance of secreted lipases during the interaction of *S. schenckii* with human and animal tissues as immunological response. These enzymes could also serve as a basis for investigations of new drug targets.

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COMPARAÇÃO DA ATIVIDADE LIPOLÍTICA DE *Sporothrix schenckii* UTILIZANDO ÓLEO DE OLIVA-RODAMINA B E TWEEN 80

Resumo

- O objetivo deste trabalho foi identificar a atividade lipolítica de cepas de *Sporothrix schenckii* utilizando Rodamina B como indicador da hidrólise de triglicerídios (trioleína do óleo de oliva) e comparar estes resultados com a utilização de Tween 80 como substrato. Ácidos graxos livres liberados da atividade lipolítica foram detectados através da emissão de fluorescência laranja em ágar, quando exposto à luz UVA, ou através de halos de precipitação em ágar Tween 80. Não houve diferenças na atividade lipolítica entre as cepas bem como meios de cultivo utilizados. A maior atividade lipolítica (fluorescência laranja) foi

observada na biomassa de 10 cepas, sem halos fluorescents ao redor das colônias, enquanto apenas seis isolados apresentaram grandes halos de precipitação. Esses ensaios em placa são úteis para a detecção de *S. schenckii* produtores de lipase na fase leveduriforme, mas ainda se desconhece se são diferentes enzimas atuando sobre os substratos ou se são variações da mesma enzima com diferentes especificidades.

Palavras-chave: Rodamina B. lipase. Tween 80;. *Sporothrix schenckii.*

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