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Rapid detection of lytic antimicrobial activity against yeast and filamentous fungi

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Abstract

A rapid method for assessing the lytic activity of antimicrobial agents against yeast and fungi has been developed. The assay is based on the release of the intracellular enzyme, maltase (α-glucosidase). The released maltase activity was measured colorimetrically by the production of *p*-nitrophenol from *p*-nitrophenyl-α-D-glucopyranoside (PNPG). The lytic activity of different antimicrobial compounds was measured against yeast cells or germinating spores of filamentous fungi. Lytic anti-yeast activity could be detected within 20 min incubation at 30 °C against *Saccharomyces cerevisiae*, *Candida albicans*, and *Cryptococcus neoformans*. Lytic anti-fungal activity appeared after 2 h of incubation at 30 °C against germinating spores of *Aspergillus niger* and *Botrytis cinerea*. Whole cells of either yeast or fungi did not hydrolyze sufficient PNPG within 3 h at 30 °C to yield a detectable color change. Lytic activity of enzymes (e.g., Lyticase™), antibiotics (e.g., Amphotericin B™), and an antibiotic-producing strain of bacteria were detected using the assay. The anti-yeast assay has been adapted to a 96-well microtiter format. Both assays provided a rapid, sensitive, and reproducible detection of lytic anti-yeast and anti-fungal activity.

Keywords: Assay; Antimicrobial; Lytic; Fungi; Yeast

1. Introduction

The incidence of fungal infections has risen dramatically over the past 15 years (Georgopapadakou and Walsh, 1994; Kurtz, 1999). This increase has included infections by both filamentous fungi and yeast (Georgopapadakou and Walsh, 1994; Kurtz, 1999). The observed increase in the number of fungal infections is likely to continue because the number of persons disposed to fungal infection (e.g., immunocompromised individuals) is expected to rise, and filamentous fungi and yeast are present and widely distributed in the human environment (Georgopapadakou and Walsh, 1994; Kurtz, 1999). Although a number of new antifungal antibiotics have been developed, there still exists a

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need for new agents for treating yeast and filamentous fungal infections Georgopapadakou and Walsh, 1994; Kurtz, 1999.

Toward the objective of identifying novel anti-fungal and anti-yeast antibiotics, we developed a method for the rapid assessment of possible lytic activity of antifungal compounds against yeast, including the pathogens *Candida albicans* and *Cryptococcus neoformans*, and filamentous fungi, including the pathogens *Aspergillus niger* and *Botrytis cinerea*. The assay was developed following a rapid coliphage detection assay (Stanek and Falkinham, 2001). In that assay, detection of coliphage is based upon the bacteriophage-induced lysis of *Escherichia coli* cells and the release of intracellular β-galactosidase (Stanek and Falkinham, 2001). For measurement of lytic antifungal activity, release of an intracellular enzyme is a consequence of the lysis of cells following exposure to a lytic compound. Maltase (α-glucosidase) was chosen as the enzyme for development of the assay because many pathogenic yeast and fungi produce it.

2. Materials and methods

2.1. Microbial strains

The strains of *Saccharomyces cerevisiae*, *A. niger*, and *Pseudomonas* strain 679-2 (Casida, 1992) were obtained from Dr. L.E. Casida, Jr. The *C. albicans* and *B. cinerea* strains were obtained from the Virginia Tech Microbiology Collection and *C. neoformans* strain H99 was obtained from Dr. W.G. Neihaus (Niehaus and Flynn, 1994).

2.2. Growth of Pseudomonas strain 679-2 and preparation of cell-free culture-filtrate

Strain 679-2 was grown in quarter-strength Tryptic Soy Broth (BBL Microbiology Systems, Cockeysville, MD) containing 0.2% sucrose at 30 °C with constant aeration by shaking at 120 rpm (Casida, 1992). Following a 48-h incubation (2×10^9 colony forming units/ml), the cells were pelleted by centrifugation ($5000 \times g$ for 30 min at 4 °C) and the supernatant culture medium collected. The supernatant culture medium was filtered through a 0.45-μm pore size filter and stored at 4 °C. The dry weight of the cell-free culture supernatant was 8 mg/ml. Antimicrobial activity of the cell-free culture-filtrate was stable for up to 4 weeks.

2.3. Growth and preparation of yeast suspension

Each yeast strain was grown in a broth medium containing 1% (w/v) yeast extract (Difco, Detroit, MI), 2% (w/v) peptone (Difco), and 2% (w/v) maltose (YEPM). *S. cerevisiae* was grown at 30 °C and *C. albicans* and *C. neoformans* were grown at 37 °C all for 48 h with constant aeration by shaking at 60 rpm. The cells were harvested by centrifugation ($5000 \times g$ for 20 min at 4 °C). The supernatant broth was discarded and the cell pellet was suspended in buffered saline gelatin [BSG; 0.1% (w/v) gelatin, 0.85% (w/v) NaCl, 0.03% (w/v) KH_2PO_4, 0.06% (w/v) Na_2HPO_4] and the turbidity adjusted to equal a No. 1 McFarland standard. Yeast cell suspensions could be stored up to 2 weeks at 4 °C without losing maltase activity or becoming very sensitive to lysis.

2.4. Growth of fungi and harvest of conidia

Fungi were grown on Potato Dextrose Broth medium (Difco) containing 1.5% (w/v) agar (PDA). Conidia were produced by spreading a suspension of *A. niger* or *B. cinerea* on PDA medium and incubating the plates at 30 °C for 48 h (*A. niger*) or room temperature for 72 h (*B. cinerea*). Conidia were recovered by flooding the surface of the agar medium with 10 ml sterile distilled water and gently suspending the conidia with a glass rod. The resulting suspension was filtered once through sterile glass wool to remove the hyphae. The conidia were suspended in BSG to equal the turbidity of a No. 1 McFarland standard (Espinel-Ingroff and Kerkering, 1991; Gehrt et al., 1995; Guarro et al., 1997). Conidia suspensions could be stored 30 days at 4 °C before losing the ability to germinate and express maltase activity.

Conidia were germinated in a maltose potato infusion broth. The potato infusion was prepared by boiling 200 g peeled and quartered potatoes in 1 l of water for 30 min. After boiling, the turbid liquid was transferred to a sterile bottle. The germination medium consisted of the potato infusion containing 1% (w/v) maltose.

2.5. Lytic agents

The following agents were tested for their ability to lyse either fungi or yeast. Lyticase™, Filipin Complex™, Miconazole, β-glucuronidase, *Cytophaga* sp. lysing enzyme, Amphotericin B™, Nystatin, lysozyme, penicillin G were all obtained from Sigma (St. Louis, MO) and polyoxin D™ was obtained from Calbiochem (La Jolla, CA). All of those agents, with the exception of lysozyme and penicillin G, were expected to exhibit lytic activity against the yeast and fungi. In addition, the lytic activity of cell-free, culture-filtrates of *Pseudomonas* sp. strain 679-2, an antibiotic-producing, maltase non-producing soil isolate (Casida, 1992) was measured.

2.6. Rapid yeast lysis assay (RYLA) tube format

To 2 ml of the maltose-grown *S. cerevisiae* cell suspension in a 16 × 125 mm screw capped tube was added 0.1 ml of the test sample at different concentrations. Tubes with only the yeast suspension and only the test sample (and BSG) were included as negative controls. The suspensions were incubated at 30 °C for 30 min with aeration (120 rpm on a rotator). After 30 min incubation, 0.5 ml of the suspension was added to the filter housing of a 0.2-μm pore size Spin-X™ tube (Costar, Cambridge, MA) that contained 0.1 ml of 5 mg *p*-nitrophenyl α-D-glucopyranoside (PNPG)/ml in the filtrate collection well. The suspension was filtered by centrifugation (16,000 × *g* for 2 min), the filter housing removed, and the filtrate incubated at 37 °C. After 30 min incubation, 0.25 ml of 1 M Na_2CO_3 was added to halt the reaction and increase the intensity of the yellow color. A positive test was signaled by the appearance of the yellow color of *p*-nitrophenol. The absorbance of the yellow color in filtrates could be measured at 450 nm.

2.7. Rapid yeast lysis assay microtiter format

Samples were tested in sterile, 96-well microtiter culture tissue plates. For each analyte sample, twofold dilutions in sterile distilled water in 100 μl total volume were prepared. Following dilution, 100 μl of the maltase-induced, *S. cerevisiae* suspension was added to all wells except for control wells to measure spontaneous- or test sample-catalyzed hydrolysis of the PNPG. Another well contained only the *S. cerevisiae* cell suspension. The microtiter plate was sealed with polyethylene sealing tape and incubated at 30 °C with orbital shaking at 60 rpm. After 30 min of incubation, the film was removed and 40 μl of filter-sterilized, 4 mg PNPG/ml water was added to each well. The plate was resealed, the contents mixed, and incubated at 37 °C without agitation. After 10 min incubation, the film was removed and 60 μl of sterile, 1 M Na_2CO_3 was added to stop the reaction and enhance the yellow color of *p*-nitrophenol. The control wells were examined to ensure that negative (no hydrolysis) and positive (hydrolysis) controls reacted as expected. The lytic ability of a test sample was reported as the highest dilution of sample yielding a yellow color judged by eye.

2.8. Maltase activity of germinating conidia

Twenty-five milliliters of the potato infusion medium containing 1% (w/v) maltose in a 125-ml flask were inoculated with an *A. niger* conidial suspension to an initial concentration of 2.5×10^4 conidia/ml. The conidial suspension was incubated at 30 °C in darkness without agitation. Samples were removed at hourly intervals samples and the germinated and ungerminated conidia were collected by centrifugation (5000 × *g* for 10 min at 4 °C). The supernatant medium was discarded and the germinated conidia washed with BSG and suspended in 8 ml BSG. Two milliliters of the suspension was transferred to a 2.0-ml plastic screw cap microcentrifuge tube and 0.1 g of 0.1-mm diameter glass beads added. The germinating conidia and conidia were broken open by five 1-min periods of breakage in a Mini-Bead Beater (Bio-Spec Products, Bartlesville, OK). Unbroken cells and conidia and debris were pelleted by centrifugation (10,000 × *g* for 30 min at 4 °C) and the clear supernatant liquid collected, taking care not to collect any of the cells. Microscopic counts showed that 90% of the germinating conidia, but only 5% of ungerminated conidia were broken. Maltase activity of the broken germinating conidial lysates was measured by adding 0.1 ml of filter-sterilized, 4 mg PNPG/ml to 1 ml of the supernatant fraction of each sample and incubating at 30 °C. Tubes with only the conidial suspension, BSG, or PNPG were included as negative controls. After 2 h incubation, 0.5 ml of 1 M Na_2CO_3 was added and examined for the appearance of the

yellow color of *p*-nitrophenol by comparison to the negative controls.

2.9. Rapid fungal lysis assay (RFLA)

Twenty-five milliliters of the potato infusion containing 1% (w/v) maltose in a 125-ml flask were inoculated with the conidial suspension to an initial concentration of 2.5×10^4 conidia/ml. The conidial suspension was incubated at 30 °C in darkness without agitation. After 16 h incubation, the germinated conidia were collected by centrifugation ($8000 \times g$ for 10 min at 4 °C). The supernatant medium was discarded and the germinated conidia washed with BSG twice and suspended in 8 ml BSG.

For the lysis test, 1 ml of the germinating conidia suspension was added to 1 ml of the test sample or diluted test sample in sterile 16×125 mm screw capped tubes. Controls containing only the germinating conidia, the test sample, or BSG were included. Tubes were incubated at 30 °C on a rotator at 60 rpm with tubes in the horizontal position. After 2 h incubation, 1 ml of filter-sterilized, 4 mg PNPG/ml was added and incubation continued under the same conditions. After 30 min incubation, 1.4 ml of sterile 1 M Na_2CO_3 was added to each tube to stop the reaction and increase the yellow color of *p*-nitrophenol. The control tubes were examined to ensure that negative (no hydrolysis) and positive (hydrolysis) controls reacted as expected. The lytic ability of a test sample or dilution was reported as the highest dilution of test sample yielding a yellow color.

3. Results

3.1. Choice of enzyme and production of maltose-grown yeast cells

A survey of the intracellular enzymes produced by *Saccharomyces*, *Candida*, and *Cryptococcus* showed that maltase (α-glucosidase) was produced by all three. Further, maltose was not metabolized by *Pseudomonas* strain 679-2. In addition, a number of colorimetric substrates of maltase (e.g., *p*-nitrophenyl-α-D-glucoside, PNPG) were commercially available. A preliminary experiment established that maltase activity was higher in *S. cerevisiae* cells grown in the yeast extract-peptone medium containing maltose (YEPM) compared to cells grown in the yeast extract-peptone medium containing either glucose or glucose and maltose (data not shown). Based on that result, cells of all three yeast were grown in YEPM medium. YEPM-grown cells, harvested by centrifugation and suspended in buffer, could be refrigerated up to 2 weeks without any loss of maltase activity and performance in the RYLA.

3.2. The rapid yeast lysis assay

The procedure for the RYLA is listed in Table 1. To identify the parameters affecting performance of the RYLA, *S. cerevisiae* was used as the target indicator and cell-free culture filtrates of *Pseudomonas* strain 679-2 were used as the lytic agent. This strain was quite effective in lysing yeast cells (Casida, 1992). The turbidity of the yeast cell suspension, the length of incubation in the presence or absence of the putative lytic agent, the temperature of incubation, and the level of aeration influenced the performance of the RYLA. The turbidity of the *S. cerevisiae* cell suspension had to be at least equal to a No. 1 McFarland standard (Table 2). Use of a suspension whose turbidity was higher than that of a No. 1 McFarland standard led to enzyme activity in the absence of lytic agent or false positive results (Table 2). Thirty min incubation of the *S. cerevisiae* cell suspension and lytic agent were required for reproducible lysis (Table 2). False positive results were also obtained if the length of incubation of the yeast suspension in the absence of any lytic agent was carried beyond 60 min (i.e., 90 and 120 min, Table 2).

Table 1
Protocol for the rapid yeast lysis assay (RYLA)

Step	Action
1	Add 0.1 ml of test sample to 2 ml maltase-induced yeast suspension in 16×125 mm screw capped tube (controls: yeast only and test sample only)
2	Incubate at 30 °C in horizontal position at 120 rpm for 30 min
3	Transfer 0.1 ml of 4 mg PNPG/ml to the collection well of a Spin-X tube and insert filter unit
4	Transfer 0.5 ml of each reaction to the filter housing of a Spin-X tube containing PNPG
5	Centrifuge at $16{,}000 \times g$ for 2 min and remove filter housing
6	Incubate Spin-X tube at 37 °C for 30 min
7	Inspect tubes for yellow color or measure absorbance at 450 nm

Table 2
Parameters influencing the rapid yeast lysis assay (RYLA)

Parameter level tested	Maltase released[a]			
	+679-2		−679-2	
	Number	Positive	Number	Positive
Turbidity of maltose-grown yeast cell suspension				
McFarland 0.5	4	2	4	0
McFarland 1	4	4	4	0
McFarland 2	4	4	4	2
McFarland 5	4	4	4	4
Duration of incubation with lytic agent				
0 min	3	0	2	0
30 min	4	3	4	0
60 min	4	4	4	0
90 min	4	3	4	2
120 min	2	2	2	2
Aeration of yeast cell and lytic agent mixture				
No aeration	3	0	2	0
Aeration	5	5	4	0

[a] Presence (+ 679-2) or absence (− 679-2) cell-free culture filtrate.

The temperature of incubation of the yeast cell suspension alone or with the lytic agent could be carried out at either 30 or 37 °C. Aeration of the yeast cell suspension was required to obtain reproducible assays. Incubation of the yeast cells suspension without aeration resulted in false-negative assays (Table 2), unless incubation was prolonged to 2–3 h, but at that point negative controls containing yeast cells alone were often positive. Addition of 0.25 ml of sterile 1 M Na_2CO_3 to a 2 ml reaction mixture was sufficient to prevent PNPG hydrolysis (data not shown).

Two approaches can be employed for detection of lytic activity. First, the maltase substrate, PNPG, can be added to control and test mixtures and appearance of the yellow color of *p*-nitrophenol scored by eye after a suitable incubation period at 30 to 37 °C. Depending on the concentration of lytic agent, that incubation period could be as short as 15 min or as long at 60 min. Because of the presence of maltase-containing yeast cells, incubation of the suspension beyond 60 min resulted in the appearance of yellow color in the yeast-only, negative control (Table 2). Thus, that approach limited the sensitivity of detection. Alternatively as listed in Table 1, the reaction mixtures could be filtered and the yeast cell-free filtrate mixed with PNPG and the appearance of yellow color scored by eye or by measuring the absorbance at 440 nm. Measurement of absorbance coupled with knowledge of the amount of lytic agent in each reaction would permit quantitative comparison of lytic activities of different compounds. Following the procedure listed in Table 1 and using cell-free culture-filtrates of strain 679-2 as the lytic agent, the RYLA demonstrated release of maltase from suspensions of both *C. albicans* and *C. neoformans* within 60 min.

3.3. The rapid yeast lysis assay: microtiter format

The RYLA was modified for use in the microtiter format (Raposo et al., 1995), using cell-free culture-filtrates of strain 679-2 and *S. cerevisiae* cell suspensions. Because the microtiter format did not include a filtration step, lytic activity could only be detected by eye. However, relatively accurate estimations of the activity of a putative lytic agent were possible because twofold dilutions of the lytic agent could be made in the 96-well format. Those factors influencing the performance of the RYLA in the test tube format also affected the results in the microtiter format. If yeast cell suspensions whose turbidity was less than a 1.0 McFarland standard were used, there was insufficient yellow color to distinguish between the negative control and test samples. Use of suspensions whose turbidity was greater than a 1.0 McFarland standard led to the appearance of yellow color in the yeast cell-only, negative control (false positive). Agitation was also required for reproducible and rapid detection of lytic activity using the RYLA in the microtiter format. The contents of the wells were agitated during the incubation of cells and lytic agent. High levels of agitation were possible if the tops of each well were sealed, though care was required when the sealing material was removed. Following the procedure described in the Materials and Methods, maltase activity was detected in suspensions of either *C. albicans* or *C. neoformans* incubated in the presence of cell-free culture-filtrates of strain 679-2 within 60 min.

3.4. Demonstration that the RYLA measures lysis

To confirm that the RYLA could be used to identify lytic agents of yeast, a number of lytic agents were tested against *S. cerevisiae* in the microtiter

Table 3
Lysis and release of maltase by *S. cerevisiae* in the presence of lytic agents

Agent	Concentration (mg/ml)	Incubation (min)	RYLA[a]	Lysis[b]
Lyticase™	1	20	+	+
	0.0005	60	+	+
β-Glucuronidase	10	30	+	+
	0.04	60	+	+
Cytophaga sp.	1	120	−	−
Lysozyme	10	60	−	−
Amphotericin B™	10	60	−	−
Nystatin	10	60	−	−
Filipin Complex™	0.1	20	+	+
Miconazole	1.0	20	+	+
	0.006	30	+	+
Polyoxin D™	0.2	120	−	−
Penicillin	1	60	−	−
		120	−	−
679-2 Culture Filtrate	8	30	+	+

[a] Appearance of yellow color in wells.
[b] Microscopic evidence of lysis.

format. Yeast cell suspensions were also examined by phase contrast microscopy for evidence of lysis. Enzymes and antibiotics expected to lyse yeast were included. Two agents, lysozyme and penicillin, that would not be expected to lyse yeast were included as negative controls. The results (Table 3) demonstrated that the enzyme preparation Lyticase™ and the enzyme β-glucuronidase were effective lytic agents and led to release of maltase from *S. cerevisiae* cells within 30 min. The experiments were not designed to identify the lowest concentration of agent resulting in release of maltase. Neither the *Cytophaga* sp. enzyme preparation, nor lysozyme resulted in release of maltase activity. Among the antibiotics, Miconazole and Filipin Complex™ resulted in release of maltase within relatively short periods of time (Table 3). Amphotericin B™, Nystatin, and Polyoxin D, like penicillin, failed to induce release of maltase (Table 3). Though it was expected that the *Cytophaga* sp. enzyme preparation, Amphotericin B™, Nystatin, and Polyoxin D would induce lysis, or at least leakage of cellular macromolecules, none did within the parameters of the assay. The only microscopic evidence of lysis was observed in reactions where maltase activity was detected (Table 3). Longer periods of incubation or higher concentrations might have uncovered lytic activity. However, longer periods of incubation would result in the appearance of activity in the yeast cell suspension itself (false positive) and higher concentrations would not be relevant to the utility of the antibiotics in clinical practice.

3.5. Choice of enzyme and production of maltose-grown germinating conidia

Maltase (α-glucosidase) is produced by many common saprophytic and pathogenic filamentous fungi, including *A. niger* and *B. cinerea*. Rather than use mycelia harvested from cultures of filamentous fungi growing in medium containing maltose, germinating conidia were chosen as the target for lytic activity. Uniform mycelial suspensions are difficult to prepare, whereas conidial suspensions can be prepared and standardized by turbidity and are preferred for susceptibility measurements (Espinel-Ingroff and Kerkering, 1991; Gehrt et al., 1995; Guarro et al., 1997). Before suspensions of germinating conidia could be incorporated into a lysis assay, the period of incubation required for the appearance of enough detectable maltase activity was identified. Accordingly, *A. niger* conidia were inoculated into potato-infusion containing 1% maltose, incubated at 30 °C, and samples withdrawn at different incubation periods and the maltase activity of lysates measured after 2 h incubation. Violent agitation in the presence of glass beads was used to break open germinating *A. niger* conidia and release intracellular maltase. The results showed that germination of conidia was complete by 15 h, which also coincided with the appearance of detectable maltase activity of lysates within the 2 h incubation

Table 4
Germination and maltase activity of germinating conidia of *A. niger*

Length of incubation (h)	Percent germination[a]	Maltase activity[b]
0	0	none
1	0	none
3	0	none
5	5	none
7	10	none
10	20	±
12	50	+
15	100	++
24	100	++

[a] Percent conidia with outgrowing hyphae.
[b] Maltase activity in 2-h incubation: ±, slight yellow, + yellow, ++, bright yellow.

period (Table 4). For the RFLA, conidia were incubated 16 h at 30 °C because it could be conveniently carried out by overnight incubation and the level of maltase activity was sufficient for detection of lytic activity. The same length and conditions of incubation for *B. cinerea* conidia resulted in a suspension of germinating conidia with sufficient maltase activity to detect lytic activity of cell-free culture-filtrates of strain 679-2 (data not shown).

3.6. The rapid fungus lysis assay

The procedure for the RFLA is listed in Table 5. To identify the parameters affecting performance of the RFLA, *A. niger* was used as the target indicator and cell-free culture filtrates of *Pseudomonas* strain 679-2 were used as the lytic agent because of its ability to lyse germinating conidia and hyphae of fungi (Casida, 1992). Variables influencing the result included (1) the pH of the suspension, (2) the turbidity of the suspension of germinating conidia, (3) the length of incubation in the presence or absence of the putative lytic agent, and (4) the level of aeration. Initial experiments in which the pH of the conidial suspension, medium, and reaction mixtures were not controlled led to negative results. Only when pH was maintained between 5 and 6 did conidia germinate and lysis occur. In addition, a pH of 5–6 was required for activity of the fungal maltase. Employment of a suspension whose turbidity was higher than that of a No. 1 McFarland standard led to false positive results (enzyme activity in the absence of lytic agent). False positive results were also obtained if the length of incubation of the germinating conidia suspension in the absence of any lytic agent was carried beyond 60 min. Aeration of both the germinating conidia and the reaction mixtures was required to obtain reproducible assays. Incubation of the conidia without aeration led to poor yields of germinating conidia and low maltase activities. Incubation of the germinated conidia and cell-free culture filtrates of *Pseudomonas* strain 679-2 or other lytic agents without aeration resulted in false-negative assays, unless incubation was prolonged to 2–3 h. However, prolonged incubation led to the appearance of maltase activity in the germinated conidia incubated in the absence of lytic agent. The temperature of incubation was not critical. Positive and reproducible results were obtained over a range of 22 to 30 °C. Higher temperatures were not investigated. Suspensions of germinating *B. cinerea* conidia could also be employed in the RFLA without any modification of the protocol.

Like the RYLA, two approaches could be employed for detection of lytic activity. The maltase substrate, PNPG, could be added to either the reaction mixtures or to filtrates. Because of the turbidity and color of suspensions of germinated conidia of *A. niger*, it was difficult to discern low levels of activity. Thus, filtrates offered the advantage of removal of background turbidity and color as well as the opportunity to measure activity levels spectrophotometrically. Attempts to adapt the RFLA to the microtiter format were unsuccessful because there was insufficient maltase activity in the small numbers of germinated conidia able to be present in a microtiter well to detect maltase activity. Although concentrated germinated conidia could be added, their turbidity and color obscured any appearance of yellow color.

Table 5
Protocol for the rapid fungus lysis assay (RFLA)

Step	Action
1	Inoculate 25 ml of 1% maltose potato infusion in a 125-ml flask with 6.25×10^5 conidia and incubate at 30 °C for 16 h in darkness without agitation
2	Collect germinating conidia by centrifugation ($8000 \times g$ for 10 min), discard supernatant medium and wash twice in BSG and suspend in 8 ml BSG
3	Add 1 ml of test sample or dilution to 1 ml maltase-induced, germinating conidia in 16×125 mm screw capped tube (controls: conidia only and test sample only)
4	Incubate at 30 °C in horizontal position at 620 rpm for 120 min
5	Add 1 ml of 4 mg PNPG/ml to each tube and incubate at 30 °C for 30 min
6	Inspect tubes for yellow color

4. Discussion

The rapid yeast and fungus lysis assays described here offer the opportunity for development of automated tests for screening a variety of compounds (Raposo et al., 1995). The RYLA appears to identify those agents that resulted in microscopic evidence of lysis of yeast cells (Table 3). Further, the RYLA and RFLA are relatively resistant to changes in experimental param-

eters. As has been reported by others (Espinel-Ingroff and Kerkering, 1991; Gehrt et al., 1995; Guarro et al., 1997; Klepser et al., 1998), the inoculum size and aeration influence the results of antifungal susceptibility measurements. Inocula higher than that described result in appearance of maltase activity in the reactions containing only *S. cerevisiae* cells or germinating *A. niger* conidia (false positive). Too few cells resulted in false negative results because of insufficient enzyme. Aeration was found to be required for detection of activity and a pH between 5 and 6 was required for germination of conidia and detection of maltase activity.

The tests can be used qualitatively or quantitatively. Quantitative measurement of lytic activity requires a filtration step. Although the RYLA in the microtiter format described here is qualitative (i.e., color read by eye), filtration of the contents of microtiter wells could be added to the microtiter protocol to allow quantitative measurement of maltase activity. The RYLA and RFLA appear to have broad applicability to screening for lytic anti-yeast or anti-fungal agents. Although the RYLA was developed primarily using *S. cerevisiae*, the pathogens *C. albicans* and *C. neoformans* served equally well as test organisms. Likewise, the RFLA could be employed for detection of lytic agents against either *A. niger* or *B. cinerea*.

The RYLA and RFLA are limited by the fact that they are capable of only detecting lytic agents. The lowest concentration of agent whose activity was detectable was limited by the fact that either yeast or fungal cells alone or the PNPG substrate yielded a positive reaction (i.e., PNPG-hydrolysis) after 4 h of incubation. Further, the assays required that the yeast or fungal strains produce maltase. However, absence of maltase is no limitation. The criteria for selection of another enzyme would be that it is intracellular, widely distributed in fungi or yeast, its substrate does not readily enter cells, and colorimetric, fluorescent, or luminescent substrates are available. Alternatively, if the RYLA or RFLA were to be used with a limited number of strains, reporter enzymes (e.g., β-galactosidase, and luciferase) could be introduced into the strains (Nevels et al., 1999).

We were surprised that the RYLA did not detect lytic activity of some of the antibiotics or enzymes that we expected to result in either lysis or leakage of intracellular contents (Table 3). For example, exposure of *S. cerevisiae* to the *Cytophaga* sp. lytic enzyme, Amphotericin B™, Nystatin, or Polyoxin D™ failed to result in the release of maltase from the yeast cells. Perhaps, the conditions of the reactions inhibited antimicrobial activity of those agents. Maltase activity could be increased by the use of a strain that produces higher levels of maltase. Further, using yeast or fungal strains that are more susceptible to lysis could increase the sensitivity of the assay. However, leakage of maltase from such mutant cells might negate their use because of the appearance of false positive reactions.

In addition to the use of the RYLA and RFLA for screening compounds or cell extracts for anti-yeast or anti-fungal activity, the assays are suitable for application to quality assurance and quality control programs. This is a consequence of their reproducibility, as shown by the fact that repeated assays of a number of different enzymes and antibiotics gave the same results (Table 2). Further, in the microtiter format, multiple agents can be screened simultaneously.

Two different problems appeared when trying to adapt the RFLA to a microtiter format. First, because of the small volumes, it was difficult to introduce a uniform and reproducible suspension of germinating conidia from the flask into a microtiter well. Second, there was an insufficient number of germinating conidia in each microtiter well to yield sufficient maltase activity upon lysis. The maltase activity of yeast cells was significantly higher than that attained by the germinating conidia. Our present efforts are focused on overcoming those two problems.

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References

Casida Jr., L.E., 1992. Competitive ability and survival in soil of *Pseudomonas* strain 679-2, a dominant, nonobligate bacterial predator of bacteria. Appl. Environ. Microbiol. 58, 32–37.

Espinel-Ingroff, A., Kerkering, T.M., 1991. Spectrophotometric method of inoculum preparation for the in vitro susceptibility testing of filamentous fungi. J. Clin. Microbiol. 29, 393–394.

Gehrt, A., Peter, J., Pizzo, P.A., Walsh, T.J., 1995. Effect of increasing inoculum sizes of pathogenic filamentous fungi on MICs of antifungal agents by broth microdilution method. J. Clin. Microbiol. 33, 1302–1307.

Georgopapadakou, N.H., Walsh, T.J., 1994. Human mycoses: drugs and targets for emerging pathogens. Science 264, 371–372.

Guarro, J., Llop, C., Aguilar, C., Pujol, I., 1997. Comparison of in vitro antifungal susceptibilities of conidia and hyphae of filamentous fungi. Antimicrob. Agents Chemother. 41, 2760–2762.

Klepser, M.E., Ernst, E.J., Lewis, R.E., Ernst, M.E., Pfaller, M.A., 1998. Influence of test conditions on antifungal time-kill curve results: proposal for standardized methods. J. Clin. Microbiol. 42, 1207–1212.

Kurtz, M.B., 1999. New antifungal drug targets: a vision for the future. ASM News 64, 31–39.

Nevels, M., Wolf, H., Dobner, T., 1999. Quantitative of β-galactosidase from yeast cell using a chemiluminescent substrate. BioTechniques 26, 57–58.

Niehaus, W.G., Flynn, T., 1994. Regulation of mannitol biosynthesis and degradation by *Cryptococcus neoformans*. J. Bacteriol. 176, 651–655.

Raposo, R., Colgan, R., Delcan, J., Melgarejo, P., 1995. Application of an automated quantitative method to determine fungicide resistance in *Botrytis cinerea*. Plant Dis. 79, 294–296.

Stanek, J.E., Falkinham III, J.O., 2001. Rapid coliphage detection assay. J. Virol. Methods 91, 93–98.