**Experimental Protocol: Susceptibility of Wangiella dermatitidis to Various Types of Antifungal Agents**

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**Purpose:** To determine the concentrations of Calcofluor White (CFW), Congo Red (CR), Bleomycin (BLE), and Hygromycin (HYG) that inhibit the growth of *Wangiella dermatitidis*

**Required Reagents/Materials:**
- Stock culture of *Wangiella dermatitidis* (ATCC 34100) [YPD agar slant]
- YPD agar plates
- YPD broth (50 ml minimum)
- Sterile, wooden transfer sticks
- Sterile, 250-ml screw-capped flask (2)
- Sterile, 50-ml conical centrifuge tube
- Phosphate buffered saline, pH 7.4 (sterile)
- YPD agar plates containing CFW, CR, BLE, or HYG at the following concentrations: 200, 100, 50, 25, 12.5, 6.3, 3.2, 1.6, and 0 µg/ml.

**Equipment Required (Procedure Specific):**
- Hemocytometer and cover slip
- YPD agar plates containing CFW, CR, BLE, or HYG at the following concentrations: 200, 100, 50, 25, 12.5, 6.3, 3.2, 1.6, and 0 µg/ml.

**Procedure:**

**Day 1**

1. From the stock culture of *Wangiella dermatitidis* (ATCC 34100), use a sterile wooden transfer stick to streak a plate of YPD agar for isolated colonies. Incubate the plate at 25°C for 3-5 days.

2. Select an isolated colony of *W. dermatitidis* from the YPD agar plate and transfer it to 25 ml of YPD broth contained in a 250 ml flask. Incubate the flask in a shaking water bath (operating at 150 rpm) at 25°C for 72 hours.

**Day 4**

3. Transfer 0.5 ml of the *W. dermatitidis* broth culture prepared in Step 2 to 25 ml of YPD broth contained in a 250 ml flask. Incubate the flask in a shaking water bath (operating at 150 rpm) at 25°C for 24 hours.

**Day 5**

4. Transfer 10 ml of the above culture to a sterile 50-ml conical centrifuge tube. Place the tube in a clinical centrifuge operating at 3,500 x g for 10 minutes at room temperature.

5. Carefully decant the supernatant into an appropriate waste container, then suspend the cell pellet in 5 ml of phosphate buffered saline (pH 7.4; PBS). Mix well using a vortex operating a high speed.
6. From the tube in Step 5 (above), remove 100 µl and transfer this to a 1.5 ml microfuge tube containing 900 µl phosphate buffered saline (pH 7.4; PBS). This tube effectively represents a 1 x 10^{-12} dilution of the cell suspension prepared in Step 5.

7. Transfer 100 µl of the cell suspension in the microfuge tube (Step 6) to a second 1.5 ml microfuge tube containing 900 µl of PBS. Mix well using a vortex operating a high speed. This tube effectively represents a 1 x 10^{2} dilution of the cell suspension prepared in Step 5.

8. Using a counting chamber (e.g., hemocytometer) and the cell suspensions prepared in Steps 6 and 7, determine the concentration of the cell suspension prepared in Step 5. **NOTE:** If you are unfamiliar with using a hemocytometer, see the resource “Using a Counting Chamber”

9. In a third microfuge tube, and using the PBS-suspended cells in Step 5, prepare a 1 ml suspension of *W. dermatitidis* at a concentration of 1 x 10^{8} cells/ml. **SPECIAL NOTE:** This will require some elementary mathematics using the traditional formula $C_1 V_1 = C_2 V_2$. As an example, suppose that you calculated that the suspension Step 5 contained 4 x 10^{9} cells per ml. This would be considered "C_1." You want a *final* concentration ($C_2$) of 1 x 10^{8} cells/ml at a *final* volume of 1.0 ml ($V_2$). Hence, you wish to calculate how much of the suspension from Step 5 ($V_1$) you will need to bring to a total volume of 1.0 ml to achieve this concentration. Apply the formula as follows:

$$C_1 V_1 = C_2 V_2$$

$$(4 \times 10^9 \text{ cells/ml}) \times (V_1 \ [\text{in ml}]) = (1 \times 10^8 \text{ cells/ml}) \times (1.0 \text{ ml})$$

$$V_1 \ [\text{in ml}] = \frac{(1 \times 10^8 \text{ cells/ml}) \times (1.0 \text{ ml})}{4 \times 10^9 \text{ cells/ml}}$$

$$V_1 \ [\text{in ml}] = 1 \times 10^8 \text{ cells/} 4 \times 10^9 \text{ cells/ml}$$

$$V_1 \ [\text{in ml}] = 1/4 \times 10^{-1} \text{ ml}$$

$$V_1 \ [\text{in ml}] = 0.025 \text{ ml or 25 µl}$$

In other words, take 25 µl of the suspension from Step 5 and add it to 975 µl PBS (for a total volume of 1.0 ml (= 1000 µl) to obtain a final cell concentration of 1 x 10^{8} cells/ml.

**Not convinced or unsure?** Well, multiply 0.025 (calculated $V_1$) by 4 x 10^{9} (suspension concentration from Step 5). What is the answer? It is 0.1 x 10^{9}, or 1 x 10^{8}. Simple! Right? Well, it should be!!!

10. Transfer 100 µl of the cell suspension in the third microfuge tube (Step 9) to a fourth 1.5 ml microfuge tube containing 900 µl of PBS. Mix well using a vortex operating a high speed. This tube effectively represents a concentration of 1 x 10^{7} cells/ml.

11. Transfer 100 µl of the cell suspension in the third microfuge tube (Step 9) to a fifth 1.5 ml microfuge tube containing 900 µl of PBS. Mix well using a vortex operating a high speed. This tube effectively represents a concentration of 1 x 10^{6} cells/ml.
12. Transfer 100 µl of the cell suspension in the third microfuge tube (Step 10) to a sixth 1.5 ml microfuge tube containing 900 µl of PBS. Mix well using a vortex operating a high speed. This tube effectively represents a concentration of 1 x 10^5 cells/ml.

13. Transfer 100 µl of the cell suspension in the third microfuge tube (Step 11) to a seventh 1.5 ml microfuge tube containing 900 µl of PBS. Mix well using a vortex operating a high speed. This tube effectively represents a concentration of 1 x 10^4 cells/ml.

14. Transfer 100 µl of the cell suspension in the third microfuge tube (Step 11) to a seventh 1.5 ml microfuge tube containing 900 µl of PBS. Mix well using a vortex operating a high speed. This tube effectively represents a concentration of 1 x 10^3 cells/ml.

15. Choose four series of YPD plates containing Calcofluor White (CFW), Congo Red (CR), Bleomycin (BLE), and Hygromycin (HYG) at concentrations of 200, 100, 50, 25, 12.5, 6.3, 3.2, 1.6, and 0 µg/ml. You will need two sets of these plates. One set of each series will be incubated at 25°C and the other at 37°C. Label these plates appropriately.

16. To each plate in the series, carefully pipet 10 µl of the cell suspensions prepared in Steps 9 through 14 to separate positions on the plate as shown in the figure below. Be sure to mix each cell suspension well using a vortex prior to pipetting the 10µl aliquot to each plate. Also, be certain to mark the proper orientation of your plates (black arrow in the above figure) so as not to confuse the position of the cell suspension spots.

1 x 10^6 (= 1 X 10^6 cells) [suspension from Step 9]  
1 x 10^7 (= 1 X 10^6 cells) [suspension from Step 10]  
1 x 10^6 (= 1 X 10^4 cells) [suspension from Step 11]  
1 x 10^5 (= 1 X 10^3 cells) [suspension from Step 12]  
1 x 10^4 (= 1 X 10^2 cells) [suspension from Step 13]  
1 x 10^3 (= 1 X 10^1 cells) [suspension from Step 14]

17. Allow each of the spots on the plates to dry before incubating one set of each series at 25°C and the other at 37°C for 3 days. Be sure to incubate the plates “bottom side up”, i.e., inverted.

Days 8-10

18. Record the relative colony growth on each of the plates after 3, 4, 5, 6, and 7 days of incubation. Determine the lowest concentration of each antifungal that completely inhibits colony formation (MIC, minimum inhibitory concentration) and the highest concentration at which none or only very slight inhibition of colony growth occurs (HIC, highest inhibitory concentration). NOTE: When observing the plates, do so quickly. Place them back into the incubators as soon as possible so that temperature fluctuations are kept to a minimum.