

Screening for Antibacterial, Antifungal, and Anti quorum Sensing Activity

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Abstract

The plate-hole diffusion assay is an invaluable screening tool to evaluate the antibacterial potential of natural products. It relies on the diffusion of test material from pre-cut wells through agar seeded with bacteria. Samples that are capable of inhibiting bacterial growth will produce a clear zone surrounding their well. For the evaluation of antifungal activity of natural products, we describe the broth microdilution method. This assay is performed using a 96 well microtiter tray containing fungal inoculum, test medium and natural product material. Samples demonstrating antifungal activity will prevent any discernible growth as detected visually. A disk diffusion assay, utilizing the pigmented indicator strain *Chromobacterium violaceum*, is described here for the screening of natural products for anti quorum sensing activity. Inhibition of quorum sensing results in growth of non-pigmented bacteria.

Key words Antimicrobial, Antibacterial, Antifungal, Anti quorum sensing, Screening, Disk diffusion, Plate-hole diffusion, Broth microdilution, Natural product, Extracts

1 Introduction

There are several methods reported in the literature to evaluate antibacterial activity of natural products. They involve either introducing the test compounds to a finite area of set agar (disk diffusion [1] and plate-hole diffusion assay [2]) or incorporating the test compounds into the molten agar or liquid media (broth [3] and agar dilution [4] assays). The advantage of using the diffusion methods for screening is that they require only a small volume of test material and the color or opaqueness of a sample will not interfere with interpretation of results. The plate-hole diffusion assay involves the inoculation of molten agar with test bacteria which is then set in a petri plate. Wells are cut into the agar which are then filled with the natural product material and suitable controls. Following incubation, zones of inhibited bacterial growth surround wells containing antibacterial compounds. The diameter of this inhibition zone is

affected by the rate of diffusion of the test material through the agar and therefore serves only as a qualitative interpretive result [5]. However, it is still an effective method for screening processes.

Broth dilution methods, rather than agar-based methodology, are considered the most reliable for the screening of natural products for antifungal activity [3, 6]. Here, we describe the broth microdilution method [3, 7]. This assay uses a 96-well microtiter tray which allows several samples and/or fungal isolates to be screened simultaneously. The procedure first involves the preparation of a conidial suspension from the test fungi containing approximately 10^5 conidia per milliliter. This inoculum is then used to prepare the microtiter tray with the test material. Samples demonstrating antifungal activity will prevent any discernible growth as detected visually.

One approach to bacterial inhibition that is gaining interest in the field of natural products research is inhibition of quorum sensing (QS). Multicellular behaviors controlled by QS include swarming, bioluminescence, and biofilm formation which are often involved in bacterial pathogenesis [8]. Therefore, QS inhibitors (QSI) can potentially reduce the virulence of bacteria without necessarily affecting their growth. This is a significant advantage compared with conventional “killing” chemotherapeutics as it is less likely to result in changes to the host microbiota and the development of resistance to treatments [9, 10]. Anti quorum sensing (AQS) activity has been reported in samples from natural product sources such as plant essential oils and solvent extracts [8, 11–13], and fungal secondary metabolites [9, 14]. The method detailed here is based on the antibiotic susceptibility method, described in 1966 [1], and takes advantage of the indicator strain *Chromobacterium violaceum* which produces a purple pigment (violacein) under *N*-acylhomoserine lactone mediated QS control [12, 15].

2 Materials

2.1 Antibacterial Assay: Plate-Hole Diffusion

1. Mueller Hinton agar (MHA) (Becton, Dickinson and Company, Sparks, MD, USA).
2. Sterile petri dishes, 9 cm diameter.
3. Overnight cultures of test bacteria, adjusted to 1×10^8 CFU/mL (0.5 McFarland standard).
4. Cork borer (*see Note 1*).
5. Natural product test material (*see Note 2*).

2.2 Antifungal Assay: Broth Microdilution

1. Filamentous fungi for susceptibility testing (*see Note 3*).
2. Sterile loop.

3. Sterile saline.
4. Potato Dextrose Broth (PDB).
5. Sterile 96 U-shaped well microtiter trays.
6. Natural product test material.
7. Antifungal agent such as amphotericin B.

2.3 Anti quorum Sensing Assay: Disk Diffusion Assay

1. Petri plates containing 15 mL of Mueller Hinton Agar.
2. Overnight cultures of *Chromobacterium violaceum* (UNSW collection 040100), adjusted to 1×10^8 CFU/mL (0.5 McFarland standard).
3. Natural product test material.
4. Sterile filter paper disks, 6 mm diameter.
5. Glass spreader.
6. Forceps.

3 Methods

3.1 Plate-Hole Diffusion

1. Prepare MHA in purified water as per the manufacturer's directions.
2. Heat the solution with frequent agitation and boil for 1 min to ensure that the powder has completely dissolved.
3. Decant the solution into small glass bottles [15 mL per petri dish (*see Note 4*)] and sterilize by autoclave (121 °C, 15 min).
4. Allow agar to cool to approximately 45 °C (*see Note 5*).
5. Aseptically inoculate each aliquot with 150 µL of bacterial suspension.
6. Mix the contents by gently inverting the bottle four times and then pour each into a sterile petri dish.
7. Allow the plates to set for 5–10 min and then use the cork borer to stamp wells into the agar (*see Note 6*).
8. Remove and dispose of the agar plugs (*see Note 7*). Label each well.
9. Pipette 20 µL of test material and controls into each well (*see Note 8*). Leave the plates at room temperature for 30 min to allow evaporation of any solvents and pre-diffusion of material into the agar.
10. Incubate plates according to test bacteria requirements (*see Note 9*).
11. Record the diameter of zone of inhibition from the underside of the plate with a ruler or sliding calipers (*see Note 10*).

Table 1
Example of variation in optical density and correlating inoculum sizes
between fungi [3, 16]

Species	OD ₅₃₀ range	10 ⁶ CFU/mL range
<i>Aspergillus niger</i>	0.20–0.50	0.5–4.5
<i>A. fumigatus</i>	0.09–0.11	0.6–5
<i>Fusarium oxysporum</i>	0.15–0.17	0.8–5
<i>Scedosporium apiospermum</i>	0.15–0.17	0.4–3.2

3.2 Broth Microdilution

1. Flood the PDA plate with 1 mL of sterile saline. Using the loop, lightly probe the surface of the mycelia to introduce conidia (and hyphal fragments) into the solution.
2. Transfer the solution to a sterile tube and vigorously vortex the suspension for at least 1 min to produce a homogenous solution.
3. Allow it to stand for 3 min and then transfer the upper portion of liquid to a sterile tube.
4. Using a spectrophotometer, adjust to the optical density that equates to a stock solution of $0.4\text{--}5 \times 10^6$ viable conidia or sporangiospores per milliliter (CFU/mL) (*see Note 11*) (Table 1).
5. Dilute the fungal suspension 1:50 in PDB to create a $0.8\text{--}1 \times 10^5$ CFU/mL range.
6. Fill microtiter tray wells with 100 μL of fungal suspension and 100 μL of natural product test material.
7. Growth control (GC) wells contain 100 μL of fungal suspension and 100 μL of distilled water (*see Note 12*).
8. Add the antifungal agent to the fungal suspension for a positive control and the test material solvent to the fungal suspension for a negative control.
9. Incubate the tray without agitation at 35 °C for up to 70 h, checking for visible growth in the GC wells after 24 and 48 h.
10. Natural product material demonstrating antifungal activity will prevent any discernible growth of the fungi.

3.3 Disk Diffusion Assay

1. Add 20 μL of each test material to a sterile paper disk (*see Note 13*).
2. A tetracycline or gentamycin disk (10 μg per disk) can be used to compare AQS activity with antibiotic effect. Purified halogenated furanone can be used as a positive control for AQS activity.
3. Allow the disks to dry for 10 min.
4. Using a sterile glass spreader evenly spread 150 μL of *C. violaceum* onto the surface of a petri plate.

5. Allow the inoculated plate to dry for 5 min.
6. Using sterile forceps, place each disk on the surface of the agar. Gently press the disk with the forceps to ensure complete contact with the agar (*see* **Note 14**).
7. Leave the plates at room temperature for 30 min to allow pre-diffusion of material into the agar.
8. Incubate the agar plate at 30 °C overnight.
9. Inspect the bacterial growth surrounding each disk (*see* **Note 15**).

4 Notes

1. A cork borer is a hollow metal tube that is often used in chemistry labs to bore holes in cork or rubber stoppers for the insertion of tubing. Choose a cork borer with a diameter of 5–7 mm and use this same tool for all subsequent assays.
2. Test material may be in the form of a crude extract, a separated fraction or a purified compound. If required, concentrate the material (via lyophilization or rotary/centrifugal evaporation) or dilute (in a carrier solvent) prior to testing.
3. The fungal suspension is prepared from cultures of filamentous fungi grown on PDA plates. Incubation conditions will depend on the species chosen. For example, *Aspergillus* spp. will generally produce conidia after 7 days growth at 35 °C.
4. We have found that McCartney bottles (28 mL volume) are an appropriate vessel for these aliquots.
5. If the temperature of the agar is too high when you add the bacterial suspension, you will risk damage to the cells. If the temperature is too low, the agar will begin to solidify before it is poured into the plate, which will result in very uneven growth. Place the bottles into a 45 °C water bath following autoclave and allow them to cool. Bacteria must, therefore, be heat stable at 45 °C.
6. When stamping wells into the agar, make sure that the cork borer comes straight down into the agar, not on an angle. Sterilize the cork borer before each plate assay by swilling the end in 75 % (v/v) ethanol, shaking off the excess liquid and placing over a Bunsen burner flame. Make sure that your finger is not covering the open hole at the top of the tool. Allow the cork borer to cool before using again to ensure a clean well is cut in the agar.
7. With one hand, open the lid of the petri plate from the far side. The lid will serve as protection in case you flick the agar plug (containing bacteria) towards yourself. With the other hand, use a wire loop to carefully lift each agar plug from the plate. Dispose of as contaminated culture media.

8. Use the test material solvent as a negative control. A standard antimicrobial agent such as ampicillin can be used as the positive control.
9. Incubation conditions must be kept consistent for subsequent assays. Do not stack more than five plates together, to ensure that all assays reach the incubation temperature at approximately the same time.
10. A clear zone surrounding the well signifies antibacterial activity by the test material. Keep in mind that this assay is a screening method only and that for a quantitative evaluation of antibacterial activity, concentration dependent methods will need to be carried out.
11. Due to the variation in spore sizes between species, an optical density value will equate to different inoculation densities (CFU/mL) depending on the fungal species chosen.
12. The growth control well/s confirms that the fungal isolate is viable and demonstrates 100 % growth of the isolate for comparison with test wells after incubation.
13. Keep disks in an empty sterile petri plate that is labelled to avoid confusion between different test materials.
14. Avoid placing disks closer than 20 mm together (center to center) as zones may overlap. Do not place disks too close to the edge of the petri plate. Once in contact with the agar, do not move disks (even if they have been placed in the wrong position) as the test material will have already started diffusing into the agar.
15. Magnification of the agar surface with an adequate light source will help to differentiate between colorless growth (indicating interruption of quorum sensing), purple growth (no effect), and no growth (indicating antibacterial activity).

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