Measuring fungal anti-E. coli activity using the zone of inhibition (ZOI) assay

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We use this protocol and it's working

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ABSTRACT

In this protocol, we describe our use of the zone of inhibition assay to assess the anti-*E. coli* activity of fungi grown on different media, including Czapek Solution Agar (CSA), Czapek Yeast Extract Agar (CYA), Malt Extract Agar (MEA), Oatmeal Agar (OA), Potato Dextrose Agar (PDA), Rice Extract Agar (REA), and Water Agar (WA).

GUIDELINES

Ensure you have the appropriate biosafety approvals for the work.

MATERIALS

Plasticware

	Description	Catalogue number	Supplier
	90mm Petri Dishes	LAB-021MR	Medi'Ray
	Falcon 50mL Conical Centrifuge Tube	BDAA352070	In vitro technologies
	Technoplast 5 mL flat bottom yellow screw cap tube	S5016SU	Mediray, New Zealand
	BRAND Semi micro cuvette	BR759015	Sigma-Aldrich, New Zealand
	Sterile cotton swab 150mm	LABMC2744S	Jackson Allison Medical and Surgical Ltd
	Pipette tips		

Growth media and chemicals

	Description	Catalogue number	Supplier
	Mueller Hinton II Broth Cation Adjusted	212322	Fort Richard, New Zealand
	Difco Potato Dextrose Broth	254920	Fort Richard, New Zealand
	Difco Oatmeal Agar	255210	Fort Richard, New Zealand
	Difco Malt Extract Agar	211220	Fort Richard, New Zealand
	Difco Czapek Solution Agar	233910	Fort Richard, New Zealand
	BBL Rice Extract Agar	211567	Fort Richard, New Zealand
	Bacto Yeast Extract	212750	Fort Richard, New Zealand
	Agar, Granulated	214530	Fort Richard, New Zealand

Equipment:

- Pipettes – various sizes
- 6mm Biopsy punch (we use Paramount)
- Spectrophotometer (to measure optical density of bacterial culture)
- Sterile scalpel handle and blade
- Germinator
- Biological Safety cabinet (Herasafe KS12)
- Ruler

Initial culturing of fungus from stocks

1 Subculture fungus onto a Potato Dextrose Agar (PDA) plate from either frozen stocks or an

existing culture.

2 Seal the plate with parafilm and store it in a plastic box at room temperature. Allow fungus to reach at least 50% radial growth before subculturing onto respective media plates.

Culturing of fungus onto different media

3 Using a sterile scalpel plate, cut a small section (0.5cm) from the growing edge of the mycelium and inoculate this segment onto fresh media. In our experiments, we routinely subculture fungi onto a selection of different media.

4 Monitor radial growth of the fungus and start the ZOI assay when it reaches 20, 50, and 100% growth.

Setting up *E. coli* 25922 lux and ZOI plates

5 Inoculate 10 mL of Mueller Hinton II broth in a 15 mL tube with *E. coli* and grow overnight at 37 degrees C with shaking at 200 rpm. We use a bioluminescent derivative of the antibiotic-testing E. coli ATCC 25922 strain designated 25922 lux.

6 In these assays, we perform the ZOI on the same media the fungus has been growing on after confirming that *E. coli* 25922 lux growth is not impacted. Prepare the necessary number of plates of each media depending on how many fungal isolates, and biological and technical replicates of each isolate, you need to test. Once dry, section the back of each plate into six equal segments. Label each with the fungal isolate name/number and the date the fungus was cultured (age).

7 Measure the optical density of the overnight bacterial culture at 600nm (OD_{600}). To do this dilute overnight cultures 1:10 in a 1.5 mL cuvette with Mueller Hinton Broth (MHB) (720 µL broth + 80 µL bacteria). Dilute the bacterial culture with MHB to give a final OD_{600} of 0.01 which is equivalent of ~10^6 bacteria per mL.

8 Add bacteria to each Petri dish. This can be done by pipetting 50ul of diluted overnight bacterial culture and spreading it over the plate using an L-shaped spreader. Alternatively, dip a sterile cotton swab into the diluted bacterial solution and gently streak the agar plate half a section at a time rotating 45 degrees each time to ensure an even lawn of bacteria.

Allow plates to dry before applying fungal plugs.

Adding fungal plugs to ZOI plates

9 Use a 6mm punch biopsy to remove mycelium from the growing edge of the fungus. Place the plug fungus-side down on your labelled plate. Repeat for all biological/technical replicates.

Use a sterile punch biopsy when changing fungi to avoid cross-contamination.

Also prepare a no-fungi control by adding an agar-only plug to the bacterial lawn. Ensure the agar is the same as that the fungus was grown on.

10 Incubate the plates upside down at 37 °C in a standing incubator overnight.

ZOI measurements

11 The following day, use a ruler to measure the diameter of any zones of inhibition formed. You may need to hold some plates up to the light to get accurate measurements. A diagram of the zone of inhibition results is shown in Figure 1.

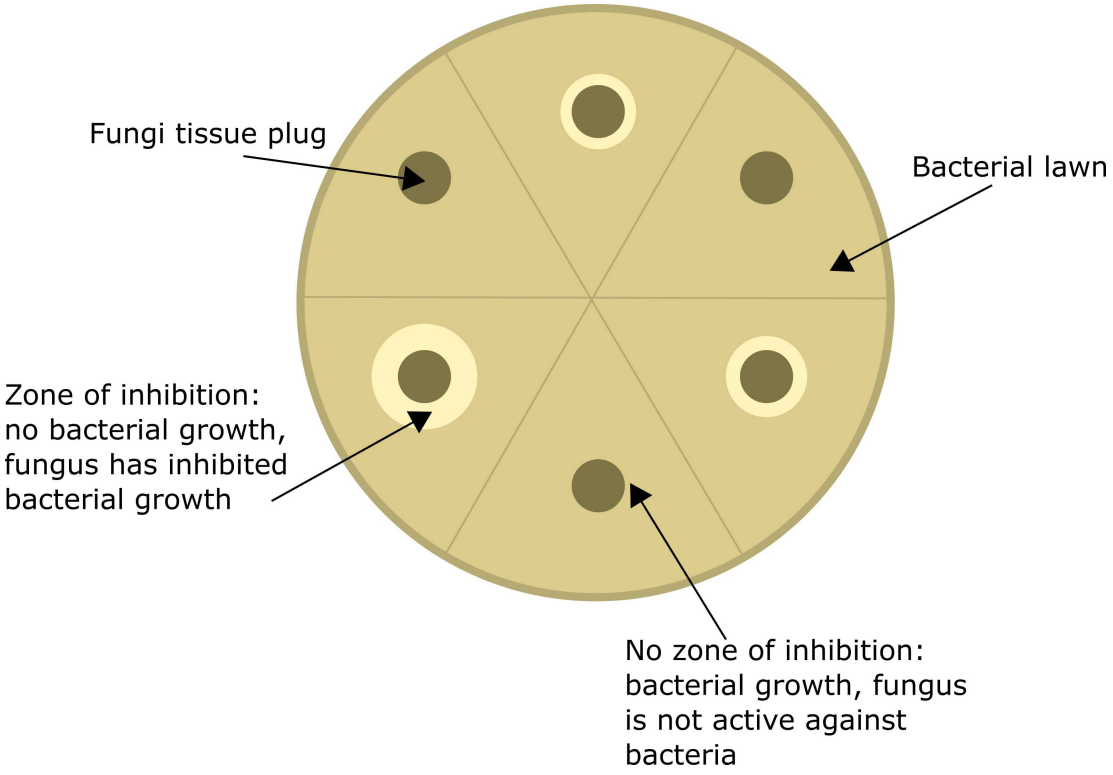

Figure 1: Schematic of a zone of inhibition plate