

Microbiology Methods for Bioprospecting

The extraction of compounds from plant materials is a practice that underpins much of organic chemistry and its derivative, bioprospecting. As the term suggests, bioprospecting is concerned with mining nature for “gold”. It involves the extraction, screening, and isolation of novel therapeutic compounds from living organisms with a view to commercialising the compounds. Bioprospecting is a core activity of many international pharmaceutical companies. This practical uses a disc diffusion method for testing potential antimicrobial agents extracted from plants.

Materials

- nutrient agar
- sterile Petri dishes
- sterile spreaders
- sterile plastic transfer pipettes
- paper discs
- culture broths of suitable bacterial species
 - *Escherichia coli*
 - *Staphylococcus epidermidis* (also known as *Staphylococcus albus*)
 - *Micrococcus luteus* (also known as *Sarcina lutea*)
 - *Bacillus subtilis* (*lawns of this species is often less uniform than others*)

Preparation of Plant extracts

Equipment and materials

- 1 g fresh leaf or vegetable matter
- 1 g sand
- Sterile water
- Mortar and pestle
- Test tubes with caps or stoppers
- Test tube rack
- Plastic transfer pipettes
- Spatula
- Filter paper (e.g. Whatman #4)
- Retort stand and clamps
- Funnel

Method

1. Combine the vegetable material and sand in a mortar and grind to a paste with the pestle.
2. Transfer the paste to a 10-mL tube and add 2 mL of sterile water.
3. Cap the tube and extract for 5 min with regular shaking.
4. Fold a circle of filter paper into quarters and press open to create a cone. Insert the cone into a funnel held in position in a retort stand.
5. Transfer the mixture from the tube to the filter paper and collect the filtrate in a fresh tube.
6. Proceed to serial dilution.

Serial dilution

Serial dilution is a fundamental procedure in microbiology and biotechnology and is used, in this case, to set up a test for the dose-dependent effects of soluble compounds on microbes.

The following method describes how to set up 1/3 and 1/9 dilutions. You can adjust the volumes of sterile water to produce alternative dilutions if required.

Equipment and materials

- Solution of compound to be tested against microbes
- Sterile water (negative control)
- Dettol or other known antibacterial reagent (positive control)
- Test tubes with caps or stoppers
- Plastic transfer pipettes

Method

1. To each of two test tubes add 1 mL of sterile water.
2. Carry out the serial dilution in the following sequence:
 - a. **Undiluted sample:** This is a tube of the solution to be tested directly. It could be an extract (as prepared above) or a commercial product. If this is a commercial product it should be prepared according to the manufacturer's specifications (e.g. Dettol would be diluted 1 part in 20 parts of water).
 - b. **A 1/3 dilution:** Transfer 0.5 mL of the undiluted sample to a tube containing 1 mL of sterile water. Cap and mix thoroughly.
 - c. **A 1/9 dilution:** Transfer 0.5 mL of the 1/3 dilution to a tube containing 1 mL of sterile water. Cap and mix thoroughly.

Note that the volumes of sterile water can be adjusted to produce alternative dilutions. For example, tubes containing 2 mL of water would be used to generate 1/5 and 1/25 dilutions of the original solution.

Testing the Antimicrobial Properties of Substances

In this experiment, the antimicrobial activity of the commercial or manually extracted substances will be tested on microbes. This is examined by placing the test substance (or a control) on a nutrient agar dish swabbed with microbes and measuring the resulting zone of inhibition. The zone of inhibition is the area around the substance in which the microbes do not grow: the bigger the zone, the more inhibitory the substance. If the substance does not inhibit the growth of the microbes, the microbes grow right up to the substance and there is no zone of inhibition.

You will set up at least one plate for each bacterial species being tested. The plate is divided into quarters, or sectors, allowing you to test the antimicrobial properties of your selected substance at three different concentrations together with a control (sterile water) against the bacterium.

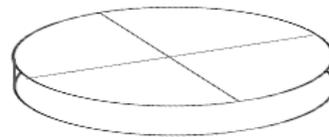
Equipment and materials

- Nutrient agar plates
- Bacterial broths
 - *Escherichia coli*
 - *Staphylococcus epidermidis* (*S. albus*)
 - *Micrococcus luteus*
 - *Bacillus subtilis*
- Shallow dishes/caps containing the test solutions and their dilutions
- Sterile water (control)
- Beaker with water (for rinsing forceps between different samples)
- Plastic transfer pipettes
- Sterile swabs (or spreaders)
- Paper discs
- Forceps
- Marker pen
- Ruler
- Biological waste bin

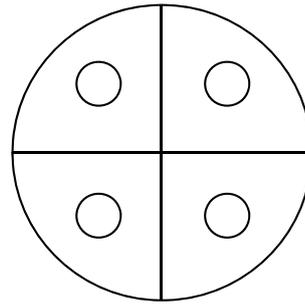
Method

1. On the base (agar side) of the nutrient agar plates use the marker pen to draw to divide the dish into 4 sectors. Label each sector with the corresponding treatment or control.
2. Turn the nutrient agar plates back over. Throughout the procedure, keep the lid on the plate between actions. When working with the plate, use the lid to shelter the agar from the air above.
3. Using a sterile transfer pipette, transfer 0.2mL of the bacterial broth onto the surface of the agar of the plate. Discard the transfer pipette in the biological waste.
4. Using the sterile swab or spreader, spread the bacterial broth over the entire surface of the agar. Take care not to break the gel. Discard the spreader in the biological waste.
5. Repeat steps 3 and 4 for other bacterial broths (if more than one species is being used).
6. Use forceps to immerse a paper disc in the dish containing sterile water (the control) for 5 seconds. Allow the solution to drain from the disc so as to avoid dripping. Position the disc in the centre of the corresponding sector on the agar plate.
7. Rinse the forceps in the beaker of water and repeat step 6 for each test solution.

Remember to rinse the forceps between each different solution



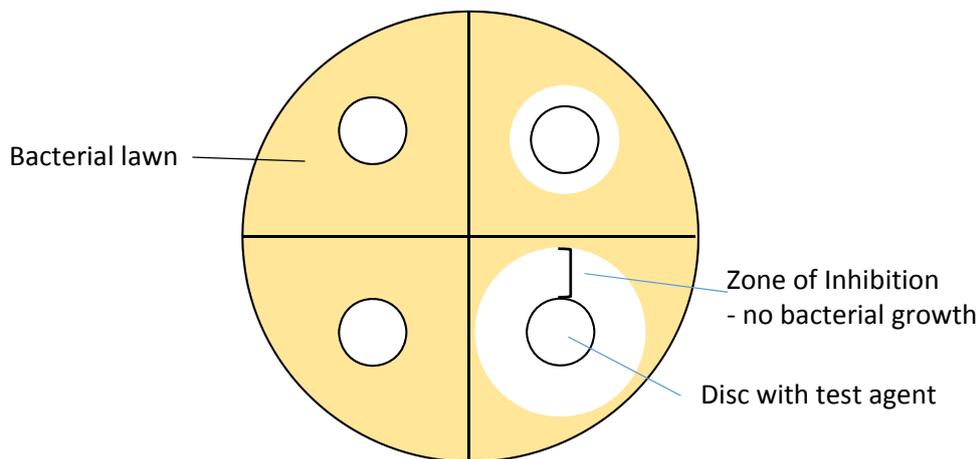
- Incubate the agar plates at 37°C (or at room temperature if you do not have an incubator) to allow the bacteria to grow.
- Use a ruler to measure the zone of inhibition. This is the distance from the edge of the disc to where the bacteria are growing. Record the result.
- When the experiment is complete, discard the plates in the biological waste.



The Zone of Inhibition

The bacterial lawn grows as an opaque layer over the agar. Compounds from the solution diffuse away from the disc into the agar. If the agent kills bacteria or slows its growth, there will be no bacterial growth in this area. In this case there is a clear zone around the disc. This is called the Zone of Inhibition. The more effective the agent at killing bacteria, the larger the Zone of Inhibition.

The Zone of Inhibition is a clear area around the disc where bacteria have been killed.



Sourcing Microbiological Materials

Item	Supplier	Cost 2015
	Southern Biological	
Nutrient agar	Various options possible: the cheapest for large numbers of plates is to buy powder and sterile petri plates and prepare your own plates	
Powder	MED3.10 - Nutrient agar powder 100g MED3.20 - Nutrient agar powder 500g 28g makes 1L broth Agar plates ~ 20-30mL per plate	\$69.50 for 100g \$218.00 for 500g
Gel ready to melt & pour	MED20.30 - Nutrient agar gel	\$9.90 for 100mL (about 5 plates)
Pre-poured plates	MED1.30 - Plain agar plates 10 plates	\$34.00
Bacterial broth cultures	All are in risk group 1 – suitable for schools Cultures can also be purchased as plates or slopes and broth cultures in sterile nutrient broth established from colonies	
<ul style="list-style-type: none"> <i>Escherichia coli</i> 	B1 - Escherichia coli, K-12 strain, live broth	\$18.80
<ul style="list-style-type: none"> <i>Staphylococcus epidermidis (S. albus)</i> 	B2 - Staphylococcus epidermidis (previously Staphylococcus albus), live broth	\$18.80
<ul style="list-style-type: none"> <i>Micrococcus luteus (Sarcina lutea)</i> 	B3B - Micrococcus luteus (previously Sarcina lutea), live broth	\$18.80
<ul style="list-style-type: none"> <i>Bacillus subtilis</i> 	B5B - Bacillus subtilis, live broth	\$18.80
sterile Petri dishes	E4.102 - Petri dishes, plastic, sterile, 92 x 16mm (pk 20) E4.202 (pk 160) E4.212 (pk 480)	\$10.80 \$52.00 \$102.00
sterile swabs	E6.10 - Swabs, sterile, rayon tipped, individually wrapped 20swabs	\$9.10
Paper discs	E1.70 - Blank discs pk50 Ejectors are also helpful for these discs	\$15.20
Paper discs - Make your own INEXPENSIVE	Whatman filter paper Hole punch Place in glass petri dish or beaker and autoclave	
Sterile plastic transfer pipettes	Various suppliers Alternatively autoclave glass Pasteur pipettes	
Disposal of plates and cultures		
Autoclave bags	Various suppliers	
Bleach	General suppliers	