Combinatorial Chemistry and Drug Discovery Lab

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Overview

• In this lab students will identify a drug that kills bacteria by producing libraries of compounds based on the A-B model.

• They will test the mixtures for antibiotic activity and then isolate the individual compound(s) which possess antibiotic properties.

• Students will screen the mixtures by utilizing techniques used to conduct Kirby-Bauer and Ouchterlony tests.
<table>
<thead>
<tr>
<th>Subject Area</th>
<th>Content Standard</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Chemistry</strong></td>
<td>2.a. <strong>Chemical Bonds</strong> – formation of ionic and covalent (peptide) bonds</td>
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<td>3.g. <strong>Stoichiometry</strong> – redox reactions, dehydration synthesis (condensation)</td>
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<tr>
<td></td>
<td>10.b. <strong>Organic Chemistry</strong> – bonding characteristics of carbon</td>
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<td></td>
<td>10.e. <strong>Functional Groups</strong> – formation of a hydrazone from an aldehyde and a hydrazine, identification and analysis of amine groups</td>
</tr>
<tr>
<td><strong>Biology</strong></td>
<td>1.a. <strong>Membrane Regulation</strong> – membrane structure and function</td>
</tr>
<tr>
<td></td>
<td>1.c. <strong>Prokaryotic and Eukaryotic Cells</strong> – structure and function</td>
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<td></td>
<td>1.h. <strong>Macromolecules</strong> – structure and function</td>
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<tr>
<td><strong>Genetics (Molecular Biology)</strong></td>
<td>4.c. <strong>Mutations</strong> – antibiotic expression</td>
</tr>
<tr>
<td></td>
<td>4.e. <strong>Proteins</strong> – structure</td>
</tr>
<tr>
<td></td>
<td>4.f. <strong>Proteins</strong> – function/chemical properties</td>
</tr>
<tr>
<td><strong>Genetics (Biotechnology)</strong></td>
<td>5.c. <strong>Biotechnology</strong> – production of novel biomedical and agricultural products</td>
</tr>
<tr>
<td><strong>Evolution</strong></td>
<td>7.a. <strong>Natural Selection</strong> – phenotype vs. genotype</td>
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<td>7.d. <strong>Genetic Variation</strong> – influence of environmental factors on the natural selection of adaptive traits</td>
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<td>8.a. <strong>Natural Selection</strong> – selective fitness; differential survival of groups</td>
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<td><strong>Physiology</strong></td>
<td>10.b. <strong>Immune Response</strong> – antibody/antigen response</td>
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<td>10.d. <strong>Bacterial Infections</strong> – use of antibiotics in treating bacterial infections; use of antibacterial agents to control the growth of bacteria</td>
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# National Science Education Standards

<table>
<thead>
<tr>
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| **Unifying Concepts and Processes** | **Change, constancy and measurement** – process of generating chemical libraries and screening for effective compounds, combinatorial data  
**Evidence, models, and explanation** – Kirby-Bauer & Ouchterlony Tests  
**Form and function** – cells, macromolecules, prokaryotes (E.coli ) |
| **Science as Inquiry** | **Abilities necessary to do scientific inquiry** – production of mixtures (libraries) of compounds using the A-B model; deconvolution (separation) of the mixtures to identify the compound(s) with antibiotic properties |
| **Life Science** | **The Cell** – types of cells, cell membrane structure and function  
**Biological evolution** – development of antibiotic resistance  
**Behavior of organisms** – growing bacterial cultures (lag vs. log phase), interpretation of bacterial plates (deconvolution) |
| **Science and Technology** | **Understandings about science and technology** – process and design in combinatorial chemistry; bringing a new pharmaceutical to market |
| **Science in Personal and Social Perspectives** | **Personal and community health** – drug discovery, pharmacology, diagnosis of human disease and course of treatment  
**Natural and human-induced hazards** – aseptic (sterile) technique, safety protocols including Material Safety Data Sheets (MSDS) |
| **History and Nature of Science** | **Science as a human endeavor** – biomedical research, clinical trials, bioethics of the biotechnology industry |
Materials
Preparing Overnight Culture of E. coli

1. Transfer 10ml of sterile LB broth in a culture tube.
2. To obtain a sample of E.coli, take the inoculating loop and dip into the frozen E. coli stock. Be certain that some of the stock has adhered to the loop.
3. Dip the inoculating loop into the culture tube containing the 10ml of LB broth. Stir the broth using the loop to ensure that the E. coli is thoroughly mixed in the broth.
4. Cap the tube and incubate the E. coli culture overnight at 37°C with agitation.
Prepare stock solutions

You have been provided 6 uniquely labeled conical tubes, each containing a specific chemical. Add 12 mL of deionized water to each tube and shake vigorously for approximately ten seconds.
If mixtures are not completely dissolving using a hot water bath may be effective.
Combinatorial Chemistry and Drug Discovery Lab

Student Protocol
Lab Safety

Tips for handling E.coli:

1. Wipe down the lab bench or station with a 10% bleach solution or 70% isopropanol solution at the beginning and end of each laboratory session.
2. When creating mixtures and transferring chemical solutions or liquid bacterial cultures, keep nose and mouth away from the opening of the tube to avoid inhaling any aerosols that may be created.
3. All spills should be reported to your instructor and cleaned up immediately according to the Material Safety Data Sheets (MSDS) for each chemical used in the experiment. Be sure to wear proper footwear (closed toe) to prevent injury.
4. Dispose of any materials that have come in contact with bacterial cultures (i.e. tubes, pipettes) in special waste containers as provided by your instructor.
5. Wash hands with soap and water before leaving the lab.
This lab protocol was adapted from the original work of Scott Wolkenberg and Andrew Su of The Scripps Research Institute in La Jolla, CA. The experiment was originally published in the June 2001 issue of the Journal of Chemical Education.

Combinatorial Chemistry is a technique used to synthesize a library of compounds and screen for a desired property. Instead of screening one compound at a time, the compounds are screened more efficiently in mixtures.

CITATION:
Wolkenberg, Scott E.; Su, Andrew I. J Chem. Educ. 2001 78 784
When microorganisms are introduced into fresh culture medium usually no immediate increase in cell number occurs therefore this is referred to as the LAG PHASE.

During the EXPONENTIAL or LOG PHASE bacteria are growing and dividing at the maximal rate given their genetic potential, nature of the medium and conditions under which they are growing.

In the STATIONARY PHASE, the total number of viable bacteria remains constant. This may result from a balance between cell division and cell death or cells may cease to divide while remaining metabolically active.

Detrimental environmental conditions such as lack of nutrients and waste buildup lead to the decline, usually logarithmic, in the number of viable cells. This is characteristic of the DEATH PHASE.

What limiting factors would cause a microbial population to enter the stationary phase?
Kirby–Bauer Test

- Disk-diffusion method used for routine testing in a clinical laboratory in which an isolated microbe is tested for susceptibility to numerous antibiotics.

- The isolated organism is uniformly placed on an agar plate with paper disk of fixed concentrations of antibiotics.

- Growth of the organism and diffusion of the antibiotic occur simultaneously resulting in a circular zone of inhibition if the antibiotic has antibacterial properties.
Ouchterlony Test

• A double diffusion technique developed by Organ Ouchterlony more than 40 years ago.

• A technique in which reaction partners, antigen and antibody, are allowed to diffuse to each other in an agar gel in a precipitation reaction.

• Classical procedure used to detect the presence of antibodies and determine their specificity by visualization of "lines of identity" or precipitin lines.
Student/Group Lab Set-up

- 3 Luria Broth (LB) agar plates
- 6, 15mL conical tubes containing stock solutions:
  • A1: 2-nitrobenzaldehyde
  • A2: 5-nitro-2-furaldehyde
  • A3: 3-nitrobenzaldehyde
  • B1: 4-bromophenylhydrazine hydrochloride
  • B2: 4-cyanophenylhydrazine hydrochloride
  • B3: aminoguanidine bicarbonate
- 1 cryotube (orange cap) containing 1.0 mL E. coli
- 1 cell spreader
- 15 disposable transfer pipettes or P-1000 micropipette
- 9 eppendorf tubes
- 1 plastic straw, wrapped
- Conical tube rack
- Sharpie Marker
- 1 sterile wrapped transfer pipette
1. Label 6 of the transfer pipettes A1, A2, A3, B1, B2, B3. These will be used to prepare your compounds.

2. Label 9 transfer pipettes M1, M2, M3, M4, M5, M6, A#B1, A#B2, A#B3. These will be used to transfer your compounds onto the plates. Each student or group will be assigned mixtures to test for confirmation of antibiotic activity (i.e. # = 1, 2, or 3 for A1B1, A1B2, A1B3; A2B2… ).
Label epitubes
Create wells in plates

Label Petri dishes

Each student or group will be assigned mixtures to test for confirmation of antibiotic activity.
(i.e. # = 1, 2, or 3 for A1B1, A1B2, A1B3; A2B2...).
Carefully invert the tube containing 1.0 ml of thawed E. coli several times before opening. Spread the E. coli culture evenly on the surface of each agar plate.
1. Make sure that the proper transfer pipette is used for each solution to avoid contamination.

2. Add solutions in the order indicated in the table. Record your observations.

### Prepare mixtures (libraries)

**NOTE:**
- 15 drops = ~ 750µL
- 5 drops = ~ 250µL
- 1 drop = .05 mL = 50 µL

<table>
<thead>
<tr>
<th>Tube #</th>
<th>Add 5 drops</th>
<th>Then 5 drops</th>
<th>Then 5 drops</th>
<th>Then 15 drops</th>
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</thead>
<tbody>
<tr>
<td>M1</td>
<td>B1</td>
<td>B2</td>
<td>B3</td>
<td>A1</td>
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<tr>
<td>M2</td>
<td>B1</td>
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<tr>
<td>M4</td>
<td>A1</td>
<td>A2</td>
<td>A3</td>
<td>B1</td>
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<tr>
<td>M5</td>
<td>A1</td>
<td>A2</td>
<td>A3</td>
<td>B2</td>
</tr>
<tr>
<td>M6</td>
<td>A1</td>
<td>A2</td>
<td>A3</td>
<td>B3</td>
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Add compounds to wells and wait approximately 15–20 minutes for liquid to absorb.
Incubate at 37ºC or store at room temperature overnight. (48 hours is optimal for room temperature option)

1. If no incubator is available: Plates can be stored overnight at room temp. with the agar side down.

2. If incubator is available: Allow compounds to absorb into agar then incubate agar side up.
A carbonyl group at the end of the carbon skeleton indicates that the compound is an **ALDEHYDE**

The amino group (-NH₂) consists of a nitrogen atom bonded to two hydrogen atoms; the compound is a **HYDRAZINE**

A special type of covalent bond called a peptide bond forms between the carbon and the nitrogen creating a newly synthesized compound called a HYDRAZONE.

The oxygen from the carbonyl group of the aldehyde leaves to bond with the hydrogen from the amino group of the hydrazine to form water. This represents a condensation reaction or dehydration synthesis.
Combinatorial Chemistry and Drug Discovery Lab

Post-lab Discussion
Autoclaving is the most effective and most efficient means of sterilization. All autoclaves operate on a time/temperature relationship. The usual standard temperature/pressure employed is 121°C/15 psi for 15 minutes.
Three Major Classes of Antibacterial Agents

Controlling the growth of microorganisms usually involves the use of physical or chemical agents which either kill or prevent the growth of microorganisms.

1) **Bactericidal** – agents that kill bacteria are called *cidal* agents; also referred to as bactericides.

2) **Bacteriostatic** – agents which inhibit the growth of cells (*without killing them*) are referred to as *static* agents.

3) **Bacteriolytic** – agents that have the ability to *lyse* or break apart, dissolve, and destroy bacteria by the use of an enzyme or other agent.

*Bacteria have the ability to develop *resistance* following repeated or subclinical (insufficient) doses, so more advanced antibiotics and synthetic antimicrobials are continually required to overcome them.*
Variations in bacterial cell wall structures not only cause differences in staining but the anatomy of each cell wall also leads to differences in the susceptibility of bacteria to antibiotics. Some antibiotics easily penetrate Gram-positive cell walls while others are more capable of penetrating Gram-negative cell walls.
Antibacterial Sites of Action

- Inhibition of cell wall synthesis (Penicillins, cephalosporins, bacitracin, vancomycin)
- Inhibition of protein synthesis (Chloramphenicol, erythromycin, streptomycin, tetracyclines)
- DNA
  - Replication
  - Transcription (Rifamycin) → mRNA
- Inhibition of nucleic acid synthesis
- Enzymatic activity
- Injury to plasma membrane
- Inhibition of enzymatic activity by antimetabolites (Sulfanilamide, trimethoprim)
Antibacterial Sites of Action

Growing Polypeptide

Chloramphenicol inhibits formation of peptide bond

Erythromycin prevents translocation

Streptomycin

Tetracycline interferes with attachment

70S prokaryotic ribosome
Concept Review

BACTERIAL GROWTH & DEATH: ANTIBIOTICS EFFECTS

• Analyze the graph by describing the phase of growth being represented by lines A thru E: LAG, LOG, STATIONARY, or DEATH.

• If the graph shown is the result of antibiotic resistance testing, what is the effect of the antibiotics on the growth of the bacteria at A thru E: NORMAL, BACTERIOSTATIC, RESISTANT or BACTERICIDAL?