Biotechnology in North Carolina Today

LESSON 3

The Synthesis of Aspirin Demonstration

Before the demonstration, discuss with students how drugs are manufactured.

Ask them how they think the biomanufacturing process differs from traditional chemical drug manufacturing. Have them use their prior knowledge about chemical reactions.

Ask how many of them have ever taken a pain reliever. Is a pain reliever only good for one kind of pain?

Safety Precautions:

Wear splash goggles and gloves. Make sure students are well back from the demonstration table, use a transparent splash shield or have them also wear goggles.

Acetic Anhydride: Corrosive, Flammable and Eye Irritant
Concentrated Sulfuric Acid: Very Corrosive
Ferric Chloride Solution: Corrosive
Salicylic Acid and Aspirin: Respiratory System Irritants- keep the dust away from your face
Ethanol: Toxic, Flammable

Procedure

1. Place 1.00g of salicylic acid and 2 mL of acetic anhydride in a test tube.
2. Add 5 drops of phosphoric acid to the tube while stirring constantly. The reaction will be exothermic. *Have students note the temperature change in the chemical reaction.*
3. Continue stirring (use a stirring rod) for about 5 minutes after all of the acid has been added.
4. Next heat the tube in a hot water bath for 5 minutes. *Ask students why they think that it is necessary for you to heat the tube .Discuss reaction directionality, review exothermic and endothermic terms.*
5. Transfer the reaction mixture to a 50 mL Erlenmeyer flask containing 10 mL of deionized water.
6. Rinse the test tube with an additional 5 mL of water and add that to the Erlenmeyer flask as well.
7. Swirl the flask and then cool it thoroughly in an ice water bath. If no crystals form, scratch the inside of the flask with a glass stirring rod to induce crystallization. *Ask students why they think that you must cool the solution to get crystallization to occur. Review guidelines regarding solubility of solids.*
8. Hold a black paper behind the flask to show students that the crystals have formed.
9. *Ask students if they think it would be easier or more difficult to make a drug using biomanufacturing. Ask for reasons. Accept all answers.*
10. (OPTIONAL) You can verify that you have created aspirin by conducting ferric chloride test or a heat test. To do this, you will have to collect and dry the crystals. This can be done by pouring the reaction mixture through filter paper and allowing it to dry overnight.

Ferric Chloride Test

1. Place 1 mL of ethanol (ethyl alcohol) in each of three small test tubes.
2. Add one drop of 1% aqueous ferric chloride solution to each tube.

3. In the first test tube, place a few crystals of aspirin.
4. In the second test tube, place the same amount of the reaction product
5. Place nothing in the third tube- it will be the control.
6. Shake each test tube. *Ask students to record the results and discuss what is happening and what it tells you about how pure the product of the reaction is.*

Heat test

1. To a small test tube, add a few crystals of the reaction product.
2. To another, an equivalent amount of pure aspirin.
3. Heat both tubes on low heat until the crystals melt.
4. Remove from the heat and note the odor of the escaping vapor by carefully wafting the vapors. *Have students walk by and waft the melted crystals. Have students note the odor and discuss what it means about the purity of the reaction product.*

Works Cited

Schneider, R. F. (2006, 10). *Synthesis of Aspirin*. Retrieved 9 13, 2008, from Chemistry 134 Lessons: http://www.ic.sunysb.edu/Class/che134/susb/susb028.pdf

Staff, X. U. (n.d.). *24. Synthesis of Aspirin*. Retrieved 9 13, 2008, from Organic Chemistry Lessons: http://www.xula.edu/chemistry/department/organic/Labbook/24Aspirin.pdf

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LESSON 3

Media Preparation: Teacher Notes

Media Preparation is the key for successful cell culture. A lack of sterilization and aseptic technique can easily result in contamination of the culture. A lack of the proper nutrients in the proper amounts can result in poor cell growth. In biomanufacturing, a great amount of research is spent in optimizing the growth of cells by providing them with both the very best media and the very best growth conditions. This is what students will be simulating in this lesson.

Sterilization techniques:

For equipment to be considered sterile, it is completely free of any living cells whatsoever. Even the smallest fragment of hair, skin or dust can contain many viable microorganisms. However, people have been making yogurt and other fermented products for thousands of years by taking precautions to keep equipment and their "media" as sanitary as possible without the benefit of autoclaves or other steam sterilization equipment. However, an autoclave would be ideal to sterilize all of your glassware and your media. Sanitization results in all but the most

Options for equipment include:

- Using the heated dry/sterilize setting on your dishwasher at home
- Boiling submerged in a water bath for thirty minutes
- Heating in a pressure cooker at 15 pounds of pressure for 15 minutes.
- Rinsing with a 10% bleach solution and allowing equipment to air dry away from contact with airborne contaminants (open ends down on a sterile surface that has also been washed and treated with the bleach solution).
- Plastic, glass, wood and cloth can be sterilized in the microwave. Most organisms will be killed after 3 minutes on high power. Include a container of water to provide a heat sink. Do not use black plastic, it heats up too much and may melt. Monitor items sterilized using this method carefully as they heat. Wooden items should be soaked in water before heating.

Once media has been made, it will have to be sterilized/ sanitized. Sterilize/sanitize before you add your cells or you will kill the cells you want to grow, too.

- Boil in a hot water bath for 60 minutes, cool. Repeat after 24 hours, twice. (3 boils total. Called Tyndalization)
- Heating in a pressure cooker at 15 pounds of pressure for 15 minutes. Repeat.
- Microwave the ingredients or finished media for 5-10 minutes. Watch carefully for boil-over. Smaller amounts of media should be microwaved for less time, larger amounts more. The literature on the effectiveness of this is mixed, but most seem to agree that it kills virtually all microbes that are not spore-forming. I would repeat this at least once after 1 day, for safety.

Media Recipes for growing yogurt bacteria (Eddleman)

Milk carbohydrate = mostly lactose, some glucose if it is heated. Trace galactose

Milk protein = mostly caseinogen (suspended particles- makes milk white), some lactalbumen.

Skim Milk Media (Eddleman)

- 1 L distilled water (or water that has been left out in an open container so the chlorine can dissipate)
- 100 g skim milk powder (bacteria have trouble with milk fats)

Litmus Milk (Eddleman)

- 1 L distilled water
- 100 g skim milk powder
- 5 g litmus powder
- Glucose fermentation → faint pink color
- Protein breakdown to alkaline products + no lactose fermentation → blue/lavender color
- Lactose fermentation → Pink to red color

Methylene Blue Milk (Eddleman)

- 1 L distilled water
- 100 g skim milk powder
- 1 g Methylene Blue
- This medium is used to identify enterococci. Up to 5 mg of Methylene Blue may be used. If enterococci are present the blue will disappear.

I would recommend having students prepare the skim milk media and then choosing an additive to test in different concentrations, if their independent variable is a media additive.

To save time and ensure sterility, the teacher could prepare the base media and then students could choose to supplement with different concentrations of additives (like corn syrup).

Media additives that increase the growth and volume of cells include autolyzed yeast extract and glucose. You can get yeast extract at Whole Foods or other health food stores. A good substitution for yeast extract would be Wyler's Low Sodium Chicken broth. It produced good growth results in my test cultures. Corn syrup is a good source of glucose. Table sugar will not work as well, but students may choose to try it. There are many other things that would make a good experiment, like different concentrations of salts, gelatin, or other sweeteners, like nutrasweet, molasses or splenda.

Works Cited

Eddleman, Harold. First Microbiology Experiments. Feb 1998. July 2008 <http://www.disknet.com/indiana_biolab/b021.htm#Safety>.

—. Making Milk Microbiology Media. February 1998. 17 July 2008 <http://www.disknet.com/indiana_biolab/b028.htm>.

Inquiry Lesson in Growing Living Cells

Problem: What factors influence the growth of cells in sterile media?

Background:

This experiment models how different variables can affect cell growth in a bioreactor. Media provides the nutrients that cells need to grow under optimum conditions. In this inquiry, you will pick one independent variable in the growth of your cells to influence. What you do with your cells will be entirely up to you and your research partner. Everyone will use the same types of containers for media growth and the same techniques.

Procedure:

The class will be growing yogurt bacteria in skim milk media. Yogurt bacteria have been cultured for centuries across the world and originated from wild bacteria. Yogurt bacteria produce lactic acid which denatures and precipitates milk proteins. Lactic acid will be the "product" we are trying to make in our biomanufacturing process.

You will be growing your culture in tubes. Every tube will receive 20 mL of sterile milk media and be inoculated with 2 mL of "Mother" culture.

You will have one control group that you will do nothing to and you must have at least 3 test groups that you will use to test your independent variable in different amounts.

You will be evaluating the growth of your bacteria in three ways.

1) You will measure the pH before you inoculate, after you inoculate, after 24 hours and after 48 hours.
2) You will note changes in the culture using qualitative observations after 24 and 48 hours.
3) You will complete a serial dilution of one of your cultures and evaluate the growth on agar plates.

Hypothesis:

If I change _____,
then the bacteria will grow (faster/slower)_____,
because _____ .

Independent Variable: _____

Test Groups: Control, 1. _____, 2. _____, 3. _____

Things to keep constant (things I won't change between the groups):_____

_____

Procedure:

Materials:

4 culture tubes "Mother" culture Skim Milk media _____

pH paper Sterile 20 mL and 2 mL measurement tools _____

Sterile swabs

1. Label 4 culture tubes with initials, class number and date. Label 1. "Control", 2. "Group 1," 3. "Group 2," 4. "Group 3."
2. Add 20 mL of sterile milk media to each tube.
3. Add 2 mL of the mother culture to each tube.
4. To group 1, I will _____
5. To group 2, I will _____
6. To group 3, I will _____
7. .
8. .
9. Cap and mix well by inverting several times.
10. Using a different sterile swab for each tube, dip into the culture and place some of the culture solution onto pH paper and record the starting pH. Record any observations of the culture.
11. Loosen the caps to allow air flow and place your culture tubes in a cup in the incubator.
12. After 24 hours, repeat step 10 and 11.
13. After 48 hours, repeat step 10 and 11 and complete the "Serial Dilution" procedure.
14. Discard cultures as directed by your teacher.
15. Record bacterial counts from your serial dilution.

Data:

Quantitative Observations:

Group	Starting pH	24 hours pH	48 hours pH
Control			
1			
2			
3			

48 hour Bacterial Count for Group_____ =_____

Qualitative Observations:

Group	Starting observations	24 hours obs.	48 hours obs.
Control			
1			
2			
3			

Line Graph: pH vs. Time

pH					
	14				
	13				
	12				
	11				
	10				
	9				
	8				
	7				
	6				
	5				
	4				
	3				
	2				
	1				
	0				
		Starting pH		24 hour pH	48 hour pH

☐ Control ☐ Group 1
☐ Group 2 ☐ Group 3

Data Analysis:

What happened to the pH for each of the groups?

Was it the same for all of the groups? If not, how was it different?

How were the observations for each of the groups different?

Was there bacterial growth in the tube you chose to sample? How many bacteria were present?

Conclusions:

Were there any differences between the control and the test groups?

Did your independent variable (what you tested) affect the dependent variable (cell growth)? How do you know?

Was your hypothesis supported by the data?

What mistakes did you make?

What other variables do you think could affect cell growth?

What would you change if you had a chance to do this experiment again? What do you think would help the cells grow or keep them from growing more than what you did?

What have you learned about the challenges of growing cells in a bioreactor to make a biological chemical?

Method of preparation of Gram stain slide

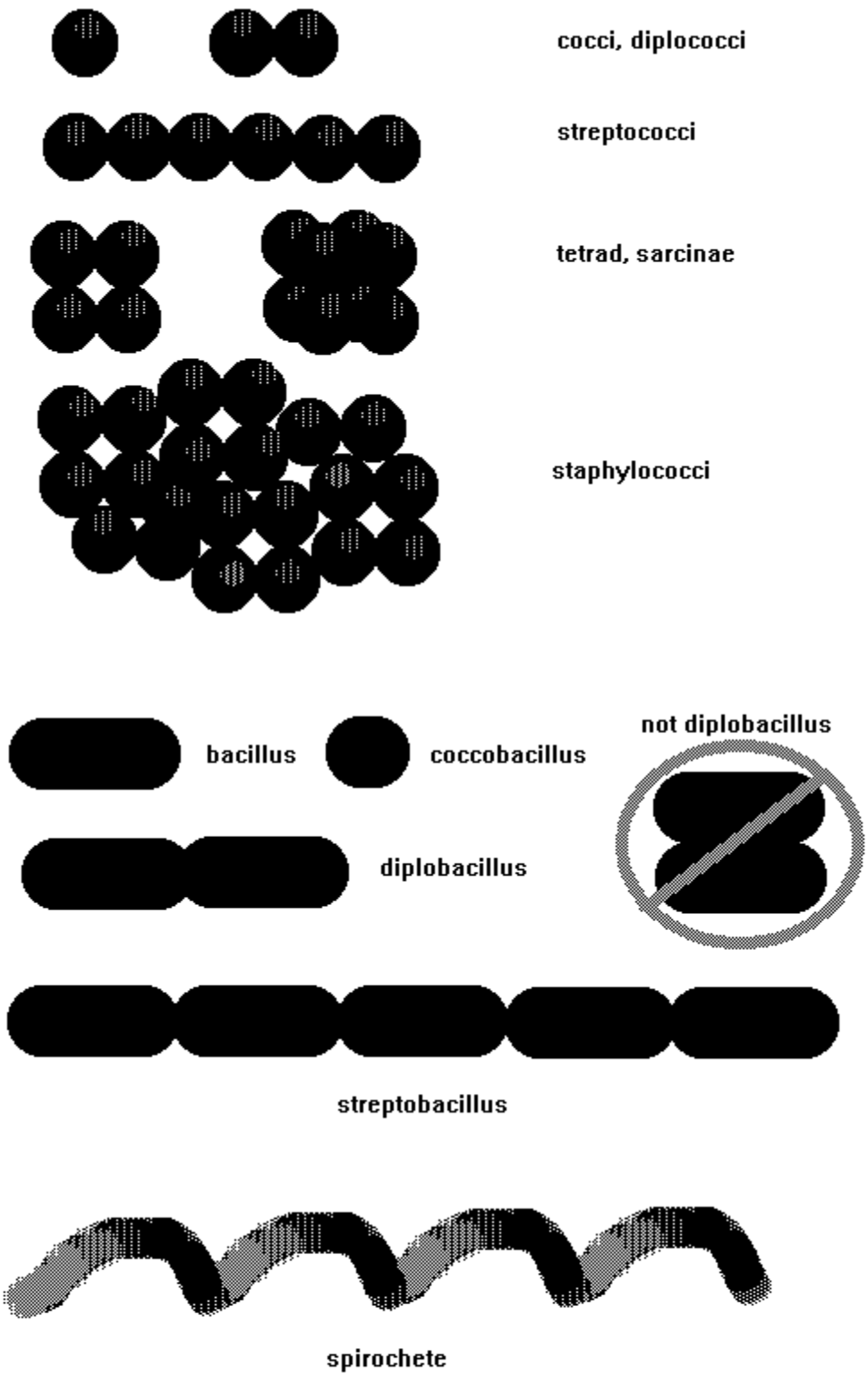
1. Centrifuge 15mL of sample in a 15mL centrifuge tube at 1000rpm for 5 min.
2. Pour off 10mL of supernatant and re-suspend pellet in remaining 5mL.
3. Place a drop of suspension on a microscope slide using a disposable inoculation loop and smear to a thin layer.
4. Allow slide to dry completely.
5. When dry, pass slide over Bunsen burner 3 times to fix cells to slide.
6. Flood slide with crystal violet, let stand for 1 min.
7. Pour off crystal violet (Blue). Flood slide with iodine, let stand 1 min.
8. Pour off iodine. Flush slide briefly with decolorizer, then immediately flush briefly with water.
9. Flood slide with safranin (Red), let stand for 1 min.
10. Briefly flush slide with water then let slide dry.
11. Place a small drop of immersion oil on the slide in an area that is visibly stained.
12. Place slide on the oil immersion microscope, adjust the mechanical stage so that the oil drop is directly below objective lens (Ensure that the 100X objective lens is in the vertical position). Turn the microscope light on. Adjust lamp brightness to high.
13. Using the coarse adjustment knob, rotate clockwise until objective lens touches the oil drop.
14. Look into the eye-piece and start to turn the fine adjustment knob clockwise slowly until you see pink or purple objects.
15. A microbiological contamination should be clearly visible using this method.

If a contamination exists, bacteria will fall into two main categories:

1. Gram positive. (Purple to dark blue objects)
2. Gram negative. (Pink to red objects)

Bacteria will then fall into 3 sub categories:

1. Cocci. (perfectly spherical small objects that clump (look like grapes) or are isolated from each other).
2. Rod. (cigar shaped. Often form chains or could be separated)
3. Spirochete. (Very rarely seen, looks like a spring.)


cocci, diplococci
streptococci
tetrad, sarcinae
staphylococci
bacillus
coccobacillus
not diplobacillus
diplobacillus
streptobacillus
spirochete

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Procedure for Counting Bacterial Cells via Serial Dilution

Background: Bacterial cells are very small and can be difficult to count from a slide under a microscope. They also grow to a high density in a liquid culture, which can also make counting them rather tricky. When a bacterial culture in liquid media is introduced to an agar plate, each individual viable bacterium will form a colony, which will be visible to the eye (assuming the agar provided the nutrients this bacterium needs). The colonies can then be counted and you will then be able to extrapolate the number of viable bacteria in the entire culture.

To avoid contamination by wild bacteria, the equipment used should be as close to sterile as possible. Follow the instructions under "Making Media" to sterilize your media and equipment.

Materials:

3 agar plates 3 sterile swabs

20 ml sterile media 2 sterile tubes

1 sterile transfer pipette

1. Label 3 agar plates on the bottom: 1. Direct Count, 2. 1/10 Dilution, 3. 1/100 dilution. Label all plates with your initials, class and the date.
2. Take the culture you wish to test, close the lid and invert it 3 times to make sure it is well mixed.
3. Dip a sterile swab into your culture, press it against the side of the tube to squeeze out excess liquid, gently rub the wet swab over the surface of the agar plate labeled "direct count."
4. Label the two tubes 1/10 and 1/100.
5. Using the transfer pipette, fill each of the 2 empty sterile tubes with 9 ml of sterile media. Be careful not to touch the sides of the tubes or the table with the pipette.
6. Withdraw 1 mL of your culture and add it to the tube labeled 1/10. Mix this well by swishing the solution in and out of the pipette. Withdraw 1ml of this solution and add it to the tube lalbeled 1/100.
7. Dip a sterile swab into the tube labeled 1/10, press it against the side of the tube to squeeze out excess liquid, gently rub the wet swab over the surface of the agar plate labeled "1/10 dilution."
8. Dip a sterile swab into the tube labeled 1/100, press it against the side of the tube to squeeze out excess liquid, gently rub the wet swab over the surface of the agar plate labeled "1/100 dilution."

9. Discard the tubes containing your dilutions according to your teacher's instructions.

10. Incubate the plates for 48 hours, then count and record the number of colonies on each plate. If there are more than 200 colonies, record the data as TNTC (too numerous to count).

11. Calculate the number of bacteria from the swab of your culture using the formula:

    - Number of bacteria = number of colonies/dilution factor