Lab 6: Assay Development, Day 1

This lab is the first of three labs in which we will be performing different types of assays. Conducting assays is an essential part of the biomanufacturing process, because they allow technicians to monitor each step during the production of a biotechnology product.

Definition of an ASSAY: a test to determine the presence, concentration, and/or biological activity of a substance (such as an enzyme, growth factor, drug, vitamin, hormone, etc.)

We've already done two assays in class so far—do you remember what they were?

Assays are extremely important in biomanufacturing b/c they are necessary for a company to:
1.

2.

3.

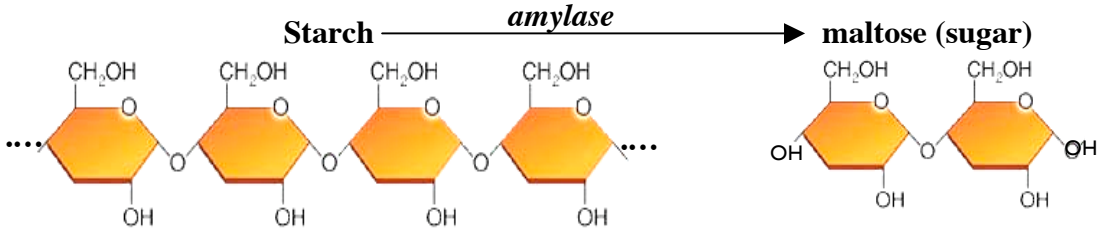
4.

Some basic information about enzymes

1. Enzymes are almost always _____.
2. The function of enzymes is to_____.
3. Enzyme terminology:
   Substrate: the reactant that an enzyme binds to and converts to product.
   Active site: the pocket in the enzyme into which the substrate binds.
4. Enzymes are very specific. Each enzyme binds to a particular substrate and catalyzes (speeds up) the rate of a particular chemical reaction.
5. Enzymes don't get used up—each time they convert a substrate to product, they are then ready to bind another molecule of substrate

Background on the Enzyme Amylase

In today's lab, we will learn two different ways of assaying for amylase activity. Below is the chemical reaction that is catalyzed by the enzyme amylase. The reactant (starch), is converted by the amylase enzyme to the product (maltose), which often continues to break down into 2 glucose molecules (not shown).



Though we can't see amylase, we can measure its activity by measuring the amount of product (_____) made over time, or the amount of reactant (_____) used over time.

In order to design an assay, we need to learn a few more terms that are very important in any experimental design:

- **Independent variable**: this is the condition being tested. The independent variable is the only factor that will differ from one group to the next. All of the other experimental conditions are the same among different samples.
  EX: measuring the effect of nitrogen concentration on grass growth, the independent variable is _____.

- **Dependent variable**: this is what is being measured, the data that is being collected. The dependent variable changes as a result of the independent variable.
  EX: measuring the effect of nitrogen concentration on grass growth, the dependent variable is _____.

- **Control**: an experimental sample in which the outcome is controlled, or known, by the researcher. The purpose of experimental controls is to allow the researcher to draw meaningful conclusions about her/his data and make sure that the experimental conditions are working in general. For example, suppose a study was performed to test a new drug for relieving headaches. Thirty people with frequent headaches are given the drug, and 20 of them report improvement in their headaches. Without a control group of 30 people that received a placebo (fake) pill, you can't say that the improvement in headaches was due to the drug—headaches may have improved in those 20 people for other reasons.

  - Negative control: this sample lacks the variable being tested. It is designed to produce a negative result. EX: when we tested for the presence of protein using NaOH and $CuSO_4$, a negative control would be a sample that had NaOH and $CuSO_4$, but substituted water for protein.
  - Positive control: This sample is designed to give a positive result. EX: when we tested for the presence of protein using NaOH and $CuSO_4$, a positive control would be a sample that had NaOH, $CuSO_4$, and a known concentration of protein.

In Activity 6a, we will learn about two different **indicators** that will help us assay for amylase activity in Activity 6b. An **indicator** is a chemical that changes color when another chemical is present. One indicator (Benedict's solution) measures the presence of some sugars, including maltose (the product of the reaction catalyzed by amylase). The second indicator (iodine) shows the presence of starch (the substrate of amylase) in a sample. Therefore, we can assay for the presence of amylase by measuring the reduction in the amount of starch and the increase in the amount of maltose.

Activity 6a
Assaying for Starch and Sugar

Purpose

In this activity, you will learn how to use two different chemical indicators to test for the presence of starch and sugar. You will then apply this information in order to test for amylase activity in Activity 6b.

Background

First, a review. Question: What kind of molecules would starch and sugar be classified as? Answer: _____.

In this activity, we will learn how to use two indicators:

- Iodine (I_3^-): indicator of starch molecules
  - when iodine is caught in long starch molecule coils, a color change occurs.
- Benedict's solution: indicator of some simple sugars, including maltose
  - contains a copper compound that reacts with a chemical group on these sugars.

Procedure

You will be conducting starch assays and aldose (sugar) assays on four different solutions:

- 2% starch
- 1% glucose
- 1% maltose
- 2% protein (gelatin)

1. To save time, I will make these solutions for you. However, to practice your solution prep calculations, first do the calculations in your lab notebook as if you were going to make 10 mL of each of the above solutions.

2. Starch assay
   a) Place 1 mL of each of the four solutions to be tested into four separate microcentrifuge tubes.
   b) Add 125 μl of iodine solution to the sample.
   c.) Mix by vortexing for 1-2 seconds.
   d) Record the color after mixing.

3. Aldose (sugar) assay
   a) Place 500 ml of the solution to be tested into four separate microcentrifuge tubes.
   b) Add 500 μl of Benedict's solution to the sample.
   c) Mix by vortexing for 1-2 seconds.
   d) Heat the tubes in a 100°C heat block for 2 minutes (note: use tube locks or put a weight on top of tubes to prevent them from exploding open).
   e) Record the color of the solution after 2 minutes.

Activity 6b
Assaying for Amylase Activity

Purpose

In this activity, you will use the starch assay (iodine indicator) and sugar assay (Benedict's indicator) to test for amylase activity. You will compare the activity of two types of amylase: recombinant amylase made in bacteria, and salivary amylase, supplied by you!

Background

Humans, like a number of other animals, make amylase in two organs in order to help break down food. Salivary amylase is produced in the mouth by our salivary glands. Pancreatic amylase is produced by the pancreas and excreted to the small intestines. In this activity, we will compare the activity of human salivary amylase (unknown activity and concentration) with a solution of bacterial amylase that has a known concentration of 10 mg/mL.

Procedure

You will set up the assay today. After 24 h, I will place each group's plates at 4°C for storage until next week. Then, we will analyze the data and record our results and observations in Activity 7a.

1. Collect ~5 mL of saliva in a clean 50 mL plastic tube (see figure). Chewing on a rubber band may increase saliva production.
2. Obtain a clean, 24-well plate. Then, read over the rest of the procedures, and label the wells that will be used. Before starting, make a diagram in your notebook of what is to be loaded into each well. Check your group's diagram with me before proceeding.
3. Place 1 mL of 2% starch solution in the first six wells of Rows 1, 2, and 3. Be sure to mix the starch solution before you take each sample.
4. To Columns 1 and 4 of the wells, add 300 µL of human salivary amylase solution to each of the starch-filled wells. To Column 4 of the wells, also add 20 µL of iodine. Mix each for 2 seconds with a pipet tip (best to use a P-1000 and a blue tip and pipet up and down to mix). Be careful not to cross-contaminate wells (though you can use the same tip for each group of triplicate wells that have the same contents).
5. To Columns 2 and 5 of the wells, add 300 µL of bacterial amylase to each of the starch-filled wells. To Column 5 of the wells, also add 20 µL of iodine. Mix each for 2 seconds with a pipet tip as you did in step 4.
6. To Columns 3 and 6 of the wells, add 300 µL deionized water to each of the starch-filled wells. To Column 6 of the wells, also add 20 µL of iodine. Mix each for 2 seconds with a pipet tip as you did in step 4.
7. Identify the positive and negative controls in this experiment. Make predictions as to what may occur in each well, including the expected color change. Record these predictions in your lab notebook.

Collecting Saliva. Wait 20 minutes after eating or drinking before collecting saliva. When collecting saliva, remember that it is a biohazard that should be treated in a mature, safe fashion. Clean all spills as necessary, and dispose of materials in the red biohazard bag at the end of the experiment.

Activity 6c
Assaying Plant Substances for Antibacterial Activity

Purpose

In this activity, you will test plant extracts to see if they possess antibacterial activity. Filter disks soaked in plant extracts will be incubated with *E. coli* bacterial cultures to determine whether the growth of the *E. coli* bacteria is inhibited by any of the plant extracts.

Background

In nature, organisms are constantly battling for resources and survival. All organisms are infected by viruses or threatened by bacterial disease. Many organisms have defense systems to combat the onslaught of foreign invaders. Numerous plants, fungi, and bacteria produce antimicrobial agents to battle the microbes. Finding and isolating an antimicrobial molecule could lead to a potential therapeutic medicine.

Isolating an active ingredient that actually has antimicrobial activity is not a trivial task. Suspect samples must be collected, sometimes from distant, remote places. Once samples are collected, extraction techniques must be determined and samples must then be processed. Then, samples can be tested for their ability to kill different microbes.

One of the ways that researchers test plant extracts for antimicrobial properties is to add extract-soaked filter paper disks to bacterial cultures spread on Petri plates. Plant extracts containing compounds effective against bacterial leave clear halos around the soaked disks in the bacterial lawns. These halos are called zones of inhibition, because they are regions where bacterial growth is inhibited or where there is bacterial death.

Procedure

The plant extracts have been prepared for you. Some of them have been store-bought. The others were prepared by your instructor by crushing the plant material with a mortar and pestle, adding water, and letting the plant extracts incubate in the water for 24h at 4°C. Then, the extracts were centrifuged so all plant solids would go to the bottom of the tube. The extracts were then filtered through a membrane to sterilize them. Sterilized filter disks were soaked in the plant extracts overnight.

In addition, there are filter disks that have been soaking in a solution of ampicillin, an antibiotic that is known to be effective in inhibiting the growth of E. coli. There are also filter disks that have been soaking in water, which has no antibacterial activity. The ampicillin-soaked disks will therefore serve as your positive control, and the water-soaked disks will serve as your negative control for this experiment.

You will choose two plant extracts to assay for antibacterial activity, in addition to your positive control (ampicillin) and negative control (water). You will assay each sample in duplicate, meaning that your group will set up two identical plates.

1. Create a list in your notebook of all the plant extracts that are available for testing. Choose the two extracts that you want to assay for antibacterial activity and record these in your notebook.

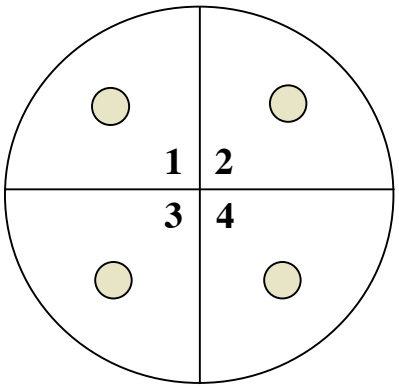
2. Obtain two nutrient agar plates from your instructor. Agar is a substance made from seaweed that is a liquid when hot, and then solidifies when it cools. The agar provides a solid support

surface on which bacterial cultures can grow. A nutrient mixture of sugars and amino acids has been added to the agar to provide food for the bacteria.

3. Use a permanent marker to draw four quadrants (sections) on the bottom of each agar plate. Number each quadrant 1-4 as shown.

4. Using a sterile pipet, transfer 1 mL of *E. coli* broth to the middle of each Petri dish. Using a sterile spreader, evenly spread the bacterial culture around the agar plate. Quickly cover the plate, and allow the culture to soak into the agar for at least 15 minutes. Make sure to dispose of the pipet and spreader (and anything that had contact with the *E. coli* culture) in the red biohazard bag.

5. Using sterile forceps (ask your instructor for help to flame the forceps in alcohol when you are ready for this step), carefully place one filter disk from the designated sample into the middle of each quadrant, about 2 cm from the outer edge of the Petri dish. Prepare the plates in duplicate (2 identical plates). The samples for each quadrant will be as follows:



quadrant #1: water
quadrant #2: ampicillin (an antibiotic)
quadrant #3: plant extract 1
quadrant #4: plant extract 2

6. Make sure that the disks are adhering well to the surface of the agar.

7. Label your plates with your group's initials and place them in the 37°C incubator upside down. The plates will be incubated for 24-48 h.

8. Record which quadrants are the positive and negative controls in this experiment. Make predictions as to what you think you will see in each quadrant next week, and record these predictions in your lab notebook.

Next week, we will measure the zone of inhibition (z.o.i.) to determine whether our plant extracts possessed any antibacterial activity against *E. coli* bacteria.

Lab 6 Homework

Name: _____

1. Lactase is an enzyme that binds to lactose molecules and breaks them down into glucose + galactose molecules. Given this information, what is the substrate for the lactose enzyme?

   _____

2. a) What type of carbohydrate is starch? _____

   b) What type of carbohydrate is maltose? _____

3. You want to design an experiment to test the effect of temperature on the growth rate of bacteria. You therefore compare the growth rates of bacterial cultures grown at different temperatures.

   a) The dependent variable in your experiment is _____.

   b) The independent variable in your experiment is _____.

4. In the assay for amylase activity that you set up in Activity 6b:
   a) Which wells would be considered positive controls? Describe the expected results for these wells.

   b) Which wells would be considered negative controls? Describe the expected results for these wells.

   c) In this assay, each condition was tested in triplicate, meaning that there were three identical wells in the 24-well plate for each condition. Why do you think each condition was tested in triplicate?

5. You are setting up an assay to test for the presence of starch in various foods.
   a) What indicator would you use to test for starch?

   b) In your experiment, you used a 2% cornstarch solution as a positive control. After performing your assay, you find that there was no change in the color of the solution in your positive control. What can you conclude about the other samples and the experiment as a whole?