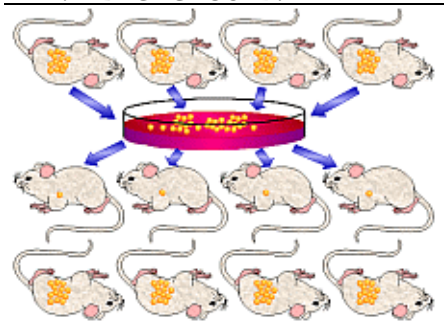
YEAST PLATE COUNT

I. OBJECTIVES:

1. To learn how to do a serial dilution.
2. To determine the number of viable yeast cells in a packet of yeast.
3. To learn sterile technique.

II. BACKGROUND:



Robert Koch, a German physician, is famous for determining the bacteria responsible for anthrax and tuberculosis. The chance observation of bacteria growing on the surface of a spoiling slice of boiled potato led Koch to devise the technique called **single colony isolation**. He correctly deduced that each spot, or colony, of bacteria had grown as a clone from a single contaminating bacterium. In this experiment, we will be using yeast (a fungus) rather than bacteria to learn some of the techniques and methods used in microbiology. We will be making use of Koch's conclusion that each viable cell (in this case yeast) that happens to land on the medium can/will grow into a small colony that we can see and count.

Koch and other researchers at that time were looking for a suitable medium upon which to grow bacteria for study. Gelatin was ruled out as a solidifying agent because (as any picnic-goer knows) a mixture of gelatin and water is not solid at body temperature (37° C). Also, because it is a polypeptide, it is digested by a number of species of bacteria, thus losing its gelling ability. A housewife friend suggested that Koch try agar, noting that many cooks used it to solidify desserts, etc. in place of gelatin. Koch subsequently developed the use of agar, a sulfuric ester of a polysaccharide (**poly** = many, **sacchar** = sugar) complex derived from certain red algae (Japanese Isinglass, *Gelidium* spp., is the best, but also derived from other genera – obtained by boiling the seaweed for six hours in dilute H_2SO_4), to solidify nutrient liquid media. This allowed the growth on the surface of the medium of a specimen spread across it, thus isolating the various microorganisms (**micro** = small) which might be present. Agar was found to be ideal as a gelling agent – it melts/dissolves only when the media are heated to near boiling, will remain melted until cooled to around 40° C, and forms a gel that stays solid at body temperature. Because it is a complex polysaccharide, it is not degraded by the vast majority of bacteria, thus the medium remains solid and does not liquify as the bacteria grow in/on it, and therefore allows strict control of growth factors which may be limiting.

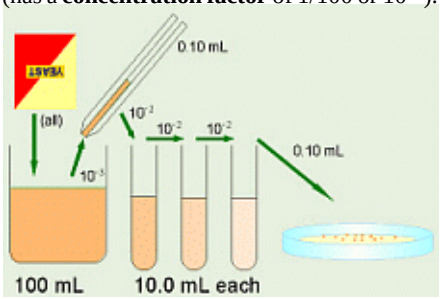
In his work with bacteria, Koch developed a series of methods for determining which species of bacterium is responsible for a given disease. In his honor, these four steps are collectively known as **Koch's Postulates**. These say that to prove that a given bacterium causes a certain disease, the researcher must:

a) find the same bacterium species in all diseased individuals investigated,
b) isolate the bacterium from diseased individuals and grow it in a pure culture,
c) induce the disease in experimental animals by transferring bacteria from the culture, and
d) isolate the same bacterium from the animal after the disease develops.

Experiments in this lab have shown that *Saccharomyces cerevisiae* (**myce** = fungus, **Ceres** = goddess of grain, **visi** = look or see) grows optimally on a nutrient agar supplemented with 4% glucose (**gluco** = sweet, – **ose** = sugar or carbohydrate). Note that glucose and dextrose (**dextro** = right) are just two names for the same thing. This medium contains nutrient broth which consists of 0.3% beef extract, and 0.5% of a pepsin digest of beef (peptone). It thus contains a broad variety of amino acids and vitamins providing a suitable medium for a wide variety of non-fastidious microorganisms (fastidious ones have very complex, specific nutritional requirements). The 4% glucose content especially encourages the growth of yeast with its ability to ferment at high rates.

By counting the number of colonies that grow on a sterile agar plate, one can determine the number of yeast cells in a packet of yeast. However, there are too many yeast cells to count them "as-is" so they must first

be diluted. In a **serial dilution**, aliquots of some solution are diluted stepwise such that the first dilution serves as the source from which an aliquot is taken for the second dilution, etc. In each of the dilutions you will be performing in this lab, 0.1 mL of yeast solution will be added to 9.9 mL of sterile dH_2O. This means that each 0.1 mL aliquot will be diluted to 10 mL thus the volume has increased 100 times, yet that 10 mL contains the same number of yeast cells as the 0.1 mL from which it was made. Therefore, the new solution is 100 times as dilute (has a **dilution factor** of 100 or 10^2). Another way to look at this is to say that it is 1/100 as concentrated (has a **concentration factor** of 1/100 or 10^{-2}).



You will be doing three of these dilutions to reach the desired dilution. In the first dilution, 0.1 mL will be diluted to 10 mL. Thus, the 0.1 mL used to make the second dilution will be 10^{-2} as concentrated as (will contain 10^{-2} times as much yeast as) the 0.1 mL you used initially. Since the second 0.1 mL is also diluted to 10 mL, then the 0.1 mL aliquot removed from that will contain $10^{-2} \times 10^{-2} = 10^{-4}$ of the yeast in the original 0.1 mL. This aliquot will, again be diluted to 10 mL, thus the 0.1 mL removed from that dilution to inoculate the medium is $10^{-2} \times 10^{-2} \times 10^{-2} = 10^{-6}$ as concentrated as (contains 10^{-6} of the yeast in) the original 0.1 mL aliquot.

Another important factor to notice here is that the "original" 0.1 mL aliquot used will not contain all of the yeast from the yeast packet. The packet of yeast will be suspended in 100 mL of water and the original aliquot will contain only 1/1000 (= 10^{-3}) of the yeast in the packet. If the final aliquot plated out only has 10^{-6} times as much yeast as the original aliquot, that means it has $10^{-6} \times 10^{-3} = 10^{-9}$ of the yeast from the yeast packet. You will also be making another plate using 0.2 mL of the last dilution, thus it will have 2×10^{-9} of the yeast from the yeast packet.

Each live, healthy yeast cell which is put onto the medium should grow by mitosis into a colony of yeast, hence it is referred to as a **colony-forming unit** or **CFU**. Thus, the number of yeast colonies which can be counted on a plate should be equal to the number of healthy yeast cells in the "final" aliquot of solution. This means that for the plate inoculated with 0.1 mL, the number of yeast colonies counted will be 10^{-9} of the yeast in the packet, or turned around, the packet contained 10^9 times as many yeast cells as are counted on the plate. For the 0.2 mL plate, the number of colonies counted will be 2×10^{-9} of the yeast in the packet, or the packet contained 5×10^8 times as many yeast cells as are counted on the plate.

PART A – MEDIUM PREPARATION
(Work in groups – 2 batches of agar per lab section of 15 to 20 people should suffice.)

III. MATERIALS NEEDED:

Nutrient broth powder, 6 g	balance	1000 mL beaker
Agar-agar, 9 g	spirit thermometer, –10 to 110° C	funnel
Glucose, 24 g	spoons or spatulas	heat source, hot pads
(opt. NaCl, 3 g)	1-L bottle with cap	autoclave
dH_2O, 600 mL	16 to 20 sterile petri dishes	incubator

IV. PROCEDURE:

WEIGH OUT THE DRY INGREDIENTS

A. Tare out the 1000 mL beaker, record apparent weight.
B. Add mass of reagent to tare weight, set balance to read that weight.
C. Add dry reagent with care to beaker until equal swings are achieved. Do not remove any excess back to the original container. Replace the cover immediately on nutrient broth container because it is **hygroscopic** (**hygro** = moist or wet, **scope** = see or watch or look; it absorbs moisture from the air and "turns into a rock," like brown sugar does in the summer). Record the actual amount added in each case.
D. Repeat steps 2 & 3 for other dry materials. (Use the new weight as the new tare, repeat the sequence of steps.)
E. Add dH_2O to the beaker with stirring, q.s. to 600 mL.

DISSOLVE AND STERILIZE THE MEDIUM

F. Stirring with a thermometer (**thermo** = heat, **meter** = to measure), heat to boiling, but do <u>not</u> allow to boil over, nor to burn on bottom. Optionally, medium may be heated in a microwave: heat 2 min., swirl, heat 2 min, swirl, heat 1 min. Have hot pads available.
G. Transfer to a 1-L bottle with a funnel. Cap loosely, label bottle with group name, and

place in autoclave. When all groups have put bottles in autoclave, autoclave at 15 lbs. pressure for 15 minutes.
H. Remove from autoclave, allow to cool to 50 to 60° C (feels hot, but possible to hold). The cooling process may be speeded up by immersion of the hot bottles of medium into a bucket of water at least 50° C, preferably 60°. (Colder water may cause the bottles to break – heat water on the stove if necessary to warm it)

POUR THE PLATES
I. On a sterile field, each student should pour two to three plates using sterile technique (your group should use up all the medium in your bottle – a total of 16 to 20 plates). To make it easier to pour the plates, position stacks of four sterile plates near the edge of the desk, remove cap and hold with little finger, flame lip of bottle, fill bottom plate first, at least half full.
J. Rinse bottle immediately after pouring last plate.
K. When plates have cooled and solidified, invert, place in 37° C incubator for 48 hours, check for sterility.

PART B – SERIAL DILUTION AND PLATE INOCULATION

III. MATERIALS NEEDED:

PER STUDENT:
- test tube rack to hold 16×150 mm test tubes (from under the sink)

A series of five stations should be set up on each side of the room as follows.

FIRST TABLE – REPIPET STATION:
- rack(s) with sterile, capped 16×150 mm test tubes (at least 3 tubes per student)
- 2 wax pencils (in drawers)
- 2 repipets with sterile dH_2O, set for 9.9 mL
- 2 Bunsen burners
- at least 1 striker
- squirt bottle of 70% EtOH

SECOND TABLE – SERIAL DILUTION STATION:
- 250 mL beaker containing 100 mL dH_2O, on magnetic stirrer
- fresh packet of bakers' yeast
- sterile 0.1 mL pipets (at least 3 per student)
- at least 2 pipet bulbs
- 2 Bunsen burners
- at least 1 striker
- used pipet container
- vortex
- squirt bottle of 70% EtOH

THIRD TABLE – SPECTROPHOTOMETER STATION:
- spectrophotometer set at 660 nm
- lens paper (in drawers)
- 2 clean cuvettes (one containing dH_2O) in plastic test tube rack

FOURTH TABLE – PLATING-OUT STATION:
- 2 turntables
- 2 spreaders, each in a beaker with 70% EtOH
- sterile 1.0 mL pipets (at least 1 per student)
- at least 2 pipet bulbs
- 2 Bunsen burners
- at least 1 striker
- used pipet container
- 2 wax pencils (in drawers)
- squirt bottle of 70% EtOH

FIFTH TABLE:
- sterile 4%-glucose nutrient agar plates from last lab
- plastic "dish" tub into which to put inoculated plates

OTHER:
- incubator set for 37° C

IV. PROCEDURE:

A. At the second station, your instructor will suspend the contents of a package of yeast in 100 mL water. This will be mixed thoroughly (using a magnetic stirrer) for at least 5 to 10 min.
B. Obtain test tube rack (under the sink).

At the first station:
C. Obtain three, sterile, capped, 16×150 mm test tubes and label them "2," "4," and "6" to represent the three serial dilutions (10^2, 10^4, and 10^6) you will be making.
D. Obtain one paper towel. Squirt some of the 70% EtOH onto the table top, and wipe to sterilize it.
E. As demonstrated by your instructor, remove the lid from a test tube, flame the lip of the tube and deliver 9.9 mL of sterile dH_2O into it using a repipet. Keep the cap off the tube the minimum time necessary and hold it with your little finger – do not set it down.
F. Flame the lip, of the test tube again, and replace the lid.
G. Repeat for your other two tubes.

At the second station:
H. Obtain one paper towel. Squirt some of the 70% EtOH onto the table top, and wipe to sterilize it.
I. Perform a 10^6 serial dilution of the yeast suspension as follows, using a fresh 0.1 mL pipet each time. Do this according to the following protocol for sterile delivery of liquids by pipet:

STERILE DELIVERY OF LIQUIDS BY PIPET

This procedure is extremely important in microbiological labs, and is one of the crucial techniques in aseptic (**a**– = not or without, **septi** = rotten or putrid) technique. While the steps may seem overly detailed in the following narrative, care in learning proper technique at the beginning establishes good technique for the rest of your life. Compare these detailed steps with the demonstration given by the professor. Patience pays off. Go slowly at the beginning, and verbally (not physically) assist your fellow students as they work through the steps. Begin with a sterile field by wiping the desktop with 70% EtOH (95% will dehydrate the bacteria rather than being absorbed to kill them). It is necessary to do this once at the beginning of the lab, but use your judgement if you think it needs to be done again (if someone leans on the table).

1. Select pipet appropriate for volume to be delivered. Hold pipet between thumb and middle finger of writing hand, control flow with index finger. Have little finger available for grasping cap of test tube. Pass pipet through flame.
2. Pick up test tube with non-writing hand, grip cap of vessel with little finger of pipet hand and remove with twisting, pulling motion. Do not lay the cap down.
3. Flame lip of test tube, then set the test tube back in the rack. Pick up pipet bulb in non-writing hand.
4. Withdraw desired volume of liquid. Then, tilt pipet almost horizontally so that fluid moves up slightly into pipet. Keep your index finger over the end! Set down bulb, pick up test tube, and reflame lip of test tube. Replace cap and return test tube to rack.
5. With non-writing hand, pick up new test tube into which fluid is to be delivered. Remove cap and flame lip as in steps 2 and 3. Release your finger to deliver the desired volume of liquid into the receiving test tube, remembering to blow out the last drop if needed. Flame the lip of the test tube, then replace the cap. After the test tube is safely sealed, place the used pipet into plastic receptacle for future cleaning.

1. Out of the 100 mL of yeast suspension that was made, you will use 0.10 mL to do the serial dilution. Note that this 0.10 mL contains 1/1000 or 10^{-3} of the yeast in the original packet (0.10/100 = 1/1000).
2. Deliver 0.10 mL of yeast suspension into the first tube (2) and mix well with vortex. This means that you have used that 0.1 mL of solution to make 10 mL of solution – 100 times as much, thus the concentration is 1/100 as strong as it was before. As you may recall, 1/100 = 10^{-2} (**concentration factor**).
3. Using proper sterile technique and a new, sterile pipet, deliver 0.10 mL from (2) tube into (4) tube and mix with vortex. You now have a solution that is 100 × 100 = 10,000 times as dilute (**dilution factor**) ($10^2 \times 10^2 = 10^4$). Note that the 0.10 mL used to make this dilution contains $10^{-3} \times 10^{-2} = 10^{-5}$ of the yeast in the original packet.
4. Again, using sterile technique and a new, sterile pipet, deliver 0.10 mL from (4) tube into (6) tube and mix with vortex. You now have a solution that is 100 × 10,000 = 1,000,000 times as dilute ($10^2 \times 10^4 = 10^6$). Note that the 0.10 mL used to make this dilution contains $10^{-5} \times 10^{-2} = 10^{-7}$ of the yeast in the original packet.

At the third station:
J. Use a spectrophotometer to determine the A_{660} of the 10^2 dilution and record that number in your lab notebook. Note that this solution is no longer sterile.

At the fourth station:
K. Obtain one paper towel. Squirt some of the 70% EtOH onto the table top, and wipe to sterilize it.

L. Obtain two, sterile, 4%-glucose nutrient-agar plates and label the **bottom** of each in small letters (you'll need to be able to see through the plate) with the date, your initials, and aliquot size (0.2 or 0.1 mL).
M. Using a 1.0 mL pipet, as explained below, plate out 0.10 and 0.20 mL of the 10^6 dilution onto the corresponding plates.
N. Note that since you are using only 0.1 mL or 0.2 mL out of the 10 mL in the last dilution, the 0.1 mL spread on the first plate contains $10^{-7} \times 10^{-2} = 10^{-9}$ of the yeast in the original packet. The 0.2 mL sample, thus, contains 2×10^{-9} of the yeast.

SPREADING TECHNIQUE FOR PLATE COUNT

1. Clean surface of plates with 70% EtOH, label with date, initials, sample source, volume plated. (Enter the same data into a table in your notebook.)
2. Flame pipet and mouth of test tube (do not place lid on desk, but rather hold in crook of little finger.). Return test tube to rack while you pipet.
3. As before, use a bulb to draw a slight excess of fluid into the pipet, and close off top of pipet with dry index finger (do not get the top of the pipet wet or control of flow is very difficult.) Adjust to desired volume.
4. Set down the bulb and pick up the test tube to flame its lip, then replace its cap, and set it back in the rack. While doing this, remember to keep the pipet closed off and nearly horizontal so that fluid does not leak out.
5. Working quickly and accurately, deliver desired aliquot of sample to the plate(s), and if needed, blow out the last drop to ensure total transfer to plate. Since you need to inoculate two plates, one with 0.1 mL and one with 0.2 mL, use a 1.0 mL pipet to obtain at least 0.3 mL of yeast solution. From this, release 0.1 mL into the "0.1 mL" plate and 0.2 mL into the "0.2 mL" plate.
6. Place pipet into used pipet receptacle. Place the 0.1 mL plate onto the turntable.
7. Obtain a spreader and shake off excess EtOH. When it is no longer dripping, pass the spreader briefly through a flame to ignite the rest. DO NOT HOLD FLAMING SPREADER OVER THE BEAKER OF EtOH!
8. When flaming has stopped, touch spreader to inside of the top of petri dish to cool (if necessary), then, turning the turntable slowly with one hand (which also holds the lid of the petri dish over the agar), rotate spreader in the opposite direction on surface of agar with the other hand. Do not press hard enough to damage agar surface. Hold petri dish top above plate to reduce airborne fallout contamination. When the fluid has been absorbed, the spreader will drag with more difficulty. Place spreader into 95% EtOH.
9. Remove 0.1 mL plate from turntable and place 0.2 mL plate on turntable. Spread the second plate as above. Invert both plates and incubate at desired temperature for two days.

O. Place inoculated plates in the designated bin for storage. They should be stored agar-side-up in the incubator so any condensation formed will be absorbed back into the agar rather than dripping onto the plate. Incubate at 37° C for 48 hr.

PART C – COUNTING YEAST COLONIES

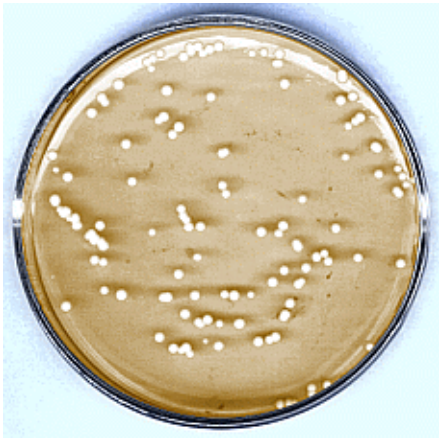
III. MATERIALS NEEDED:

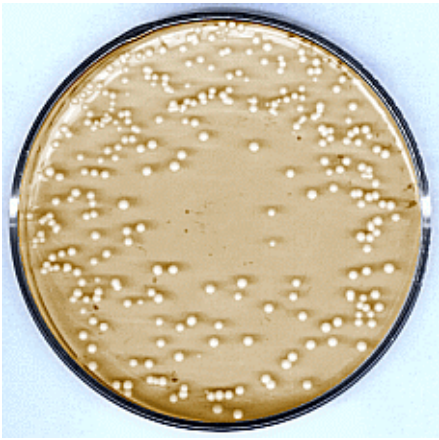
petri plates from last period
wax pencil and/or clicking counter
opt. colony counter or other light source

IV. PROCEDURE:

1. Count the number of colonies on each of your plates. In theory, the 0.20 mL plate should have approximately twice the colonies as the 0.10 mL plate. Record these numbers in your lab notebook and in the computer.
2. Assuming that one yeast cell can grow to form a colony of yeast as it grows and divides (it is a colony-forming unit), calculate the number of **colony-forming units (CFU)** in the original yeast package and also enter these data into the computer. Note that the 0.1 mL plate is 10^{-9} as concentrated as the original package. The 0.2 mL plate is 2×10^{-9} as concentrated as the original package. Thus, the package of yeast would contain $10^9 \times$ [number of colonies on the 0.1 mL plate] or $5 \times 10^8 \times$ [number of colonies on the 0.2 mL plate] CFUs (actual yeast cells).

V. DATA:





Record all data as indicated in the procedure, both into your notebook and the computer. Draw illustrations of all new equipment (autoclave, repipet, incubator, test tubes with lids, vortex, 0.10 and 1.0 mL pipets with markings, turntable and spreader, Bunsen burner, etc.), making sure to label all significant parts/features. Draw and/or xerox your final results/plates. Make sure to enter all data requested (including seat number, initials, A_{660} reading, number of colonies counted on the 0.1 and 0.2 mL plates) into the computer so that they can be xeroxed for class distribution.

In your lab notebook, do the dilution practice problems which follow.

VI. DISCUSSION:

1. Comment on your reaction to discovering the number of yeast cells in a package of yeast.
2. There is one tablespoon of yeast in a package and there are three teaspoons in a tablespoon. Based on our calculations of the number of cells/packet, how many yeast cells would there be in 1/8 teaspoon? How much of a teaspoon does one yeast cell fill? Optionally, if the weight of a yeast packet is given, how many grams does one yeast cell weigh? If you had one "mole" of yeast cells, how much would that weigh (what would the "molecular weight" of yeast cells be?

DILUTION PRACTICE PROBLEMS

Because solutions in science are often much more concentrated than are desired or can be managed for a given protocol, it is frequently necessary to dilute these solutions to a desired range. This requires a working knowledge of the principles of diluting, dilution factors, concentration factors and the calculations involved. High dilutions are usually expressed exponentially.

DEFINITIONS:

Aliquot: a measured sub-volume of sample.
Diluent: material with which the sample is diluted
Dilution factor: ratio of final volume (aliquot plus diluent volume) divided by the aliquot volume
Concentration factor: ratio of aliquot volume divided by the final volume

Example: You make a dilution by adding 0.1 mL specimen to 9.9 mL of diluent which gives a final volume of 10 mL:

Dilution Factor = final volume/aliquot volume = (0.1 + 9.9)/0.1 = 1 to 100, 1:100 or 10^2

Concentration Factor = aliquot volume/final volume = 0.1/(0.1 + 9.9) = 0.01 or 10^{-2}

To prepare a desired volume of solution of a given dilution:

1. Calculate the volume of the aliquot:
   aliquot volume = concentration factor × final volume
2. Calculate the volume of the diluent:
   volume of diluent = (final volume - sample aliquot volume)
3. Measure out the correct volume of diluent, add the correct volume of aliquot to it, mix.

SAMPLE PROBLEMS:

1. How much sample is required to prepare 10 mL of a 1 to 10 dilution, and how much diluent would you need?
2. What is the dilution factor when 0.2 mL is added to 3.8 mL diluent? What is the concentration factor?
3. What should the aliquot and diluent volumes be to prepare 5 mL of a 10^2 dilution?
4. You have 0.6 mL of sample, and want to dilute it to a fiftieth of its present concentration. How much diluent will you add, and what will the final volume be?
5. How would you prepare 20 mL of a 1:400 dilution?
6. What is the dilution factor when you add 2 mL sample to 8 mL diluent?
7. You want 1 liter of 0.1 M NaCl, and you have 4 M stock solution. How much of the 4 M solution and how much dH_2O will you measure out for this dilution?
8. You add a pint of STP gas treatment to a 12 gallon fuel tank, and fill it up with gas. What is the dilution factor?
9. You diluted a bacterial culture 10^6, and plated out 0.2 mL and got 45 colonies on the plate. What was the concentration of bacteria in the original undiluted culture?

A hard one:

10. You have 100.0 mL of dH_2O. How much glycerine would you have to add in order to make a 2.00 % v/v solution of the glycerine? (Hint: it requires a little algebra.)

Here's another "English system" one (for people who aren't interested in cars and STP?): if you are making homemade ice cream, and put 1 tsp of vanilla in a 1-gallon batch of ice cream mix, what is the dilution factor?