University of Samarra
College of science applied
Biotechnology/ lab
Department of biotechnology

Biotechnology
Principle of

Lab no.: 5

The enumeration methods of Microorganisms

Objectives: Each student should be able to describe several different ways to quantify the number of bacteria in a given sample.

The laboratory microbiologist often has to determine the number of bacteria in a given sample, as well as having to compare the amount of bacterial growth under various conditions. Which is important in dairy, food, & water microbiology.

The three most widely used methods for determining bacterial numbers are:
1- **Direct Microscopic Method (Total Cell Count).**
2- **Spectrophotometric (turbidimetric) method.**
3- The plate count method (standard, or viable counting)

1- **Direct Microscopic Method (Total Cell Count)**
In the direct microscopic count, a counting chamber consisting of a ruled slide and a coverslip is employed. A small number of bacteria in a volume is directly counted microscopically while the larger number of bacteria in the original sample is determined by preparing serial dilutions.

Materials Microscope, Hemocytometer & coverslip, Suspension of yeast

Procedure
1. Make a serial dilution series of the yeast suspension, from 1/10 to 1/10000.
2. Starting with the 1/10 dilution, use a Pasteur pipette to transfer a small aliquot of the dilution to the hemocytometer. Place the tip of the pipette into the H-shaped groove of the hemocytometer and allow the cell suspension to flow into the chamber

of the hemocytometer by capillary action until the chamber is filled. Do not overfill the chamber.
3. Add a similar sample of diluted yeast to the opposite side of the chamber and allow the cells to settle for about 1 minute before counting.
4. Refer to the diagram of the hemocytometer grid in Figure 1 and note the following.
5. The 4 outer squares, marked 1-4, each cover a volume of 10–4 mL.
6. The inner square, marked as 5, also covers a volume of 10–4 mL, but is further subdivided into 25 smaller squares. The volume over each of the 25 smaller squares is 4.0 × 10–6 mL.
7. Each of the 25 smaller squares is further divided into 16 squares, which are the smallest gradations on the hemocytometer. The volume over these smallest squares is .25 × 10–6 mL.
8. Given these volumes, the number of cells in a sample can be determined by counting the number of cells in one or more of the squares. Which square to use depends on the size of the object to be counted. Whole cells would use the larger squares, counted with 10X magnification. Isolated mitochondria would be counted in the smallest squares with at least 40X magnification.
9. For the squares marked 1–4, the area of each is 1 mm2, and the volume is .1 mm3. Since .1 mm3 equals 10–4 mL, **the number of cells/mL = average number of cells per 1 mm2 × 104 × any sample dilution.**
10. For the 25 smaller squares in the center of the grid marked 5, each small square is 0.2 × 0.2 mm2, and the volume is thus 0.004 mm3. **For small cells, or organelles, the particles/mL equals the average number of particles per small square × 25 × 104 × any sampledilution.**

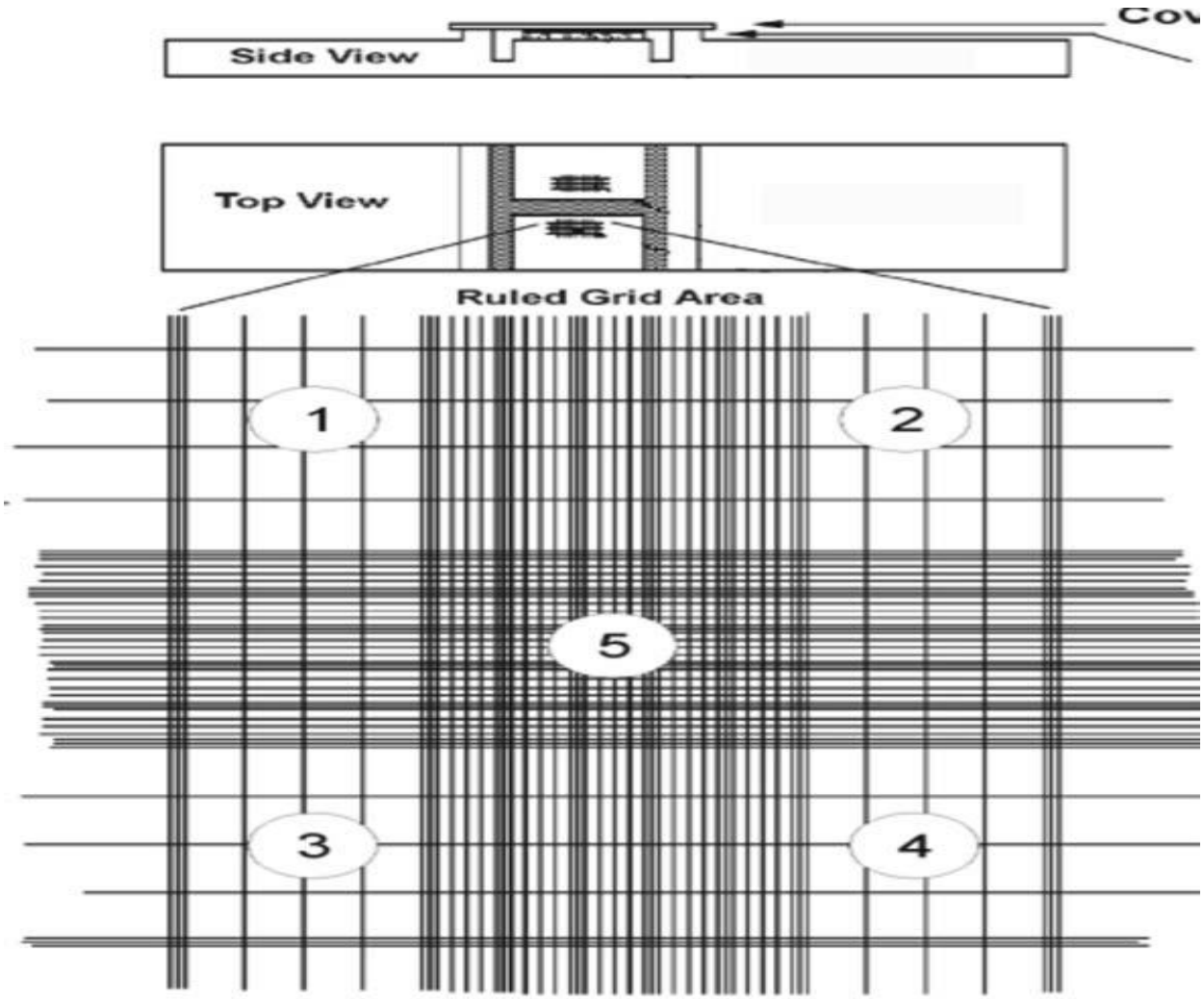

Figure 1

2- **Spectrophotometric (turbidimetric measurement) method.**
The spectrophotometric analysis is based on turbidity and indirectly measures all bacteria (cell biomass), dead or alive. The bacteria growing in a liquid culture appears turbid**.** This is because a bacterial culture acts as a colloidal suspension that blocks and reflects light passing through the culture.

The instrument used to measure turbidity is a spectrophotometer. Within limits, the light absorbed by the bacterial suspension will be directly proportional to the concentration of cells in the culture.

Procedure;

1. Twofold or tenfold serial dilutions made of a bacterial or yeast stock.
2. A counting chamber can then be used to perform a direct microscopic count on each dilution.
3. Then, a spectrophotometer that the correct wavelength in nanometers (550 - 600 nm) used to measure the absorbance of each dilution tube.
4. A standard curve comparing absorbance to the number of bacteria can be made by plotting absorbance versus the number of bacteria per ml (figure 2).
5. Once the standard curve is completed, any dilution tube of that organism can be placed in a spectrophotometer and its absorbance read. Once the absorbance is determined, the standard curve can be used to determine the corresponding number of bacteria per ml (figure 3).

Note; A tube that contains just sterile broth. This tube is called the **blank** because it has a sample concentration equal to zero. It should therefore have an absorbance of zero .

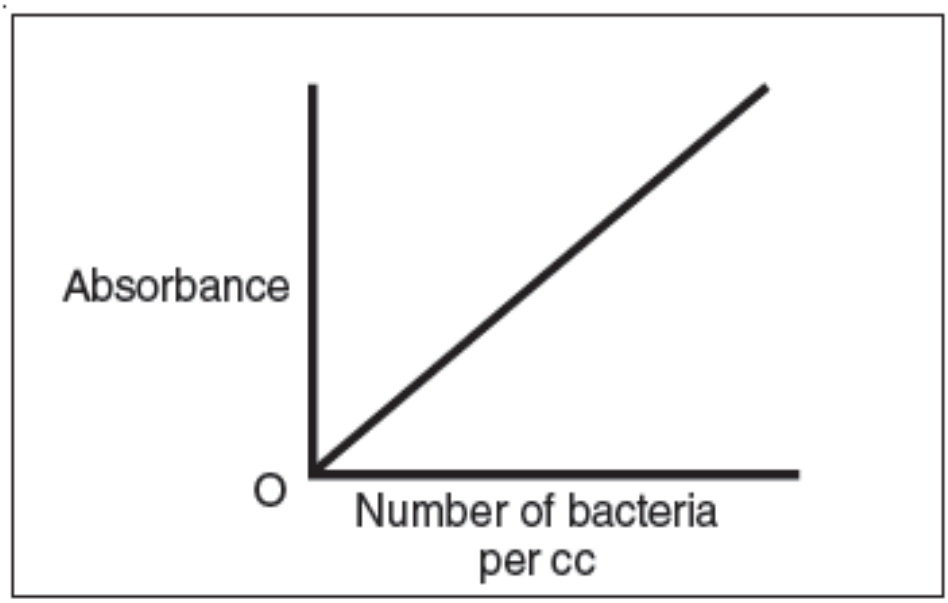

FIGURE 2: A standard curve plotting the number of bacteria per ml versus absorbance.

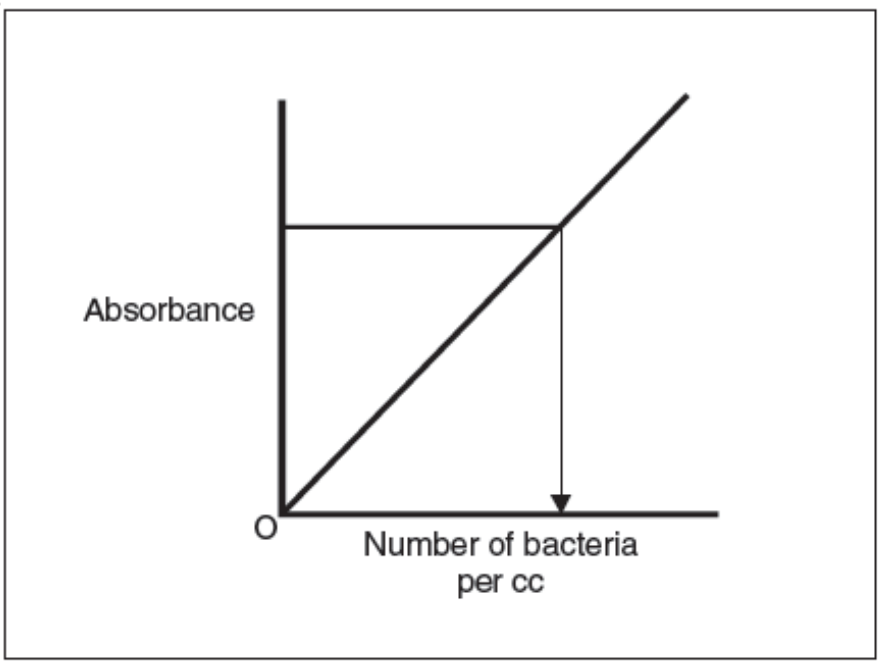

FIGURE 3 using a standard curve to determine the number of bacteria per ml in a sample by measuring the sample's absorbance.

3- The plate count method (standard, or viable counting)

It reveals information related only to live bacteria. It is an indirect measurement of cell density due to the number of bacteria in a given sample is usually too great to be counted directly, when the sample is serially diluted and then plated out on an agar surface, single isolated bacteria can form visible isolated colonies. The final plates in the series should have between 25 and 250 colonies. Each one distinguished as distinct **colony-forming units (CFUs).**

The number of colonies can be used as a measure of the number of viable (living) cells in that known dilution.

Procedure

1-The original sample is diluted several times to dilute the population sufficiently.
2- 1 ml of each dilution is then dispensed into the bottom of a petri plate.
3- Agar pours are then added to each plate. Isolated cells grow into colonies and can be used to establish pure cultures.
4- After the pour plates have cooled and the agar has hardened, they are inverted and incubated at 35°C for 24 hours or 20°C for 48 hours.
5. At the end of the incubation period, select all of the petri plates containing between 25 and 250 colonies.
6- By using a colony counter Calculate the number of bacteria (CFU) per milliliter of original sample as follows:
Number of CFUs per mL of sample = Number of colonies (30–300 plate) × the dilution factor of the plate counted.
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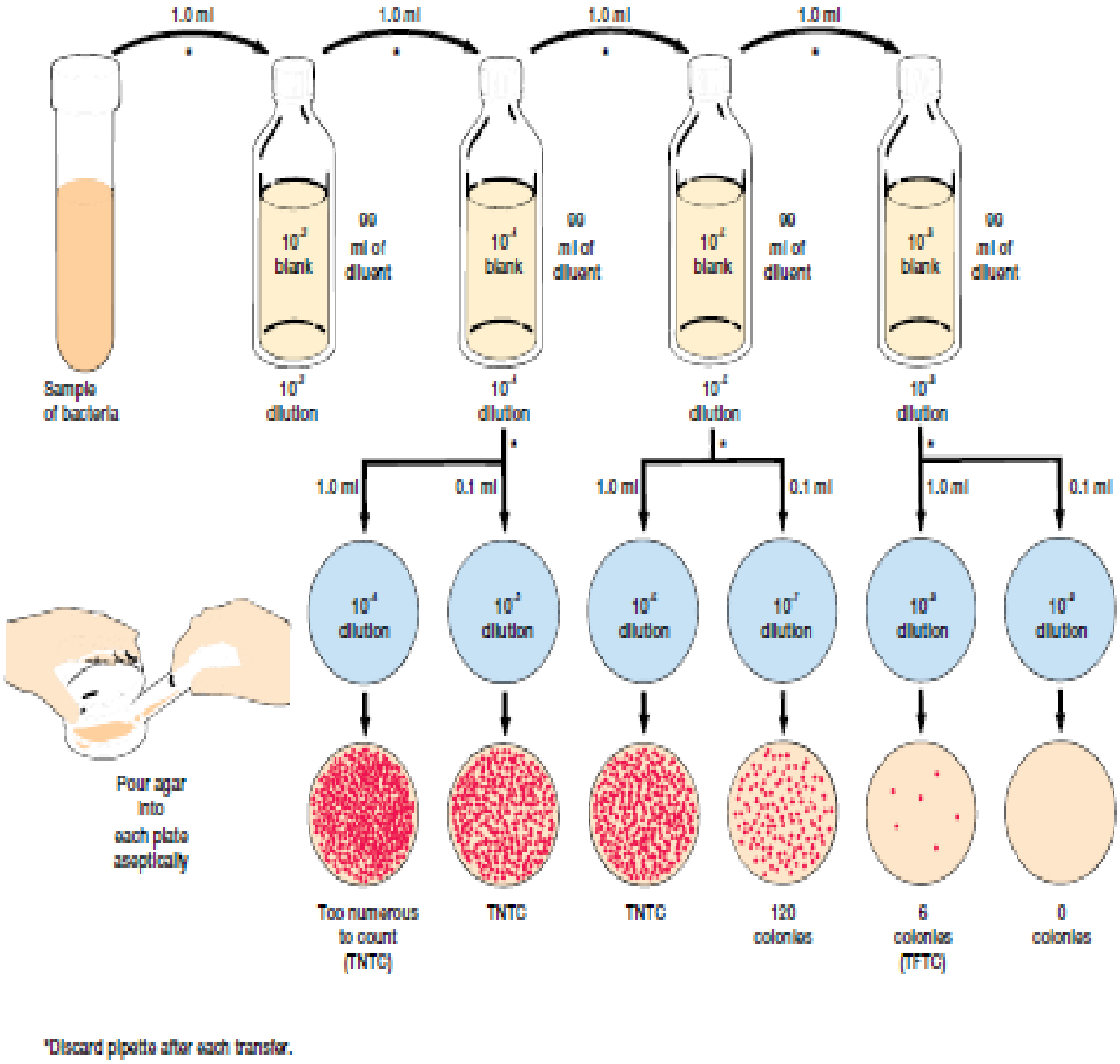

Figure 4 Quantitative Plating Procedure.