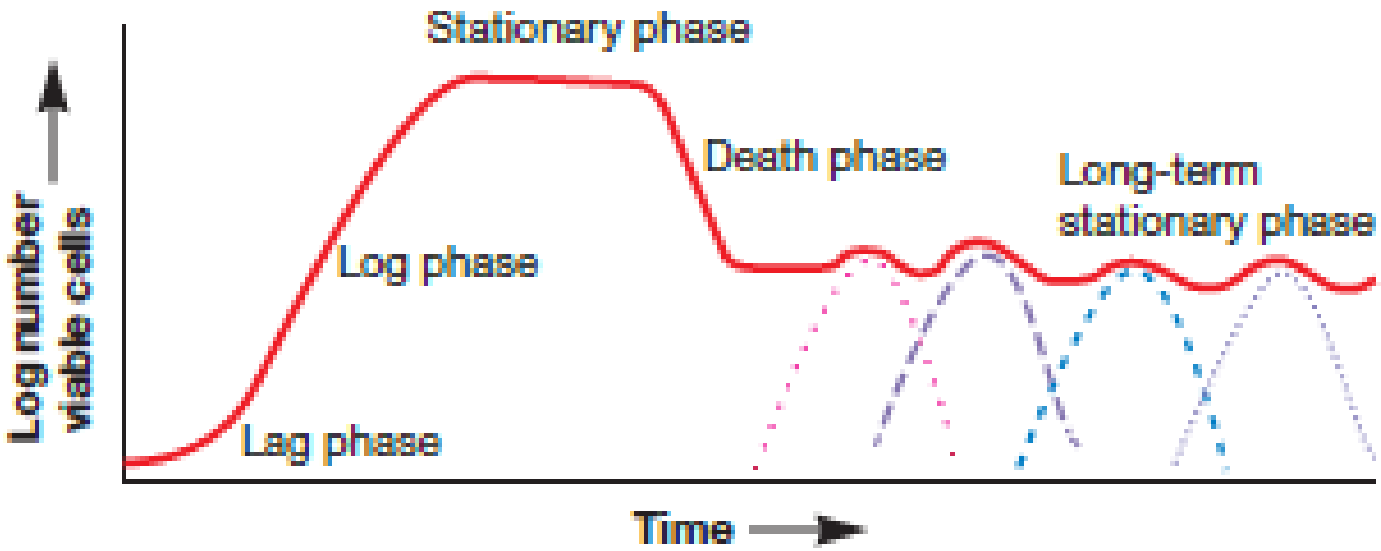
UNIT III (20 Periods)

- Microbial growth: Growth curve, Generation time, synchronous batch and continuous culture, measurement of growth and factors affecting growth of bacteria.
- Microbial Metabolism: Metabolic pathways, amphi-catabolic and biosynthetic pathways
- Bacterial Reproduction: Transformation, Transduction and Conjugation. Endospores and sporulation in bacteria.

Microbial growth: Growth curve, Generation time,



Population growth is often studied by analyzing the growthof microbes in **batch culture;** they are incubated in a closed culture vessel with a single batch of medium. Fresh medium is not provided during incubation, so nutrient concentrations decline and concentrations of wastes increase over time.

Population growth of microbes reproducing by binary fission in a batch culture can be plotted as the logarithm of the number of viable cells versus the incubation time. The resulting curve has five distinct phases:

Lag Phase

When microorganisms are introduced into fresh culture medium, usually no immediate increase in cell number occurs. This period is called the **lag phase.** It is not a time of inactivity; rather cells are synthesizing new components. The cells may be old and depleted of ATP, essential cofactors, and ribosomes; these must be synthesized before growth can begin. The medium may be different from the one the microorganism was growing in previously. In this case, new enzymes are needed to use different nutrients. Possibly the microorganisms have been injured and require time to recover. Eventually however, the cells begin to replicate their DNA, increase in mass, and divide.

Exponential Phase

During the **exponential** (**log**) **phase,** microorganisms are growing and dividing at the maximal rate possible given their genetic potential, the nature of the medium, and the environmental conditions.

Their rate of growth is constant during the exponential phase; that is, they are completing the cell cycle and doubling in number at regular intervals. The population is most uniform in terms of chemical and physiological properties during this phase; therefore exponential phase cultures are usually used in biochemical and physiological studies.

The growth rate during log phase depends on several factors, including nutrient availability. When microbial growth is limited by the low concentration of a required nutrient, the final net growth or yield of cells increases with the initial amount of the limiting nutrient present .

Stationary Phase

In a closed system such as a batch culture, population growth eventually ceases and the growth curve becomes horizontal.

This **stationary phase** is attained by most bacteria at a population level of around **10^9 cells per milliliter.**

Final population size depends on nutrient availability and other factors, as well as the type of microorganism. In stationary phase, the total number of viable microorganisms remains constant. This may result from a balance between cell division and cell death, or the population may simply cease to divide but remain metabolically active.

One reason microorganisms enter the stationary phase is nutrient limitation; if an essential nutrient is severely depleted, population growth will slow and eventually stop. Microbes enter the stationary phase for other reasons besides nutrient limitation. Aerobic organisms often are limited by O2 availability. Oxygen is not very soluble and may be depleted so quickly that only the surface of a culture will have an O2 concentration adequate for growth. Population growth also may cease due to the accumulation of toxic waste products. This seems to limit the growth of many cultures growing in the absence of O2.

For example, streptococci can produce so much lactic acid and other organic acids from sugar fermentation that their medium becomes acidic and growth is inhibited. Finally, some evidence exists that growth may cease when a critical population level is reached.

Death Phase

Cells growing in batch culture cannot remain in stationary phase indefinitely. Eventually they enter a phase known as the death phase. During this phase, the number of viable cells declines exponentially, with cells dying at a constant rate. It was assumed that detrimental environmental changes such as nutrient deprivation and the buildup of toxic wastes caused irreparable harm to the cells. That is, even when bacterial cells were transferred to fresh medium, no cellular growth was observed. Because loss of viability was often not accompanied by a loss in total cell number, it was assumed that cells

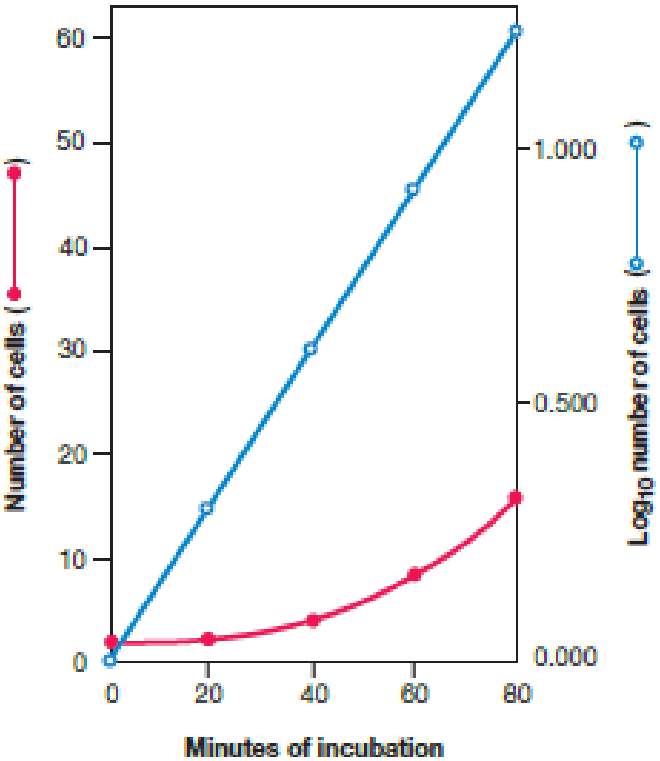
Viable but nonculturable (**VBNC**), is thought to be the result of a genetic response triggered in starving, stationary phase cells.

The second hypothesis is **programmed cell death.** In Contrast to the VBNC hypothesis whereby cells are genetically programmed to survive, programmed cell death predicts that a fraction of the microbial population is genetically programmed to

die after growth ceases. In this case, some cells die and the nutrients they leak enable the eventual growth of those cells in the population that did not initiate cell death. The dying cells are thus "altruistic"—they sacrifice themselves for the benefit of the larger population.

Long-Term Stationary Phase

Long-term growth experiments reveal that after a period of expo nential death some microbes have a long period where the popu Lation size remains more or less constant. This long-term stationary phase (also called extended stationary phase) can last months to years . During this time, the bacterial Population continually evolves so that actively reproducing cells are those best able to use the nutrients released by their dying brethren and best able to tolerate the accumulated toxins. This dynamic process is marked by successive waves of genetically distinct variants. Thus natural selection can be witnessed within a single culture vessel.



Exponential Microbial Growth. Four generations of growth are plotted directly (•—•) and in the logarithmic form (˚—˚). The growth curve is exponential, as shown by the linearity of the log plot.

Table 7.8 An Example of Exponential Growth

Time[1]	Division Number	2^n	Population[2] ($N_0 \times 2^n$)	$\log_{10} N_t$
0	0	$2^0 = 1$	1	0.000
20	1	$2^1 = 2$	2	0.301
40	2	$2^2 = 4$	4	0.602
60	3	$2^3 = 8$	8	0.903
80	4	$2^4 = 16$	16	1.204

Calculation of the growth rate constant

Let N_0 = the initial population number

N_t = the population at time t

n = the number of generations in time t

For populations reproducing by binary fission

$$N_t = N_0 \times 2^n$$

Solving for n, the number of generations, where all logarithms are to the base 10,

$$\log N_t = \log N_0 + n \cdot \log 2, \text{ and}$$

$$n = \frac{\log N_t - \log N_0}{\log 2} = \frac{\log N_t - \log N_0}{0.301}$$

The growth rate constant (k) is the number of generations per unit time $\left(\frac{n}{t}\right)$. Thus

$$k = \frac{n}{t} = \frac{\log N_t - \log N_0}{0.301t}$$

Calculation of generation (doubling) time

If a population doubles, then

$$N_t = 2N_0$$

Substitute $2N_0$ into the growth rate constant equation and solve for

$$k = \frac{\log (2N_0) - \log N_0}{0.301g} = \frac{\log 2 + \log N_0 - \log N_0}{0.301g}$$

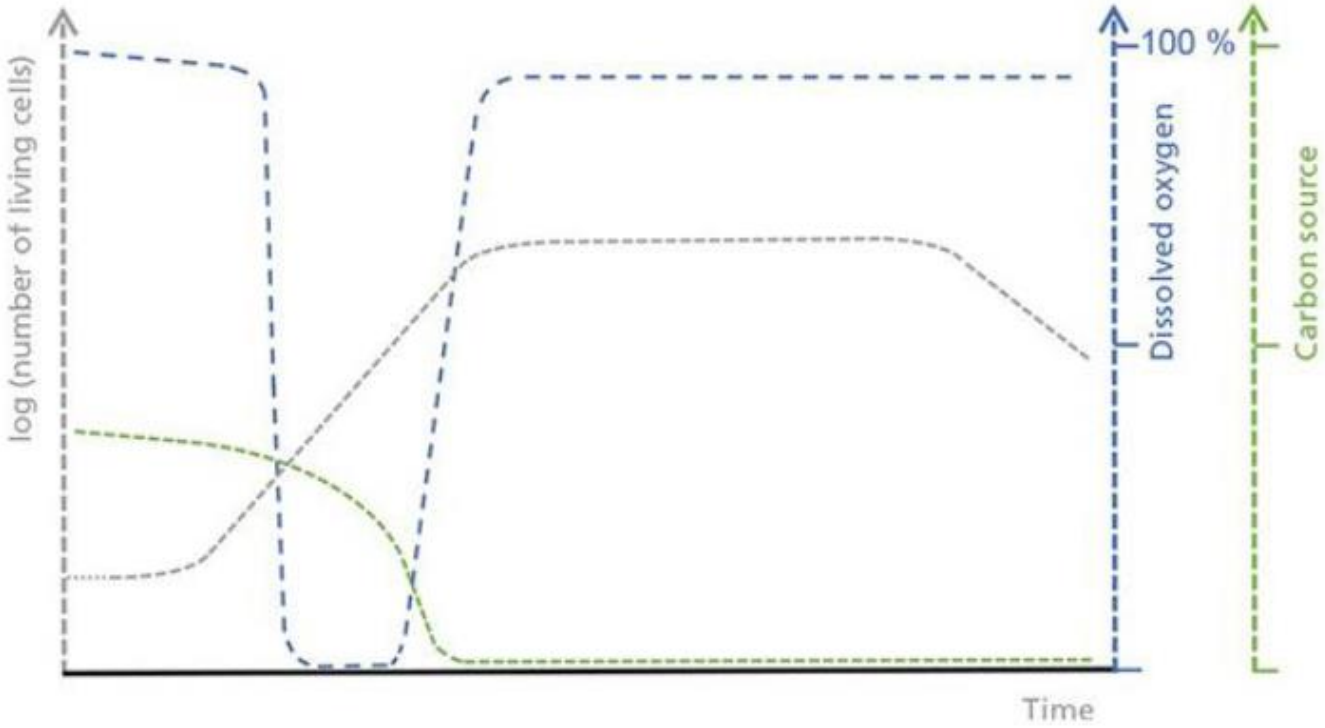
$$k = \frac{1}{g}$$

The generation time is the reciprocal of the growth rate constant.

$$g = \frac{1}{k}$$

- **Batch,** where no extra feeding is used from beginning to end of the process
- the batch process is classified as a discontinuous process,
- **Fed-batch**, where feeding with substrate and supplements can extend the duration of culture for higher cell densities or switch metabolism to produce e.g. a recombinant protein
- **Continuous culture,** where either the feed rate of a growth-limiting substance keeps cell density constant (a chemostat) or cell density determines the feed rate of the substrate (turbidostat). Cell retention can offer another, very productive option (perfusion).
- The incoming feed rate matches the rate of removal of harvest. The balanced nature of the feeding allows a steady state to be achieved which can last for days to months. This state is good for studying microbial metabolism or long-term production.
- **Repeated Fed-batch,** where harvesting all but a small residue of a completed (fed) batch and leaving the remaining cells available is to use as an inoculum for the next batch

- In a batch process, all nutrients are provided at the beginning of the cultivation, without adding any more in the subsequent bioprocess.
- During the entire bioprocess, no additional nutrients are added; it is a closed system. The bioprocess then lasts until the nutrients are consumed.
- Suitable for rapid experiments such as strain characterization or the optimization of nutrient medium.
- The disadvantage of this convenient method is that the biomass and product yields are limited.
- The microorganisms are not in the exponential growth phase for a long time.
- The advantages of a batch culture are:
- Short duration
- Less chance of contamination as no nutrients are added
- Easier to manage
- **Some disadvantages include:**
- Product is mixed in with nutrients, reagents, cell debris and toxins
- Shorter productive time



The correlations between living cell concentration, dissolved oxygen, and the limiting carbon source in batch operation

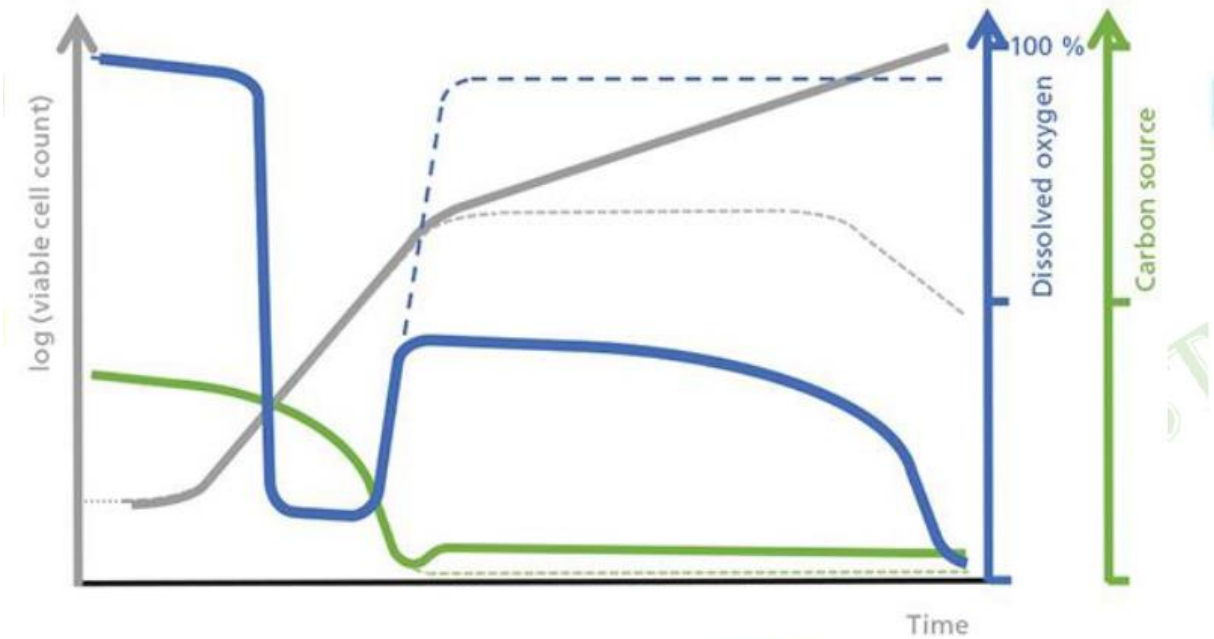
- Fed-batch process, is a partly open system, one or more nutrients are constantly supplied during cultivation.
- A fed-batch process is a semi-continuous process.
- Under specific growth conditions, the microorganisms and/or cells constantly double and therefore follow an exponential growth curve.
- Generally, the substrate is pumped from the supply bottle into the culture vessel through a silicone tube.

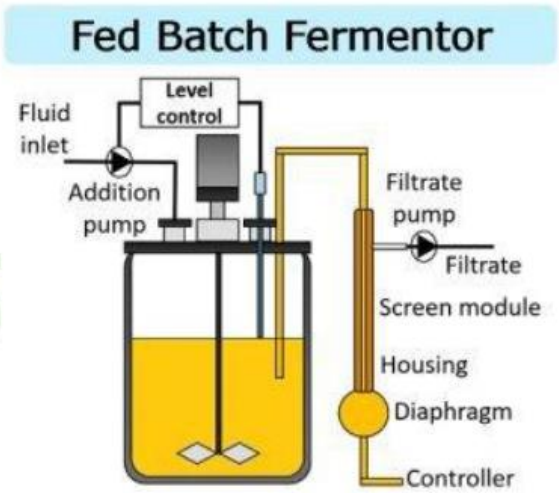
Advantages of a fed-batch culture are:

- Extends a culture's productive duration
- Can be used to switch genes on or off by changing substrate
- Can be manipulated for maximum productivity using different feeding strategies

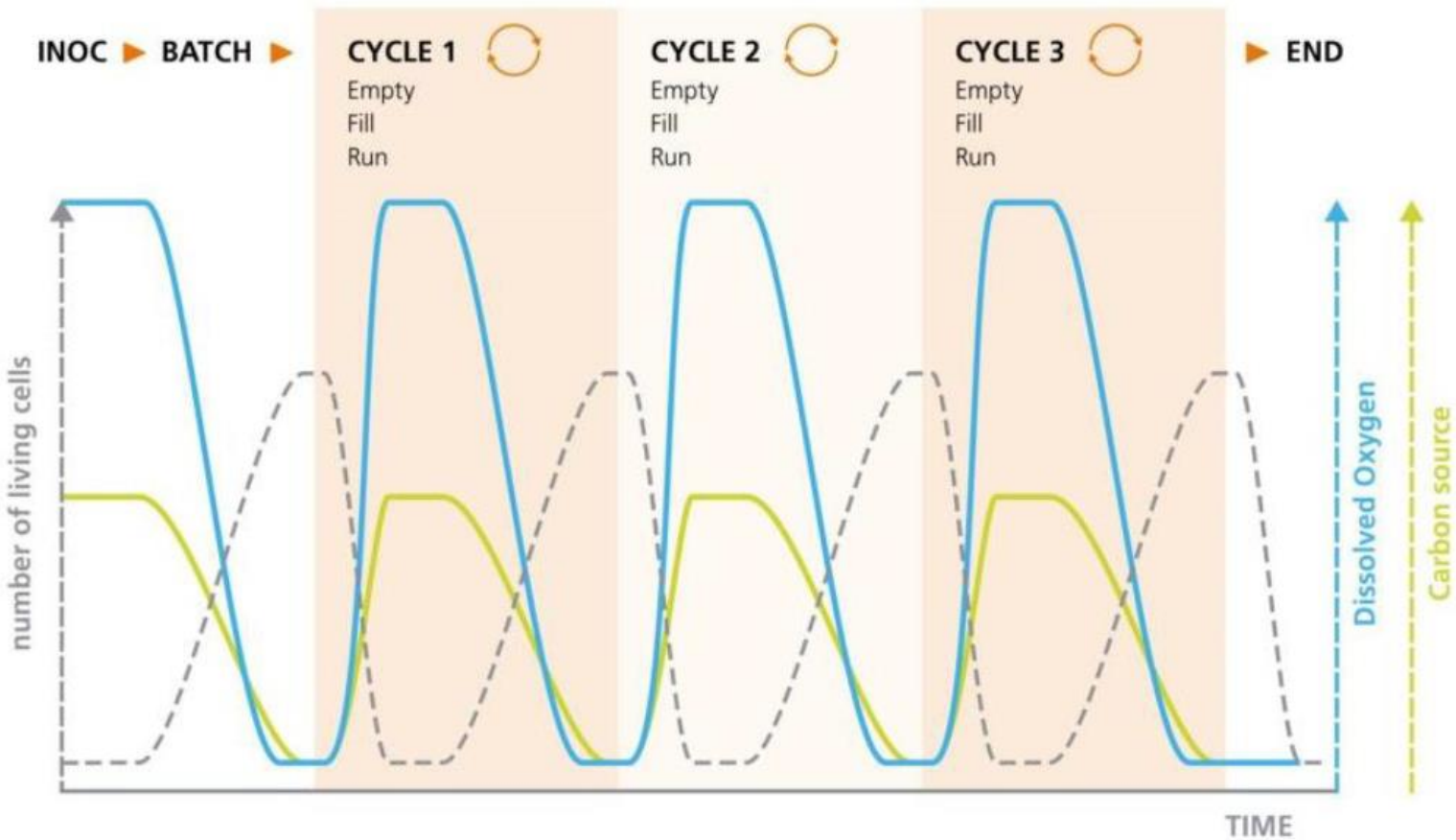
Disadvantages include:

- Allows build up of inhibitory agents and toxins
- Provides another point of ingress for contamination
- May produce high cell density numbers and product yields which are difficult to deal within downstream, creating bottlenecks in the whole process





The relationship between the living cell concentration, dissolved oxygen, and the limiting carbon source in the fed-batch process.



- The correlations between living cell concentration, dissolved oxygen, and the limiting carbon source in a repeated Fed-batch operation.
- At the end of batch cultivation, between one quarter and three quarters is harvested.
- The existing culture is used as inoculum for the next cycle and is supplemented with a fresh culture medium.

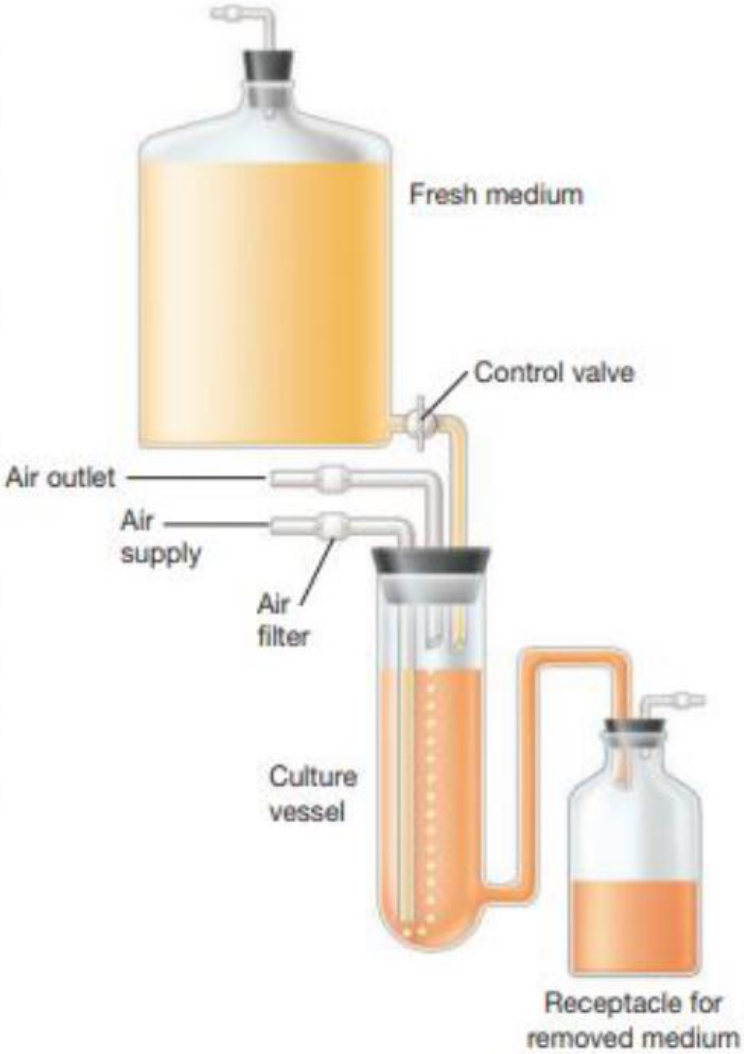
Continuous Culture System

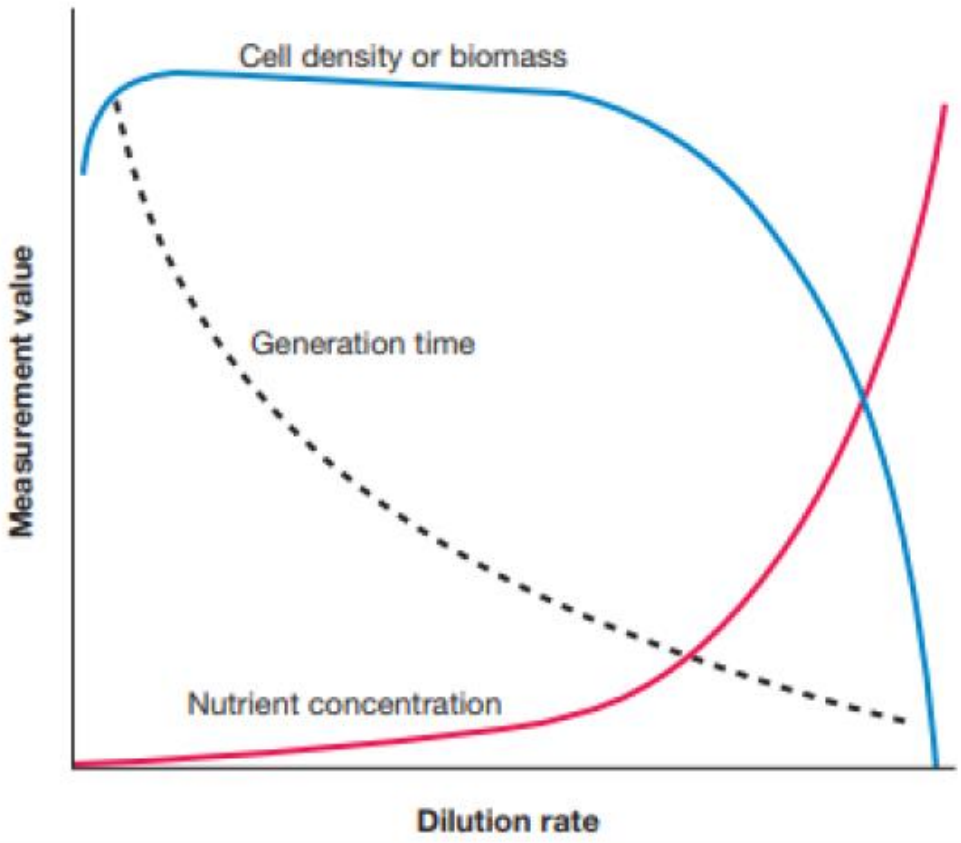
- Continuous culture system is a system where microorganisms are growing under constant environmental conditions, maintained through continual provision of nutrients and removal of wastes.
- Such a system is called a continuous culture system.
- These systems can maintain a microbial population in exponential growth, growing at a known rate and at a constant biomass concentration for extended periods.
- Continuous culture systems make possible the study of microbial growth at very low nutrient levels, concentrations close to those present in natural environments.
- These systems are essential for research in many areas.
- Continuous culture systems also are used in food and industrial microbiology.
- Two major types of continuous culture systems commonly are used: **chemostats** and **turbidostats.**

Chemostats

- A chemostat is constructed so that the rate at which a sterile medium is fed into a culture vessel is the same as the rate at which the medium containing microorganisms is removed
- The culture medium for a chemostat has a limited quantity of an essential nutrient (e.g., a vitamin).
- the final cell density depends on the concentration of the limiting nutrient.
- The rate of nutrient exchange is expressed as the dilution rate (D), the rate at which medium flows through the culture vessel relative to the vessel volume, where f is the flow rate (milliliter/hr) and V is the vessel volume (milliliter).

$$D=f/V$$

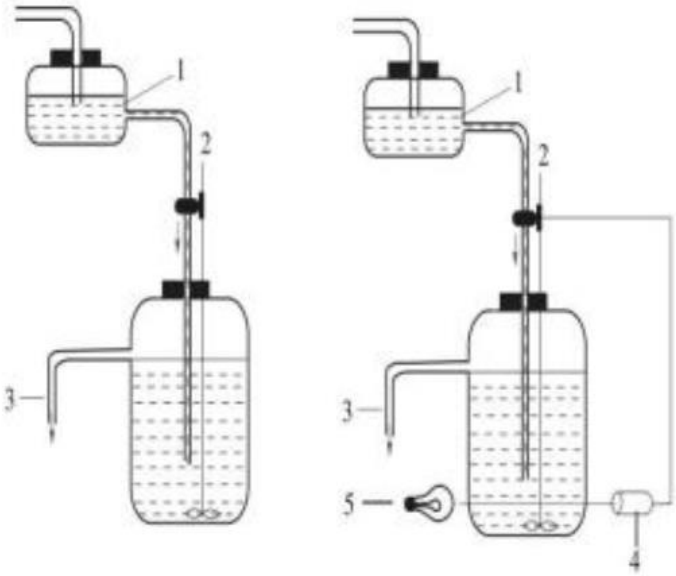




Chemostat Dilution Rate and Microbial Growth.
The effects of changing the dilution rate in a chemostat.

Turbidostats

- Turbidostats The second type of continuous culture system, the turbidostat, has a photocell that measures the turbidity (defined as the amount of light scattered) of the culture in the growth vessel. The flow rate of media through the vessel is automatically regulated to maintain a predetermined turbidity.
- Turbidostats maintains a desired cell density.
- The dilution rate in a turbidostat varies, rather than remaining constant, and a turbidostat's culture medium contains all nutrients in excess.
- A turbidostat operates best at high dilution rate

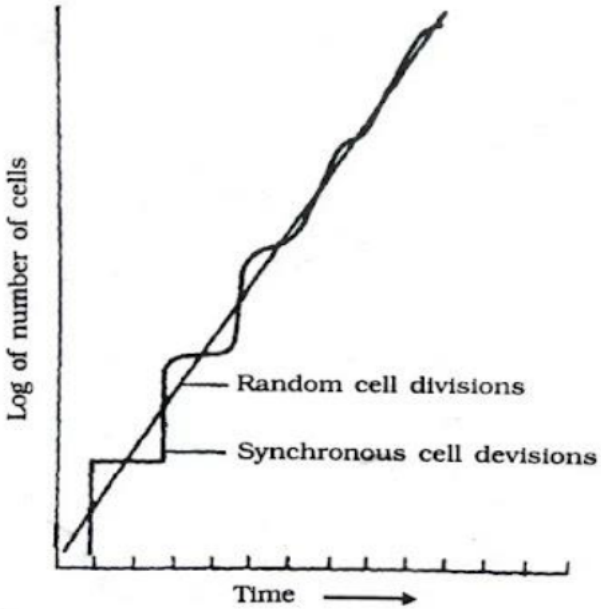


Chemostat Turbidostat

SYNCHRONOUS GROWTH

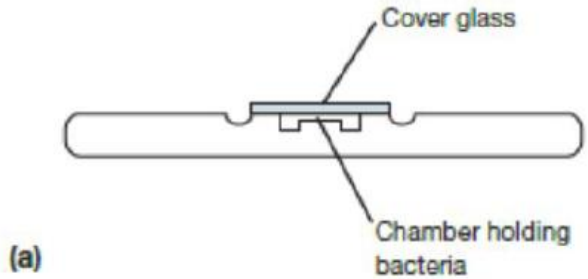
Synchronous Culture

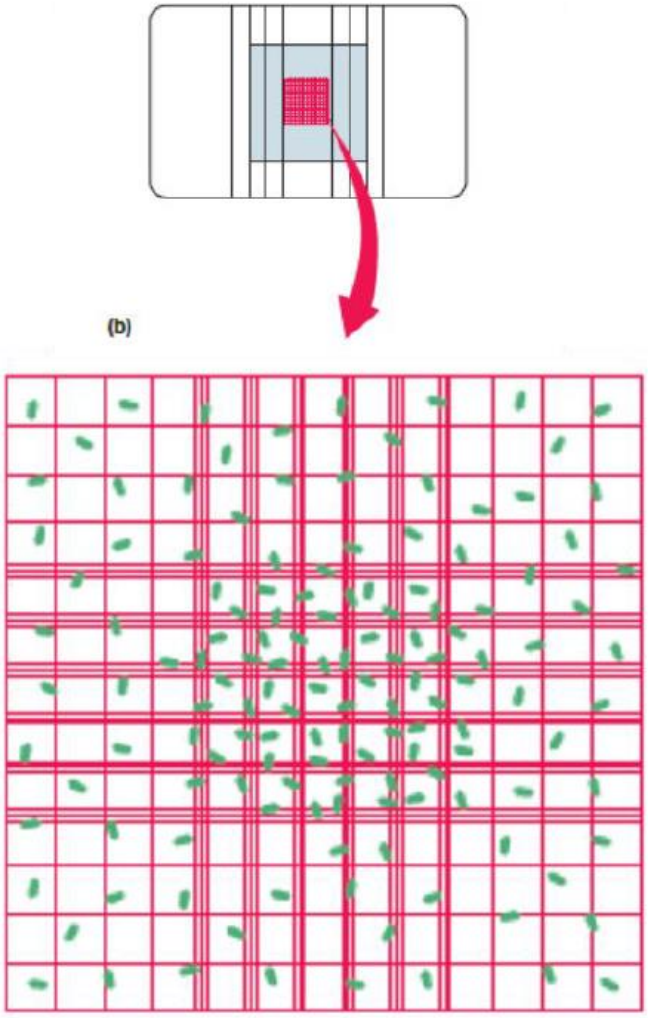
- Synchronous growth of a bacterial population is that during which all bacterial cells of the population are physiologically identical and in the same stage of cell division cycle at a given time
- Synchronous growth helps studying particular stages or the cell division cycle and their interrelations
- A synchronous culture can be obtained either by manipulating environmental conditions such as by repeatedly changing the temperature or by adding fresh nutrients to cultures as soon as they enter the stationary phase, or by physical separation of cells by centrifugation or filtration
- Most widely used method to obtain synchronous cultures is the Helmstetter-Cummings Technique in which an unsynchronized bacterial culture is filtered through cellulose nitrate membrane filter



Methods Of Microbial Growth Estimation

- **Direct**
  - **Direct Measurement of Cell Numbers**
    - Flow cytometry
    - Petroff-Hausser counting chamber
    - Coulter counter
  - **Viable Counting Methods**
    - Plate count Technique
      - Membrane Filter
      - Pour-Plate
      - spread plate
      - Streak Plate
    - Most Probable Number (MPN)
- **Indirect**
  - Measurement of Metabolic activity
  - Measurement of Cell Mass
    - Dry weight
    - Turbidity





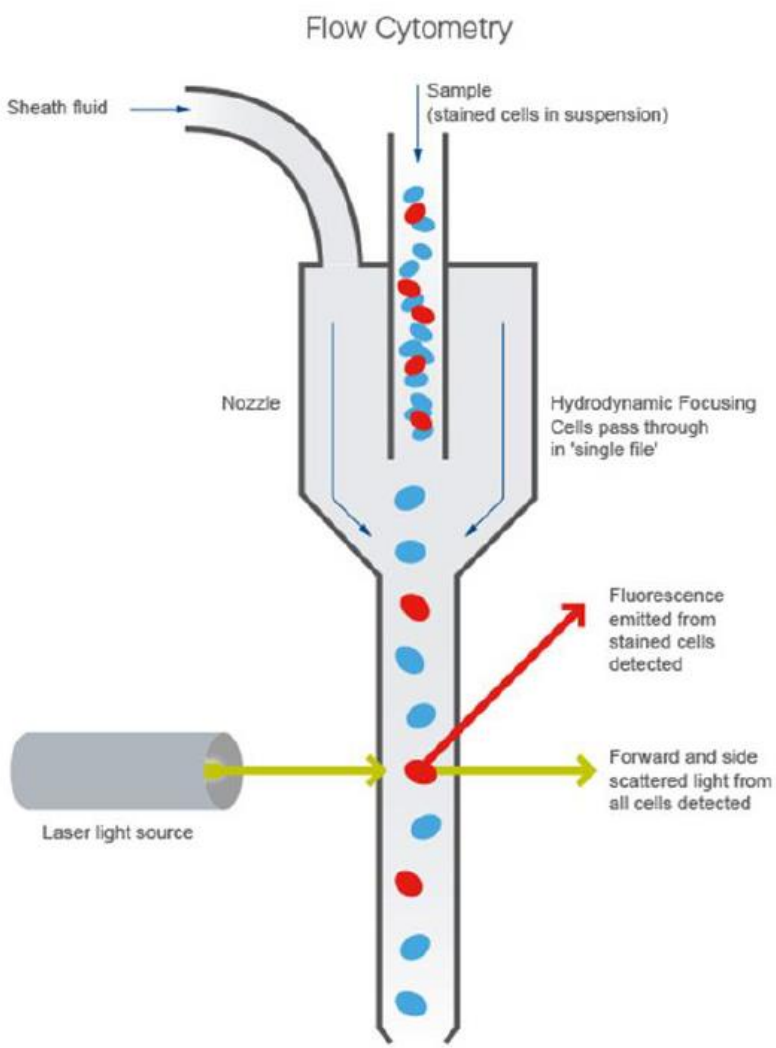
Petroff-Hausser counting chamber

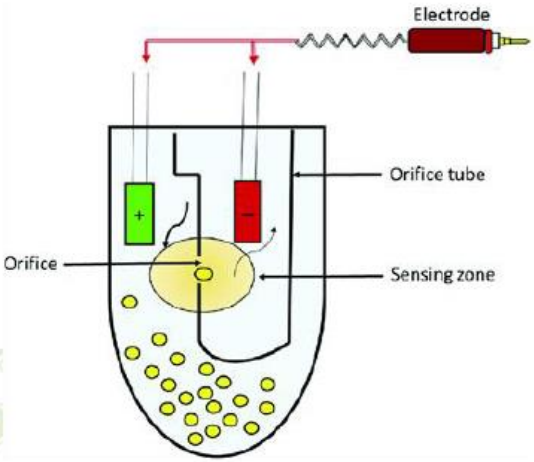
25 squares covering an area of 1 mm^2, the total number of bacteria in 1 mm^2 of the chamber is (number/square)(25 squares). The chamber is 0.02 mm deep and therefore, bacteria/mm^3 (bacteria/square)(25 squares)(50).

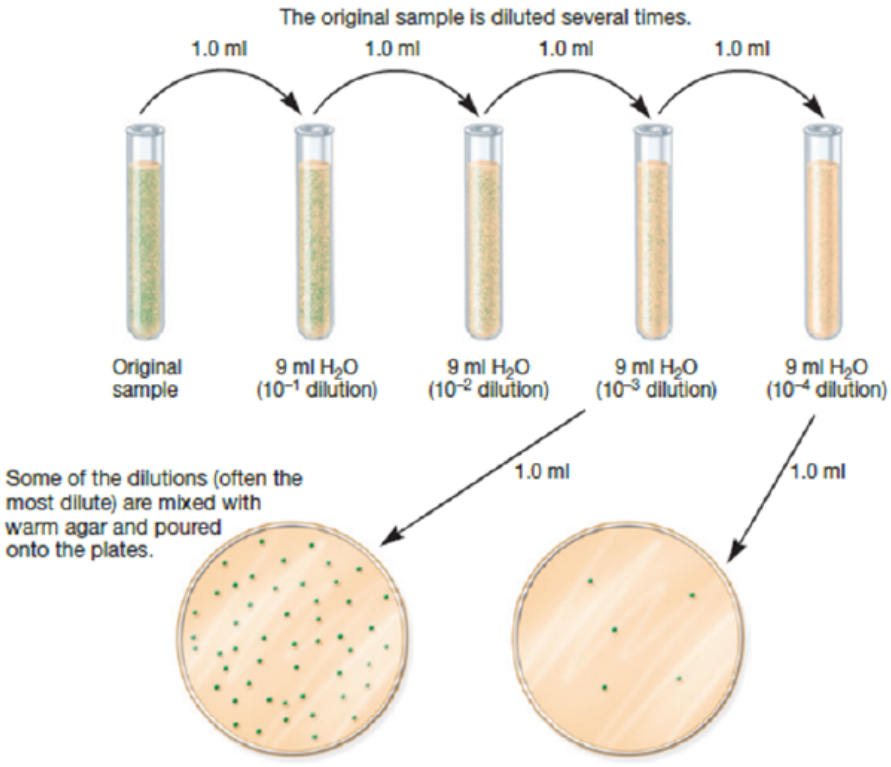
The number of bacteria per cm^3 is 10^3 times this value.

IF the average count per square is 25 bacteria: bacteria/cm^3 (25 bacteria) (25 squares)(50)(10^3) = 3.125×10^7.

* Adopted from Prescott's Microbiolog


Flow Cytometry
Sheath fluid
Sample
(stained cells in suspension)
Nozzle
Hydrodynamic Focusing
Cells pass through
in 'single file'
Fluorescence
emitted from
stained cells
detected
Forward and side
scattered light from
all cells detected
Laser light source


Electrode
Orifice tube
Orifice
Sensing zone



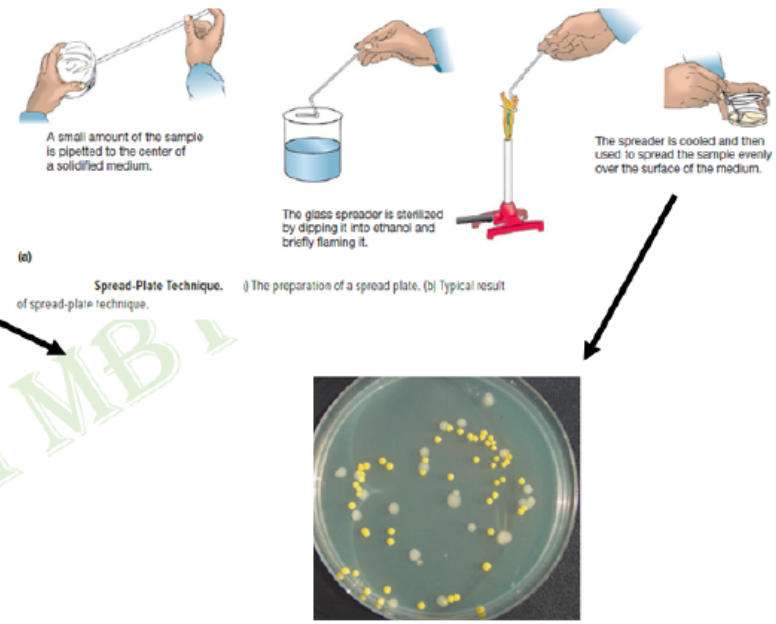
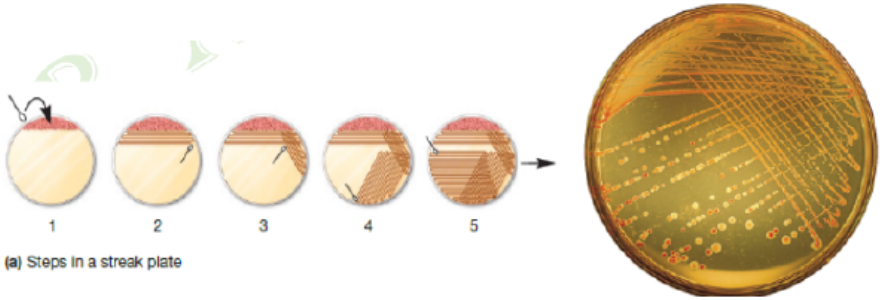


Plate count Techniques



* Adopted from Prescott's Microbiology

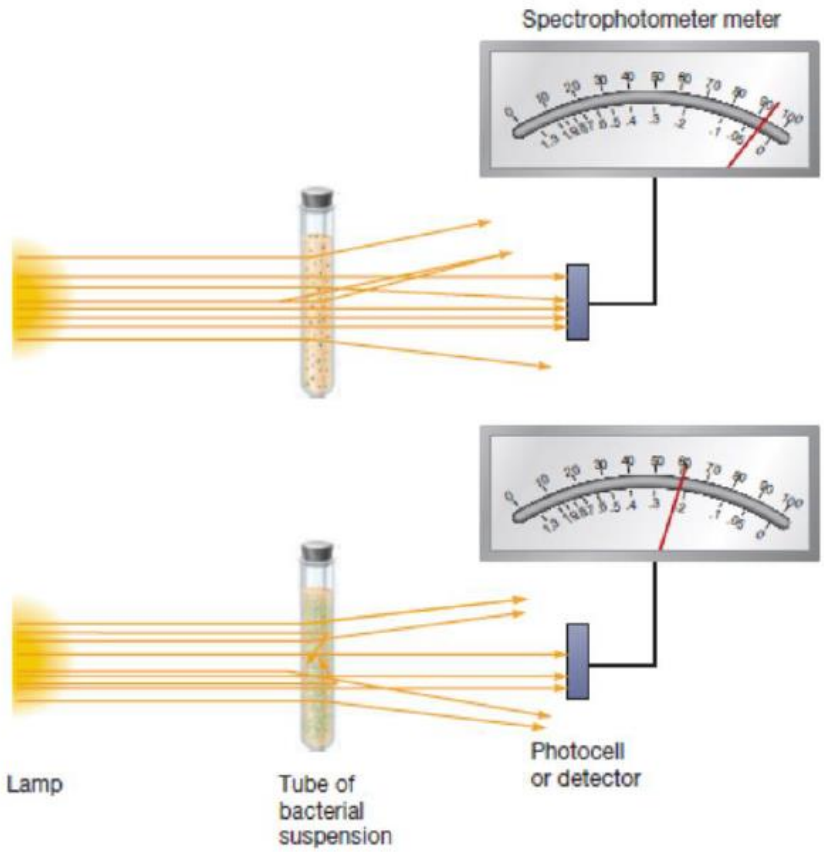
Cases

if the microbe cannot be cultured on solid r

colonies overgrow the surface of the plate

- In this method, numerous replicates of several dilutions of a cu added to tubes containing a suitable liquid growth medium.
- After incubation, each tube is examined to determine if growth occ
- It is assumed that the last tube in the dilution series that demon inoculated with between one and 10 cells, while the next tube h 100 cells, and so on.
- MPN values are most commonly used when a selective medium ca supports the growth of a specific type of microbe.

Indirect Method
Measurement of Cell Mass



Turbidity and Microbial Mass Measurement

* Adopted from Prescott's Microbiology

Measurement of Turbidity

Bacterial Culture