Bacterial growth curve

Bacteria grow by binary fission which leads to an increase in the total population. The population growth is generally studied by analyzing the growth curve of a microbial culture. The microbial culture most commonly practiced in the labs is a batch culture system. This is a closed system because microbes are incubated and grown in a single batch of media with no provision of adding fresh medium. Due to no addition of fresh medium the nutrients decline overtime and we see distinct phases of growth. These phases are lag phase, log phase, stationary phase and death phase respectively.

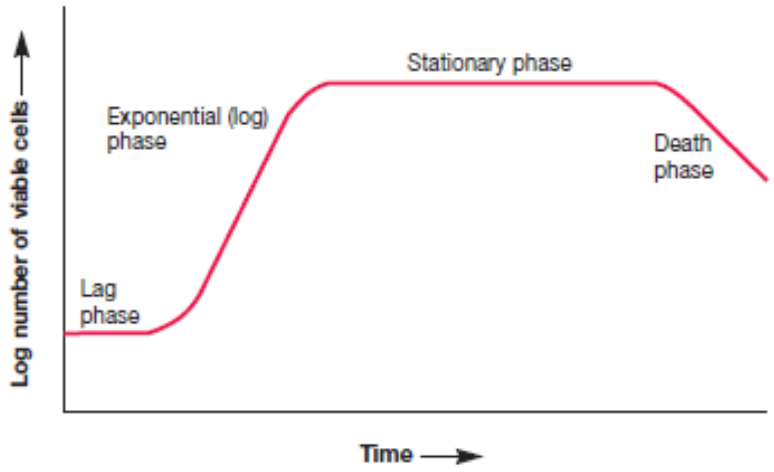


Figure showing microbial growth curve in a closed system.

1. **Lag phase:** When microbes are introduced in a fresh medium then increase in cell number and biomass starts sometime later. This gap between introduction and increase in cell numbers is called as lag phase. This phase occurs due to one or more of the following reasons:
   - Time taken to replenish ATP, essential cofactors and ribosomes.
   - Differences between the previous and current mediums.
   - Time required for recovery from injury and insults.

   The duration of lag phase is variable with the conditions. E.g. if inoculum is from an old or refrigerated culture or media is different then lag phase is usually long while if inoculum is from young and growing culture then lag phase is very short.

2. **Exponential phase:** This is also called as log phase and in this phase microbes grow at maximal rate possible given their genetic potential and growth conditions. The rate of growth is constant during log phase and microbes divide and double their number at regular interval. The curve rises smoothly than rising in discrete jumps because each individual divides at slightly different moment.
   The exponential phase cultures are mostly used for various studies because population is most uniform in terms of chemical and physiological properties during log phase.

3. **Stationary phase:** The microbial culture in labs is generally a closed system and in such systems the population growth ceases and the growth curve becomes horizontal. This phase of horizontal growth is called as stationary phase.
   In bacterial cultures stationary phase is achieved when cells reach a density of 10^9 cells/ml. This cell density is different for other organisms.
   Microbial populations enter the stationary phase due to following reasons:
   - Nutrient limitation: When an essential nutrient is depleted the population growth slows down. E.g. Aerobic bacteria are often limited in their growth by oxygen availability because oxygen is not very

soluble. This is the reason that cultures are usually kept in rotary shaker so that all levels can get access to oxygen.

- Accumulation of toxic waste products: This factor is mainly seen in anaerobic microbial cultures. E.g. streptococci produce lactic acid and other organic acids from sugar fermentation and their accumulation makes the medium acidic and halts the microbial growth. They also cease growth due to depletion of sugars.
- Cells reaching a critical level of population.

These limitations on growth are also seen in the nature as most environments have low nutrients level. In such situations, microbes reach the stage of starvation and have evolved many strategies to survive it except for endospore formation. These strategies are:

- Decrease in overall size along with protoplast shrinkage and nucleoid condensation.
- Changes in gene expression and physiology and expression of starvation proteins.

The starvation proteins tend to make the cell resistant in to damage in a variety of ways. These ways are:

- Peptidoglycan crosslinking leading to increased cell wall strength.
- Expression of Dps (DNA-binding proteins from starved cells) for protection of DNA.
- Increased expression of chaperons for preventing denaturation of proteins and renaturation of denatured proteins.

Due to these and many other changes the starved cells become more resistant to starvation itself and are harder to be killed by damaging temperature changes, oxidative and osmotic changes and by toxic chemicals such as chlorine.

There is also evidence to show that *Salmonella typhimurium* and many other bacteria become more virulent when starved.

4. **<u>Senescence and Death phase:</u>** It has been a general belief that decline in viable cells after the stationary phase is due to death of the cells. Thus, it has been described as death phase. And when bacteria from the death phase were transferred to a new medium then no growth was observed, the cells were said to be unviable.
However, the loss of viability is mostly not accompanied by loss in total cell number and it was thus believed that cells died but did not lyse.
But these assumptions are currently being challenged by two alternative views.
First is phenomena of Viable but nonculturable (VBNC). This view states that starving cells that show exponential decline in cell density have not lost their ability to reproduce beyond retrieval. These cells are temporarily unable to grow under laboratory conditions being used. These cells become viable for growth when conditions change such as temperature changes or passage through an organism.
Second is phenomena of programmed cell death and it suggests that a fraction of the microbial population is genetically programmed to commit suicide. When nonculturable cells are dead and the nutrients are leaked they enable the growth of cells in the population that did not initiate suicide. The dying cells are therefore altruistic as they sacrifice themselves for the benefit of the larger population.

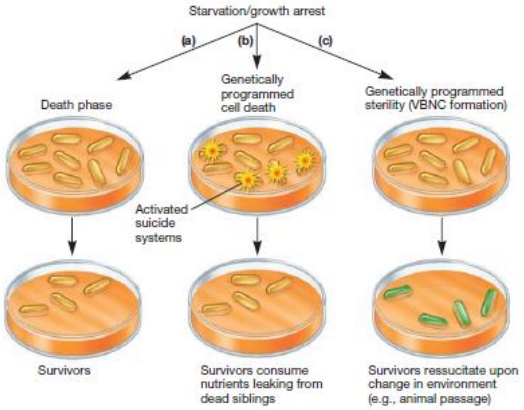


Figure showing loss of viability in the death phase by all the three mechanisms discussed above.

Mathematics of growth: The microbial growth discussed here is applicable to microbes that are in exponential phase and divide by binary fission. During exponential phase each microbe divides at a constant interval and due this the population will double in a specific length of time called as generation time or doubling time. Consider the examples given below:

- If a flask is inoculated with a microbial cell that divides every 20 minutes then population will be 2 cells after 20 minutes, 4 cells after 40 minutes and so on. Since the population is doubling at every generation, the increase in population is always 2^n where n is the number of generations.
- A cup is half filled with bacteria and the generation time for the bacteria is 30 minutes then the time needed to completely fill the cup with bacteria will be 30 minutes. It is because the population is doubling at every generation.

The observations discussed above can be put into equations as is given below:

N_0 = the initial population number.

N_t = population at time t.

n = number of generations in time t.

$N_t = N_0 X 2^n$.

If we solve for n and take $\log_{10}$ on both the sides, then we get:

$\log N_t = \log N_0 + n \log 2$

$n = (\log N_t - \log N_0) / \log 2 = (\log N_t - \log N_0) / 0.301$

In batch cultures, we use mean growth rate constant (k) to express the rate of growth during exponential phase. k is defined as number of generations per unit time and is expressed generally as generations per hour.

$k = n/t = (\log N_t - \log N_0) / 0.301t$

Using the above equation, we can calculate the mean generation time or mean doubling time (g) as given below.

$N_t = 2N_0$

Substitute $2N_0$ in the mean growth rate equation and solve for k.

$k = [\log (2N_0) - \log N_0] /0.301 g$

$k = (\log 2 + \log N_0 - \log N_0)/0.301g$

$k = 1/g$

from this we can deduce that mean generation time is reciprocal of the mean growth rate constant i.e. $g = 1/k$

E.g. A bacterial population increases from 10^3 cells to 10^9 cells in 10 hours. Calculate the mean generation time or generation time.

$k = (\log 10^9 - \log 10^3)/ (0.301 \times 10 hr)$

$= (9-3)/3.01$

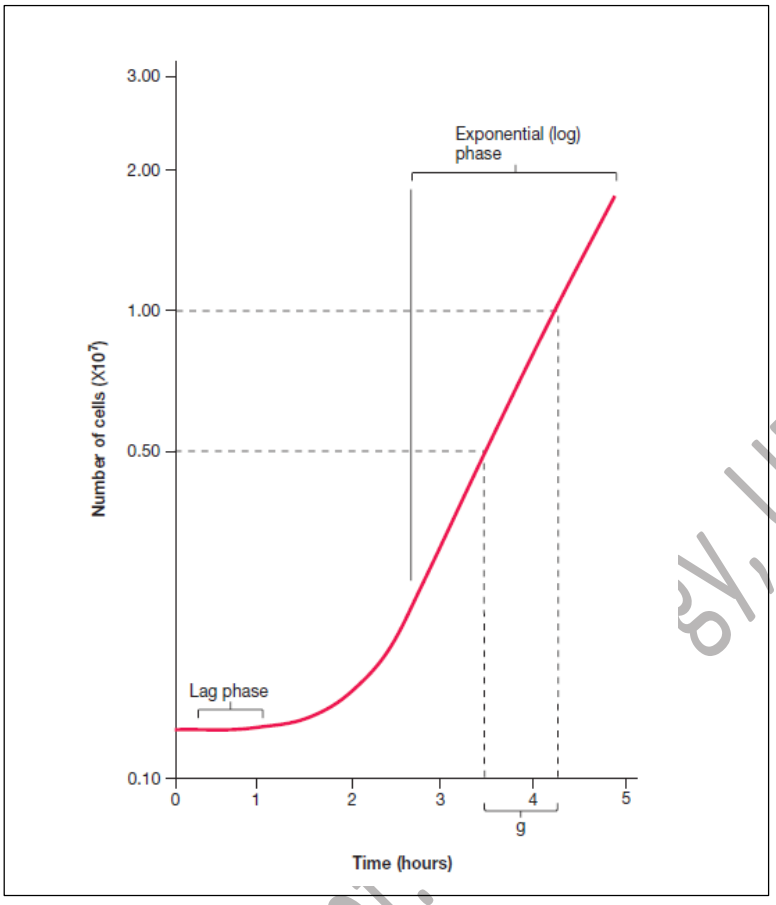
$= 2.0$ generations/hr

$g = 1/k$

$= 1 / (2$ gen./hr)

$= 0.5$ hr/gen or 30 min/gen.

The mean generation time (g) can also be determined directly from a semilogarithmic plot as is shown below.



The generation times vary among the different microbial species and it is also affected by the environmental conditions. A representative table of the generation time for different microbes is given below.

Table 6.2 Examples of Generation Times[a]

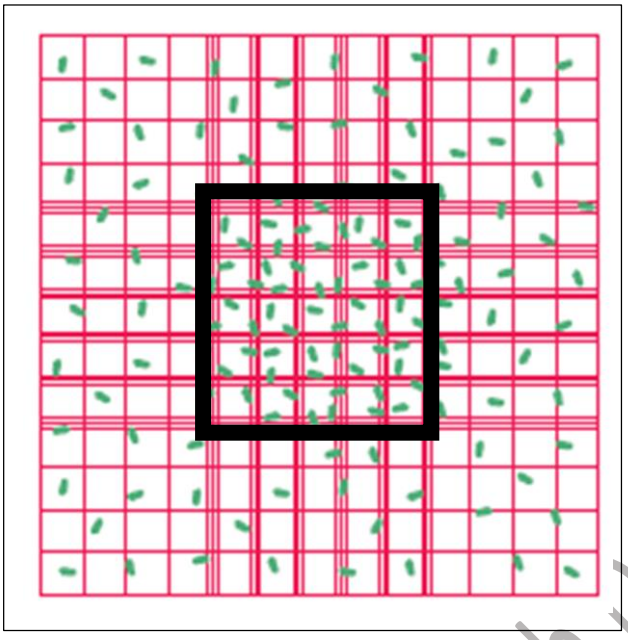
Microorganism	Incubation Temperature (°C)	Generation Time (Hours)
Bacteria		
Beneckea natriegens	37	0.16
Escherichia coli	40	0.35
Bacillus subtilis	40	0.43
Staphylococcus aureus	37	0.47
Pseudomonas aeruginosa	37	0.58
Clostridium botulinum	37	0.58
Rhodospirillum rubrum	25	4.6–5.3
Anabaena cylindrica	25	10.6
Mycobacterium tuberculosis	37	≈12
Treponema pallidum	37	33
Protists		
Tetrahymena geleii	24	2.2–4.2
Scenedesmus quadricauda	25	5.9
Chlorella pyrenoidosa	25	7.75
Asterionella formosa	20	9.6
Leishmania donovani	26	10–12
Paramecium caudatum	26	10.4
Euglena gracilis	25	10.9
Acanthamoeba castellanii	30	11–12
Giardia lamblia	37	18
Ceratium tripos	20	82.8
Fungi		
Saccharomyces cerevisiae	30	2
Monilinia fructicola	25	30

[a] Generation times differ depending on the growth medium and environmental conditions used.

Measurement of microbial cell numbers and mass: There are various methods available for this and are listed below. Methods 1-4 are used for cell number measurement and 5-6 are used for cell mass measurement.

1. Direct counting.
2. Using electronic counters such as Coulter counter and Flow cytometer.
3. Membrane filter techniques.
4. Viable counting methods such as spread-plate technique and pour-plate technique.
5. Microbial dry weight measurement.
6. Spectrophotometry.

Direct counting method: It is done using Petroff-Hausser Counting chamber. The chamber is shown in the figure below.



The bacteria are counted in the central squares which is given as marked with black square. There are 25 squares covering an area of $1mm^2$ and chamber is 0.02mm deep.

Bacteria/ml = Number of cell counted x Dilution factor x 50,000.

Why use 50,000 in the above formula?

50,000 = 50 (cell depth is 1/50 or 0.02mm) x 1000 (1000 mm^3 = 1ml)

Bactera/mm^3 = (Average number of cells per square) X 25 x 50.

Why use 50 in the above formula?

1ml = 1000 mm^3 and we take 50,000 as a multiplying factor while counting cells/ml. But when counting cells per mm^3 the measurement is being done for a 1000 fold less volume so we divide the 50,000 by 1,000.

This method has a drawback that it counts live and dead cells both and can`t provide any information about live cells. If we wish to find the number of live cells, then we use viable counting methods. For this reason, total count is always higher in direct counting methods than in viable counting methods.

Spectrophotometry:

Measurement of bacterial cell mass by this method is also called as turbidimetry. This is a rapid and sensitive method in comparison to microbial dry weight measurement. It depends on the fact that microbial cells scatter light that strikes them and the cells in the population are roughly the same size the amount of scattering is directly proportional to the biomass of the cells present and indirectly related to cell number. The extent of light scattering is almost linearly related to cell concentration at low absorbance levels. Thus, population growth can be easily measured as long as the population is high enough to give detectable turbidity.

Continuous culture of the microbes:

These systems are used to maintain a microbial population in exponential growth phase and at a constant biomass concentration for an extended period of time unlike the batch culture systems where exponential phase lasts only for few generations. This is done by creating an open system in labs wherein environmental system is kept constant through continual provision of nutrients and removal of wastes.

Since microbes are in exponential phase for long durations such culture systems are called as continuous culture systems. There are two major types of continuous culture systems i.e. chemostat and turbidostat.

Chemostat: In a chemostat, rate of sterile media addition and rate of removal for media containing microbes are same. The culture medium used in a chemostat has one essential nutrient in a limiting concentration. Due to this limiting nutrient, growth rate is determined by the rate of fresh media addition and final cell density depends on the concentration of the limiting nutrient. The rate of nutrient exchange is expressed as dilution rate (D) and is calculated as given below:

D = f/V. D = dilution rate, f = flow rate in ml/hr and V = Vessel volume.

E.g. if f = 30ml/hr and V = 100ml then D = 0.30/hr.

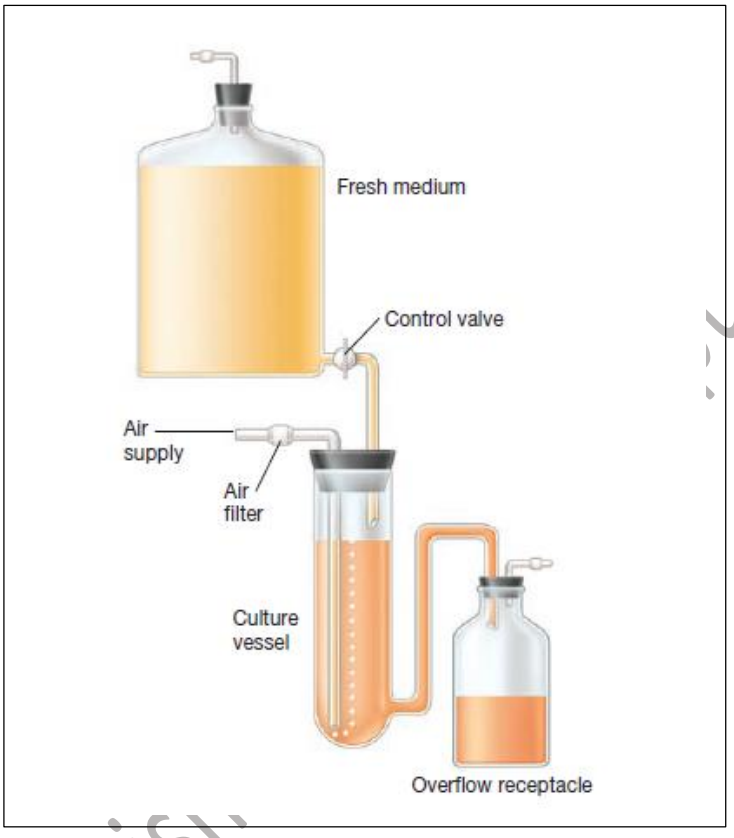


Figure: Chemostat – A Continuous culture system.

In a chemostat, microbial population level and generation time both are related to the dilution rate. The microbial population density largely remains constant over a wide range of dilution rates but the generation time decreases as the dilution rate increases.

The limiting nutrient is almost completely depleted under balanced conditions. However, if the dilution rates are higher than the maximum growth rate then microbes are washed out of the medium before reproduction. This leads to a reduction in the biomass.

When D is very low, an increase in dilution rate leads to an increase in cell density and growth rate. This is due to Monad relationship which dictates effect of nutrient concentration on the growth rate. When only a limited supply of nutrients is available such as at low D values, the maximum fraction of available energy is used for cell maintenance. But when D is increased, nutrient supply increases and now energy is available for cell maintenance as well as reproduction. This leads to an initial increase in the biomass when D is increased.

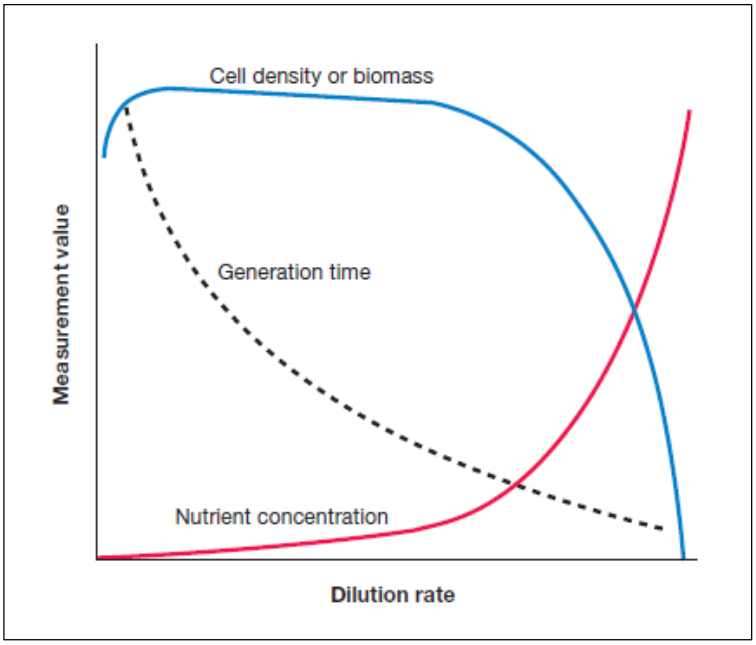


Figure: Relationship between chemostat dilution rate and microbial growth.

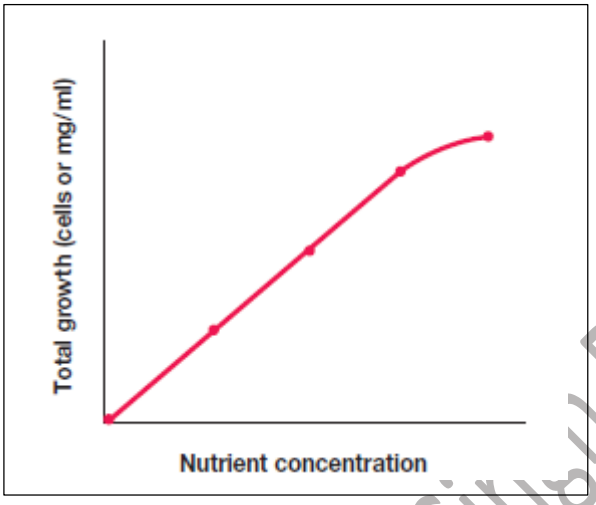


Figure: Monad relationship showing the effect of changes in limiting nutrient concentration on the total microbial yield.

Turbidostat: This system measures the absorbance or turbidity of the culture in the growth vessel and for this purpose it has a photocell. The flow rate of media is regulated automatically to maintain a predetermined turbidity or cell density.

The turbidostat differs from the chemostat in the manner that dilution rate is variable and not a constant. Also all the nutrients are in excess and none is limiting.

The turbidostat operates best at high dilution rates while chemostat is most stable and effective at lower dilution rates.

The continuous culture systems are useful as they provide a constant supply of cells in exponential phase which are growing at a known rate. This property is utilized in many industrial and research processes.