MICROBIAL BIOTECHNOLOGY

Prof. Utpal Bora
Department of Biosciences and Bioengineering
Indian Institute of Technology Guwahati

Lecture-15

Lec 15: Microbial growth kinetics

Hello everyone, welcome back to my course on microbial biotechnology. Today, we will be starting a new module, which is on the control of microorganisms. We will start with a discussion on microbial growth kinetics first, followed by the control of microorganisms by various methods like physical, chemical, and biological methods. So, in this lecture, we are going to discuss the growth requirements of microbes, then the growth dynamics, and finally the estimation of microbial growth. Before that, let us have a small discussion on why a cell must divide.

And again, before answering that question, try to look into this small cube with one unit dimension—length, breadth, and width. So, its volume will be one cubic unit, as you can understand: one multiplied by one multiplied by one. Now, it has six faces. So, the area of one face is one multiplied by one, which equals one square unit. Now, it has six faces.

So, it has a total of around six square units of surface area. Now, if you divide this surface area by the volume of this cube, we get a ratio, which we call surface area to volume. So, in this case, the ratio will be 6:1, or we can simply say the ratio is 6. Now, if we take a bigger cube, suppose five times the size of this smaller one, the volume will be 125, and the area of one face is 5 multiplied by 5, so 25 multiplied by 6 will be around 150.

And in this case, if we divide the surface area—the total surface area of these large cubes—by the volume, we get a smaller ratio of 1 to 2. Now, if this is considered as a bacterial cell, which may not be an exact shape, but something between a spheroidal and this cuboidal shape, a similar thing is going to occur. As the cell grows, the surface area will increase, no doubt, but its surface-area-to-volume ratio will actually get reduced. So, in such a case, the cell size is basically determined by genetic programming, but it is also influenced by the need for efficient nutrient and waste transport. Cell size is limited by the surface-area-to-volume ratio, which we are discussing just now.

As a cell grows, its volume increases faster than its surface area, decreasing the SA-to-volume ratio. A low surface-area-to-volume ratio will limit the cell's ability to transport nutrients and waste, which can lead to cellular stress and dysfunction. To maintain an optimal surface-area-to-volume ratio, these cells must divide, reducing their size and increasing their overall surface area. So, we are in a better position when these cubes start dividing into smaller units, maybe of the ideal size of one unit square. So, this is the reason why a cell must divide; otherwise, it won't be able to survive.

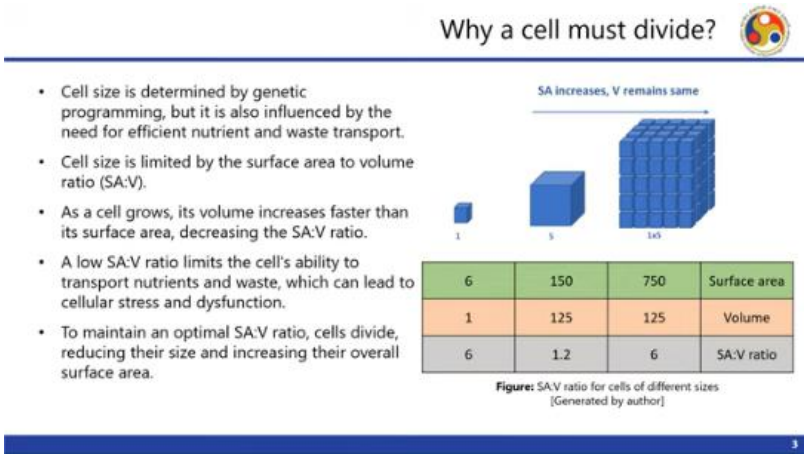


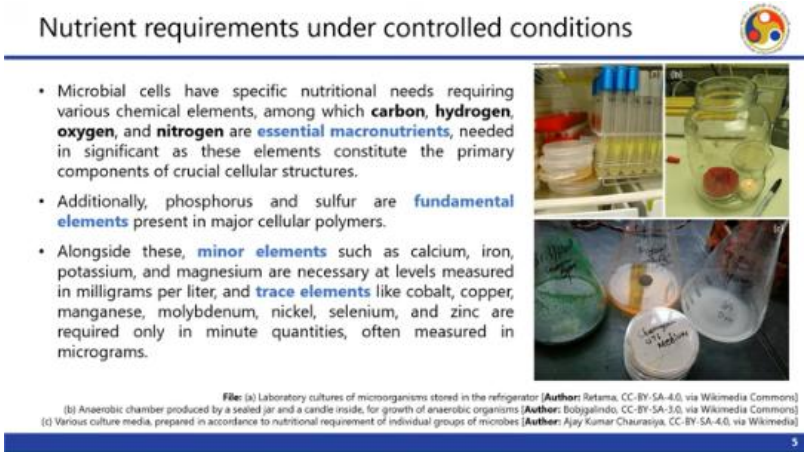
Figure: SA:V ratio for cells of different sizes [Generated by author]

Now, what are the nutrient requirements for growth under controlled conditions? Microorganisms have diverse nutrient requirements. Some bacteria, known as prototrophs, can thrive on minimal media that includes basic carbon sources, energy providers, and essential minerals. However, other microorganisms require specific compounds, either in whole or in part, for growth and reproduction.

Microbes that cannot grow without additional organic substances, such as amino acids or vitamins, are termed as auxotropes. Culture media For many organotrophic microorganisms often require vitamin and growth factor supplements, particularly B vitamins, and then which may be thiamine, riboflavin, pyridoxine, cobalamin, biotin, nicotinic acid and pantothenic acid. Only a few microbes require fat soluble vitamins, which are A, D, E and K. While vitamin C can enhance growth, it is not considered a true microbial growth factor. Micromill cells have specific nutritional needs requiring various chemical elements among which carbon, hydrogen, oxygen and nitrogen are essential micronutrients and they are needed in significant

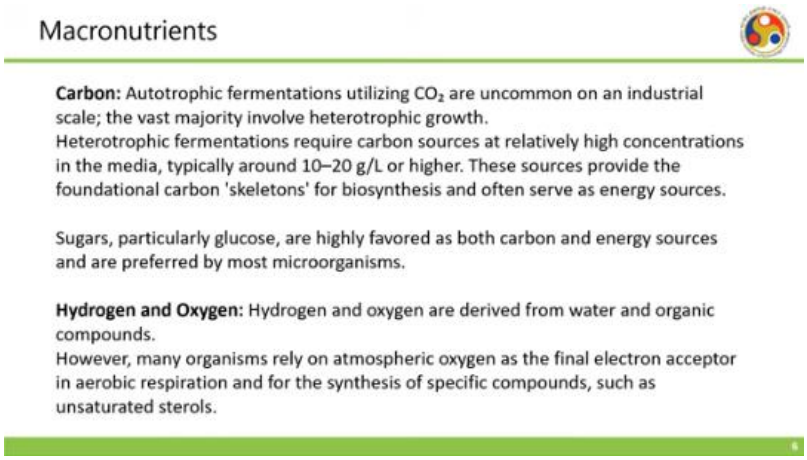
as these elements constitute the primary components of crucial cellular structures. Additionally, phosphorus and sulfur are fundamental elements present in major cellular polymers. Alongside these minor elements such as calcium, iron, potassium and

magnesium are necessary at levels measured in milligrams per liter and trace elements like cobalt, copper, manganese, molybdenum, nickel, selenium and zinc are required only in minor quantities often measured in micrograms. Let us discuss about the macronutrients. First let us start with carbon.



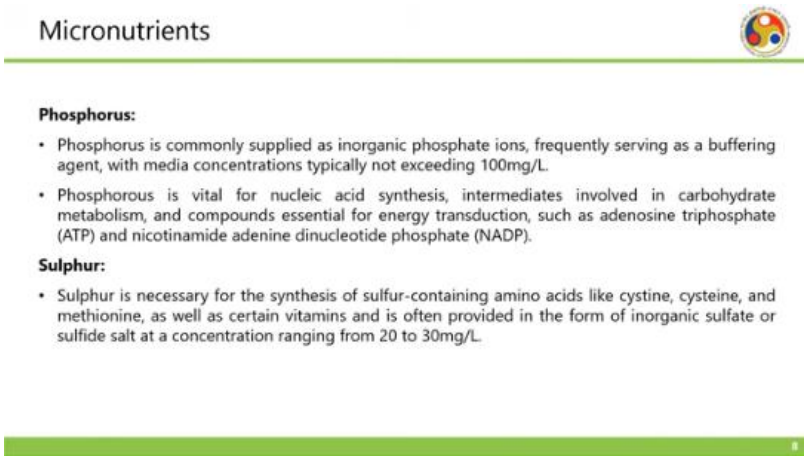
Autotrophic fermentations that utilize carbon dioxide are uncommon on an industrial scale. The vast majority involve heterotrophic growth. Heterotrophic fermentations require carbon sources at relatively high concentrations in the media, typically around 10 to 20 grams per liter or higher. These sources provide the foundational carbon skeletons for biosynthesis and often serve as energy sources.

Sugars, particularly glucose, are highly favored as both carbon and energy sources and are preferred by most microorganisms. Next come hydrogen and oxygen, which are derived from water and organic compounds. However, many organisms rely on atmospheric oxygen as the final electron acceptor in aerobic respiration and for the synthesis of specific compounds such as unsaturated sterols. Nitrogen is another important micronutrient. Microorganisms typically contain over 15% weight by weight nitrogen, primarily found within structural and functional proteins and nucleic acids.



Ammonium salts are frequently the preferred nitrogen source, although nitrate, amino acids, or nitrogen-rich compounds like urea may occasionally be used. Mobile sartans, specialized nitrogen-fixing bacteria, notably Azotobacter and Rhizobium species, can utilize molecular nitrogen. Then, the next important micronutrient is phosphorus, which is commonly supplied as inorganic phosphate ions, frequently serving as a buffering agent with media concentrations typically not exceeding 100 mg per liter. Phosphorus is vital for nucleic acid synthesis, intermediates involved in carbohydrate metabolism, and compounds essential for energy transduction such as adenosine triphosphate and nicotinamide adenine dinucleotide phosphate. Another important micronutrient is sulfur.

This is necessary for the synthesis of sulfur-containing amino acids like cysteine and methionine, as well as certain vitamins, and is often provided in the form of inorganic sulfate or sulfate salt at a concentration ranging from 20 to 30 milligrams per liter. Some of the other minor elements are calcium, iron, potassium, and magnesium. These need to be supplied in relatively modest yet crucial amounts, typically less than 10 to 20 milligrams per liter. These minor elements play essential roles in specific enzyme activities or have structural roles. For example, magnesium is essential in giving stability to ribosomes.

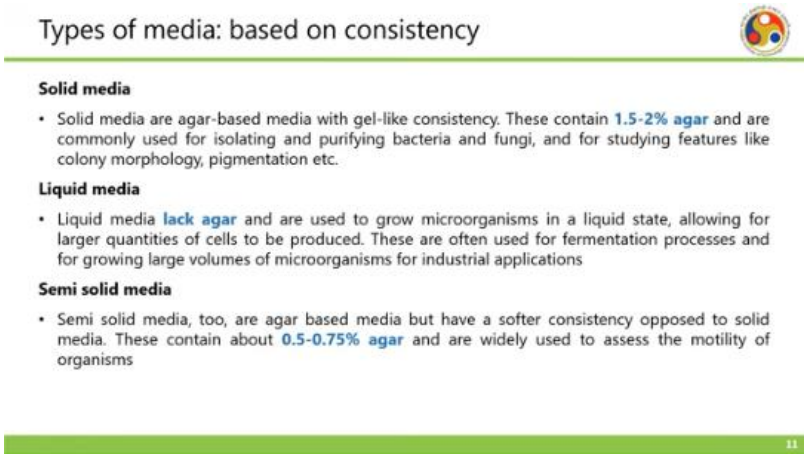


Then there is another class of elements which are very important. We call them trace elements, which include cobalt, copper, manganese, molybdenum, nickel, selenium, and zinc, and are typically needed in concentrations of 0.1 to 1 mg per liter or less to support specific enzyme functions. These elements are often present in water supplies or can be found as contaminants in other media components, thereby fulfilling the requirements. Let us now discuss microbial cultures.

Microbial culture is a technique used to increase the number of microorganisms by allowing them to reproduce in a controlled laboratory environment using a specific culture medium. And you can see here we are using different kinds of media over here to grow different kinds of microbes. Now, what is a pure culture? A pure culture consists of cells of a single type of organism or microorganism growing in the absence of other species, and these are often crucial for research and industrial applications. Microbial cultures are foundational tools in molecular biology, medical and environmental microbiology, allowing scientists to study the characteristics and behavior of different microorganisms.

So, in the figure, we can see the overnight culture of a transgenic E. coli, the culture of Pseudomonas aeruginosa, and also some cultures of E. faecalis and Staphylococcus aureus on tryptic soy agar. Then C shows the slant cultures showing variations in colony appearance, and E shows antibiotic susceptibility using antibiotic discs, while E shows Bacillus cereus streaked on nutrient agar. So, different kinds of formats and different kinds of media are being used to culture different kinds of microorganisms. Now, what are the types of media based on consistency? Number one is solid media, which is agar-based media with a gel-like consistency.

These contain 1.5% to 2% agar and are commonly used for isolating and purifying bacteria and fungi, as well as for studying features like colony morphology and pigmentation. Then we have liquid media, which lack agar and are used for growing microorganisms in a liquid state, allowing for larger quantities of cells to be produced. These are often used for fermentation and for growing large volumes of microorganisms for industrial applications. Then we have something in between, which we call semi-solid media. These are agar-based media but have a softer consistency compared to solid media.

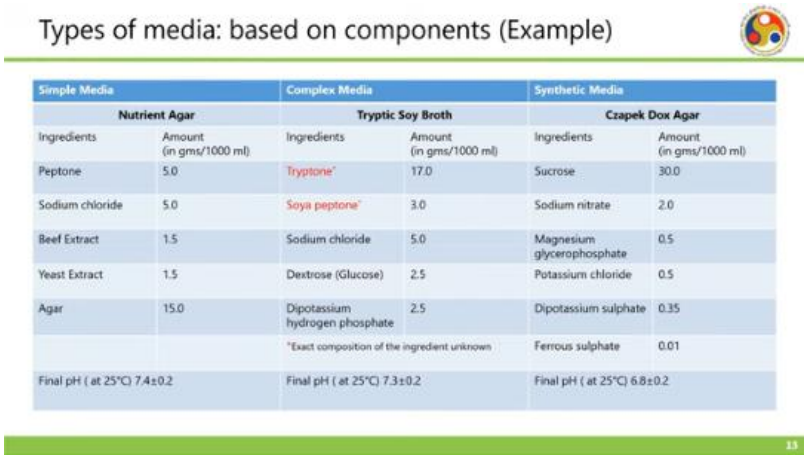


These contain about 0.5% to 0.75% agar and are widely used to assess the motility of organisms. Now, we can also classify media based on components. For example, we have simple media, which contain essential nutrients required for the basic growth of microorganisms, such as carbon, nitrogen, minerals, and water, but lack specific additional components like amino acids or vitamins. Simple media are used for culturing non-fastidious microorganisms that do not require additional growth factors, and some examples include nutrient broth, peptone water, and nutrient agar. Then we have complex

media, which are nutrient-rich and contain a variety of nutrients such as peptones, extracts, and digests from various sources like plants or animal tissues.

Being derived from various sources, the exact chemical composition of these media is not known. For example, we have LB broth or Luria-Bertani broth or tryptic soy broth under complex media. Then we have a third type called synthetic media. These are precisely formulated with known quantities of specific nutrients. Each component is precisely measured, and the composition is well-defined, unlike complex media.

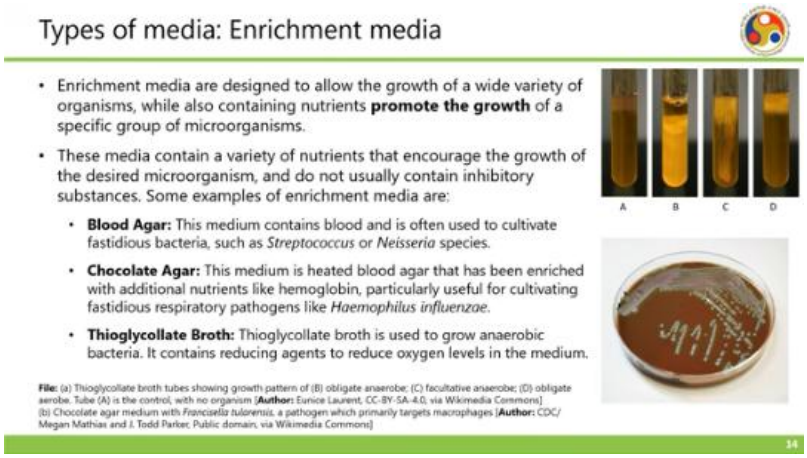
And some of the examples are chapagdox medium and simon citrate agar mineral glucose medium etc. now here we can see the composition of simple media complex media and synthetic media at one glance for comparison purpose so we have here for example peptone but we are having here again soya peptone and trypton then we have certain salt like sodium chloride which is also common over here and then we have here beef extract and then yeast extract and agar for solidification. Here also we have dextrose and then the exact composition of these ingredients for example here are not known. And then you can see here they have pH from around 7.4 or 7.3 and 6.8 with a little bit of deviation here and there.



But in the synthetic media, for example, the Chapek-Dox agar, everything is defined sucrose, sodium nitrate, magnesium, glycerophosphate, potassium chloride, dipotassium sulphate, ferrous sulphate and including the pH which is well defined. Now let us discuss another type of media which we call as the enrichment media. Enrichment media are designed to allow the growth of a wide variety of organisms while also containing nutrients. It promotes the growth of a specific group of microorganisms.

These media contain a variety of nutrients that increase the growth of the desired microorganism and do not usually contain inhibitory substances. Some examples of such enrichment media are blood agar. This medium contains blood and is often used to cultivate fastidious bacteria such as Streptococcus or Neisseria species. Then we have chocolate agar.

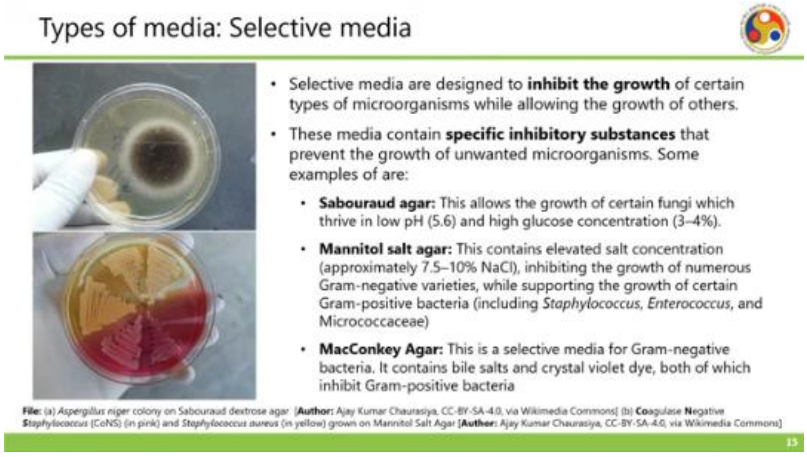
This medium is heated blood agar that has been enriched with additional nutrients like hemoglobin, particularly useful for cultivating fastidious respiratory pathogens like Haemophilus influenzae. Then thioglycolate brought, which is used to grow anaerobic bacteria. It contains reducing agents to reduce oxygen levels in the medium. So here in this file, we can see the thioglycolate brought tube showing growth pattern of obligate anaerob. Then we have facultative anaerobes and then obligate aerobes.



And tube A is actually the control with no organism. And here we can see the chocolate agar medium with Francisella tolarensis, which is a pathogen that primarily targets macrophages. Another type of media is the selective media. These are designed to inhibit the growth of certain types of microorganisms while allowing the growth of others. These media contain specific inhibitory substances that prevent the growth of unwanted microorganisms.

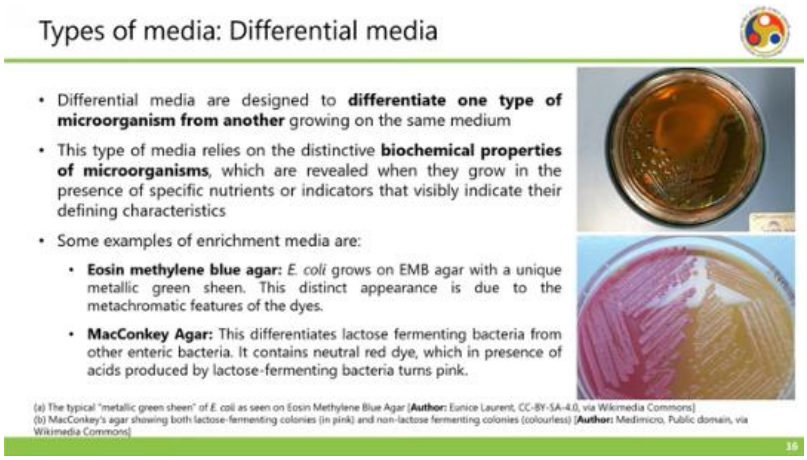
Some examples of selective media are Subarod Agar. This allows the growth of certain fungi which thrive in low pH and high glucose concentration. Then we have the mannitol salt agar. This contains elevated salt concentration, approximately 7.5 to 10% sodium chloride, and it inhibits the growth of numerous gram-negative bacteria while supporting a growth of certain gram-positive bacteria like staphylococcus, enterococcus, etc. Then we have another example, McConkey agar.

This is a selective medium for gram-negative bacteria. It contains bile salts and crystal violet dye, both of which inhibit gram-positive bacteria. Another interesting type of medium is differential medium. Differential media are designed to differentiate one type of microorganism from another growing on the same medium. This type of medium



relies on the distinctive biochemical properties of microorganisms, which are revealed when they grow in the presence of specific nutrients or indicators that visibly indicate their defining characteristics. Some examples include eosin methylene blue agar, E. coli grows on eosin methylene blue agar with a unique metallic green sheen. This distinct appearance is due to the metachromatic features of the dyes. MacConkey agar is another example of differential medium.

This differentiates lactose-fermenting bacteria from other enteric bacteria. It contains neutral red dye, which in the presence of acids produced by lactose-fermenting bacteria turns pink. So, in Figure A, we can see the typical metallic green sheen of E. coli on EMB. And in Figure B, we can see MacConkey agar showing both lactose-fermenting colonies in pink on one side and non-lactose-fermenting colonies on the other side.



Let us now try to understand bacterial growth dynamics, where we will discuss batch culture, the bacterial growth curve, and finally synchronous culture, fat-based cultures, continuous cultures, and diauxic growth. Let us first discuss batch culture. A batch fermentation system is a closed system with all medium components placed in a reactor at the beginning of the cultivation. In the course of microbial growth, there is no exchange of media, with only the passage of atmospheric gases, acid or base for pH control, and anti-foaming agents.

The composition of the medium, the biomass concentration, and metabolite concentration generally change constantly as a result of the metabolism of the cells, and the system therefore remains unsteady. Microbial metabolites may be produced at the primary or secondary stage of the microbial cultivation period. Fermentation is terminated when either all the nutrients are exhausted or the desired concentration of the product is achieved. Batch cultures are essential for the study of growth kinetics. Now, let us discuss some terminologies, such as generation time.

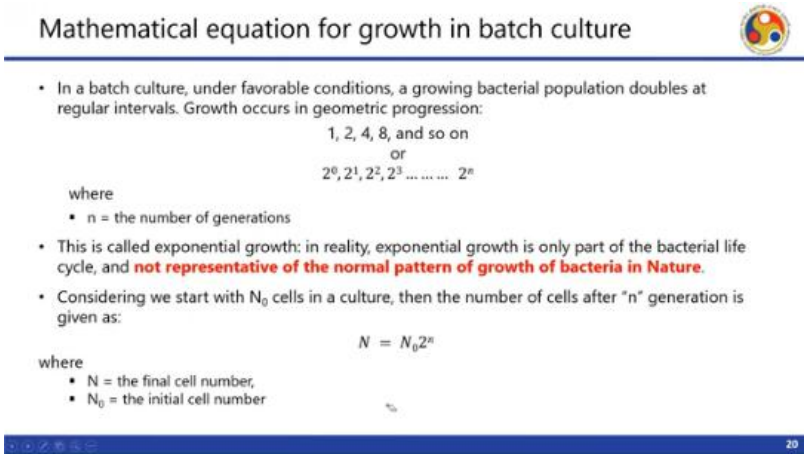
This is the interval for the formation of two cells from one, and we call it generation time. This is required for the cell population to double. The cell mass doubles during this period as well. Because of this, the generation time is also called the doubling time. In nature, however, microbial doubling times may be much longer than those obtained in laboratory cultures under ideal conditions.

This is because in nature, ideal growth conditions for a given organism may exist only intermittently. Depending on resource availability, physicochemical conditions like temperature, pH, and moisture availability, and seasonal changes, Bacterial populations in nature double only once every few weeks or may even take longer. The mathematical

equation for growth in a batch culture. In a batch culture, under favorable conditions, a growing bacterial population doubles at regular intervals.

Growth occurs in geometric progression. From one, it will become two. Then it will become 4, 8, and so on. So, we can actually represent these as 2 to the power 0, 2 to the power 1, 2, 3, and 2 to the power n. So, after n cycles of generation, the population will be 2 to the power n. Therefore, n is the number of generations. So, we call this geometric progression growth exponential growth.

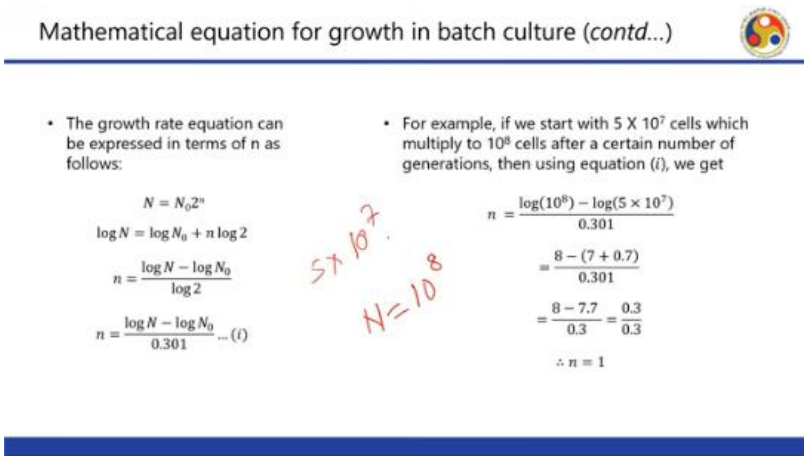
In reality, exponential growth is only part of the bacterial life cycle and is not representative of the normal pattern of bacterial growth in nature. However, considering we start with N0 cells in a culture, then the number of cells after n generations would be as given below. N will be N0 multiplied by 2 to the power n, which comes from the series we discussed. So N is the final cell number, and N0 is the initial cell number. So the growth rate equation can be expressed in terms of N as follows.



N is equal to N0 multiplied by twice N. If you take the log on both sides, we can derive that small n is the generation is equal to the log of the final cell numbers minus the log of the initial cell numbers divided by a constant 0.301. Now, let us do some calculations for an example. If we start with an initial number of cells, 5 multiplied by 10 to the power of 7, which is basically N0, and then these multiply to reach around 10 to the power of 8 cells, which is basically N. So, what would be the number of generations required

to reach 10 to the power of 8 from 5 multiplied by 10 to the power of 7? So, using this equation, we can calculate this as shown here, plotting the values of N as 10 to the power of 8 and N0 as 5 multiplied by 10 to the power of 7. By solving this, we can find that in just one division, the 5 multiplied by 10 to the power of 7 cells will become 10 to the power

of 8 cells. So, for any starting population, we can calculate the expected number of populations and the number of generations if the number of generations is given, or we can also calculate the number of generations required to reach a desired number of cells by using this simple equation.



In a batch culture, the exponential phase growth rate can be quantified using the mean growth rate constant, called K, which represents the number of generations per unit time, as given below. K is expressed as n divided by t. Substituting these into equation 1 for the value of small n, we get another equation: K is equal to log N minus log N0 divided by 0.301 t. The mean generation time, or mean doubling time G, is the time taken by the population to double in size, such that in time t equal to G, the number of cells will be twice the initial population. So, using this, we can calculate the values.

So, by replacing the value of n and t in equation number 2, we will have k equal to log twice n0 minus log n0 divided by 0.301, and here t is replaced by g. So, we get a further simplification of this as k equal to 0.301 divided by 0.301 g, or finally we will get that k is actually 1 by g. So, this is how we calculate the relationship between the mean growth rate constant k and and the mean generation time or mean doubling time g using equation number 3. Let us now discuss the bacterial growth curve. What is the utility of a bacterial growth curve?

The study of population growth is achieved by examining the growth curve of a microbial culture. This is a typical growth curve which has a slow phase in the beginning, which we call the lag phase. Then there is a sudden increase in population, which we call the log phase. And then the population remains constant or fixed for a long time, which we call the stationary phase. And then a time comes when the population starts to decline.

This we also call the death phase or senescence. Typically, microorganisms are cultivated in a closed system called a batch culture, wherein they are incubated with a fixed amount of medium, and no additional nutrients are supplied during incubation. As a result, nutrient levels decrease while waste concentrations increase. The growth of microorganisms undergoing binary fission can be plotted as the logarithm of variable cell numbers against the incubation time, revealing four distinct phases, as we have discussed recently. So, when microorganisms are introduced into a fresh culture medium, no immediate increase in cell number occurs. Although there is no net increase in mass, the cell is actively synthesizing new components.

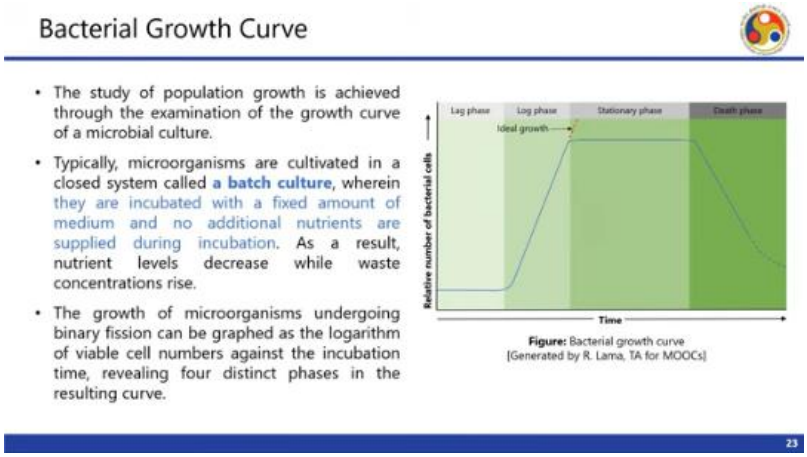
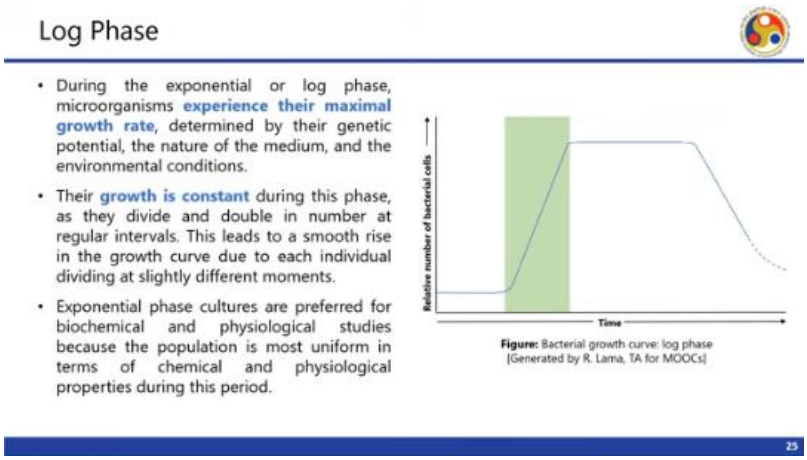


Figure: Bacterial growth curve
[Generated by R. Lama, TA for MOOCs]

For instance, the cells might be old and lacking in ATP essential cofactors and ribosomes which need to be synthesized before growth can begin. Additionally, the medium might differ from the one the microorganism was previously growing in requiring some adaptation before growth resumes. During the exponential or the log phase, microorganisms experience their maximal growth rate determined by their generic potential, the nature of the medium and the environmental condition.

The growth is constant during this phase as they divide and double in number at regular intervals. This leads to a smooth rise in the growth curve due to each individual dividing at slightly different movements. These exponential phase cultures are preferred for biochemical and physiological studies because the population is most uniform in terms of chemical and physiological properties during this period. Then we have the stationary phase. In the stationary phase of bacterial growth, the number of the viable cells remain relatively constant.



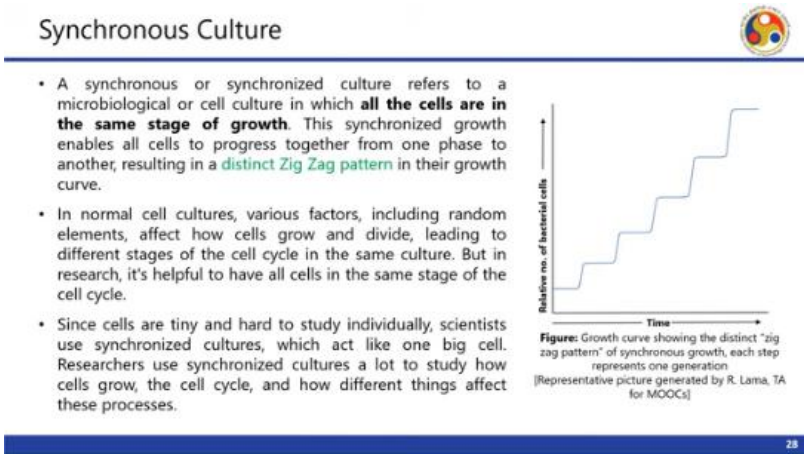
The phase is characterized by a decrease in the rate of cell division with new cell formation being balanced by cell date resulting in a plateau in the growth curve. It occurs when the growth mediums, nutrients become limited, waste products accumulate and the environment becomes unfavorable for further proliferation. Bacteria in the stationary phase may also undergo physiological changes to adapt to the stressful conditions such as forming dormant cell forms. The death phases. The death phase in bacterial growth is the stage where the number of viable cells decrease over time.

During this phase, the rate of cell death exceeds the rate of cell division, leading to a decline in the total number of bacteria in the population. The death phase is typically reached after the stationary phase. When the growth medium's nutrients are depleted, waste products accumulate, and the environment becomes increasingly hostile for bacterial survival. As a result, bacteria start to die off, and the population declines until only a few viable cells remain. Let us now move on to another topic, which is synchronous culture.

A synchronous or synchronized culture refers to a microbial cell culture in which all the cells are in the same stage of growth. This synchronized growth enables all cells to progress together from one phase to another, resulting in a distinct zigzag pattern in their growth curve, as shown in this figure. In normal cell cultures, various factors, including random elements, affect how cells grow and divide, leading to different stages of the cell cycle in the same culture. But in research, it is helpful to have all cells in the same stage of the cell cycle. Since cells are tiny and hard to study individually, scientists use synchronized cultures, which act like one big cell, representing the features at a particular point in time uniformly.

So, researchers use synchronized cultures a lot to study how cells grow or phenomena like the cell cycle and how different things affect these processes. What are the various methods

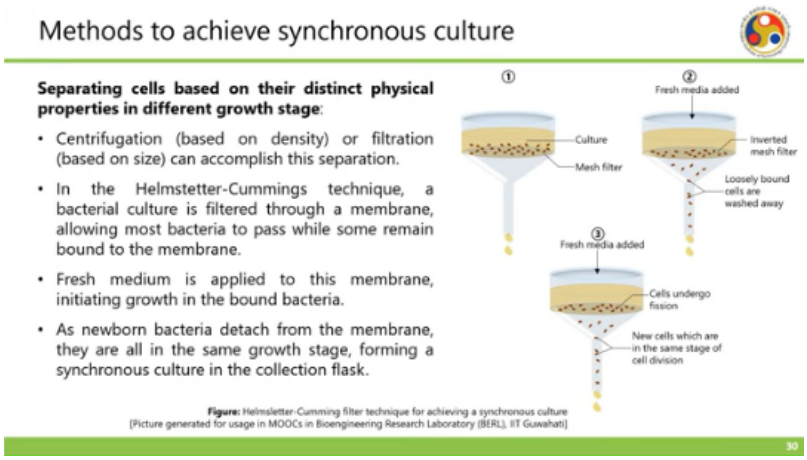
to achieve a synchronous culture? We can do it by changing external conditions. Altering external conditions to halt the growth of all cells within the culture, followed by restoring these conditions to prompt growth again. As growth resumes, all newly growing cells initiate growth at the same stage, achieving synchronization.



For instance, in photosynthetic cells, light exposure can be interrupted for a period and then reintroduced. Alternatively, removing an essential nutrient from the growth medium and later reintroducing it can synchronize the growth stages. So, we have chemical inhibitors which may be used to arrest cell growth. Once growth has ceased entirely for all cells due to the inhibition by the inhibitor, removing the inhibitor from the culture prompts synchronous growth to commence. So, another way is to separate cells based on their distinct physical properties in different growth stages, which can be by centrifugation based on density or filtration based on size. We can separate the cells which are in various phases of their growth.

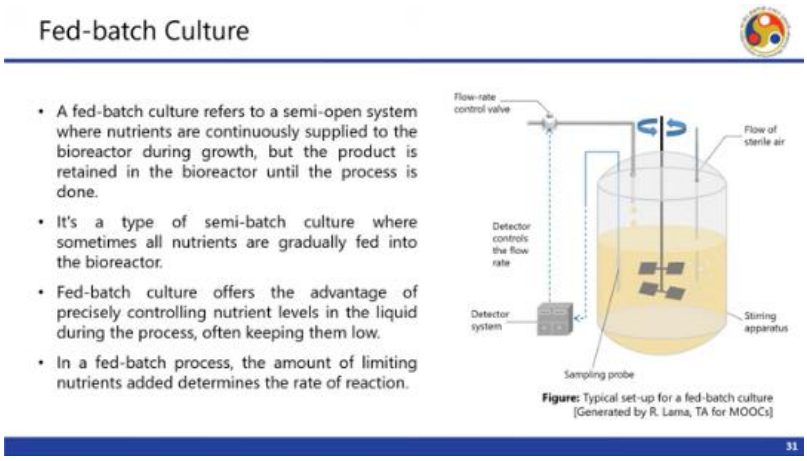
In the Hempsteader-Cummings technique, a bacterial culture is filtered through a membrane, as you can see in this figure, 1 to 3, allowing most bacteria to pass while some remain bound to the culture. Fresh medium is applied to this membrane, initiating growth in the bound bacteria, as you can see in step 2. Then, this mesh is inverted so the loosely bound cells are washed away. Now, what are these loosely bound cells? These are basically the newly dividing cells.

As newborn bacteria detach from the membrane, they are all in the same growth stage, forming a synchronous culture in the collection flask. Fed-batch culture. A fed-batch culture refers to a semi-open system where nutrients are continuously supplied to the bioreactor during growth. But the product is retained in the bioreactor until the process is completed.



It's a type of semi-batch culture where sometimes all nutrients are gradually fed into the bioreactor. So here we have a flow rate control valve and then you have also certain sampling probe which will tell us about the media conditions like temperature, pH, etc. And then there is a stirrer which will keep on mixing the media from time to time and it is supplied with sterile air. So, there is a special air supply system through which this air will be injected into these

system. Fat-based culture offers the advantage of precisely controlling nutrient levels in a liquid during the process, often keeping them low. In this process, the amount of limiting nutrients added determines the rate of reaction. So, the fermentation will continue for a long time as we do not allow nutrient depletion. What are advantages of fed-based culture?



The primary benefits of fed-based cultures over batch culture are prolonged product synthesis over an extended period, enhanced process efficiencies by boosting cell count, and subsequent product quantity directly linked to biomass concentration, heightened yield and productivity via controlled sequential nutrient additions, utilizing the bioreactor for

production during otherwise non-profitable periods instead of the typical preparation phase for the next batch. Then we have the continuous culture, which is a method used in microbiology to maintain microorganisms in a steady state of growth under controlled conditions. Unlike batch culture where nutrients are added only once at the beginning and then depleted over time, continuous culture allows for a constant supply of fresh nutrients and the removal of waste products, creating an environment of balanced growth. The continuous culture system typically consists of a bioreactor or fermenter with an inflow of fresh medium

and an outflow of the culture broth, which carries away the waste. The inflow rate of fresh medium and the outflow rate of culture broth are regulated to maintain a constant volume in the bioreactor. These constant flows of nutrients and removal of waste products enable the microorganisms to grow at a steady state, reaching a balanced state between cell growth and death. Let us discuss the chemostat now. A chemostat is designed in a way that sterile medium is continuously fed into the culture vessel where an equal amount of medium containing microorganisms is removed.

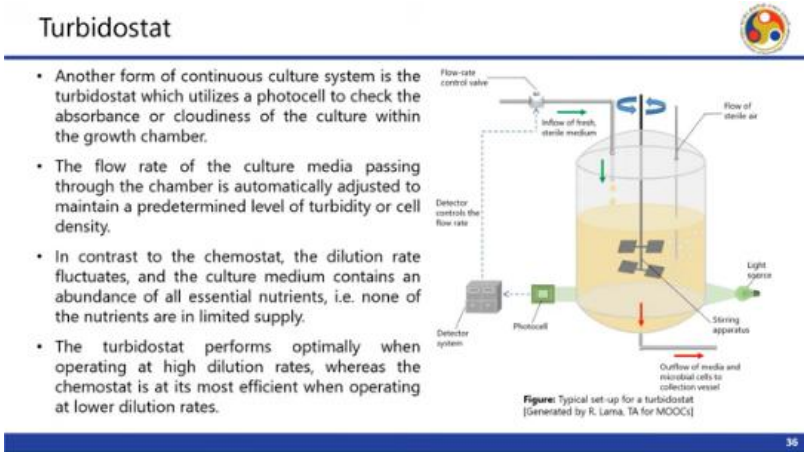
The culture medium used in a chemostat contains a limited quantity of an essential nutrient, which becomes the limiting factor for growth. The growth of the microorganisms is determined by the rate at which new medium is supplied, and the final cell density depends on the concentration of the limiting nutrient. The rate at which medium flows through the vessel relative to its volume is called the dilution rate, and this is given by the equation D is equal to F by V, where F is the flow rate in ml per hour and V is the vessel volume in ml. Now, what are the various experimental uses of the chemostat? It is used to study particular enzymes, particularly their activity, which may be quite lower in stationary phase cells than in exponential phase cells, and thus chemostat-grown cultures prove to be ideal settings.

It is also used in microbial ecology, enrichment, and isolation of bacteria, then constant supply of cells in exponential phase growing at a known rate. Then study of microbial growth at very low nutrient concentrations close to those present in natural environments. Then study of interactions of microbes under conditions resembling those in aquatic environments. And then finally, it is also used in food and industrial microbiology.

Let us now discuss the turbidostat step. This is a form of continuous culture system which utilizes a photocell to check the absorbance or cloudiness of the culture within the growth chamber. So here you can see the photocell, and there is a detector system and a light source

which will travel through this reaction vessel, and the detector will detect this. And then, using this feedback, it will control the flow rate of fresh sterile medium into the system. So, on this side, there is the inflow of fresh sterile medium, and on this side, there is the outflow of media and microbial cells to the collection vessel.

The flow rate of the culture media passing through the chamber is automatically adjusted to maintain a predetermined level of turbidity or cell density. In contrast to the chemostat, the dilution rate fluctuates, and the culture medium contains an abundance of all essential nutrients; that is, none of the nutrients are in limited supply at any given point in time. The turbidostat performs optimally when operating at high dilution rates, whereas the chemostat is at its most efficient when operating at lower dilution rates. Let us look into the significance of continuous culture. Continuous culture systems offer significant utility by ensuring a consistent provision of cells in the exponential growth phase, characterized by a known rate of proliferation.



They enable the investigation of microbial growth even under conditions of extremely low nutrient levels, mirroring the concentrations found in natural surroundings. These systems play a vital role in various fields of research, such as the examination of interactions between different microbial species within environments that resemble the conditions of freshwater lakes or ponds. So here is a representation of the different modes of culture maintenance. In A, we have the batch culture; in B, we have the fed-batch culture where we are supplying nutrients. And then in C, we have the continuous culture where we are supplying nutrients as well as removing the spent media.

But in the case of a fat-based culture, there is no taking out of the spent media. So, the relative cell numbers will vary from the batch culture to fed batch to the continuous culture, as you can see in these diagrams, which is represented by n. Also, the total volume will be

different; it will remain constant in the case of batch culture and continuous culture. But in the case of fed-batch culture, the total volume will increase because we are feeding in additional media but not taking it out. And then the substrate concentration—you can see the dynamics in the three situations will be different, as represented by the curve with respect to time.

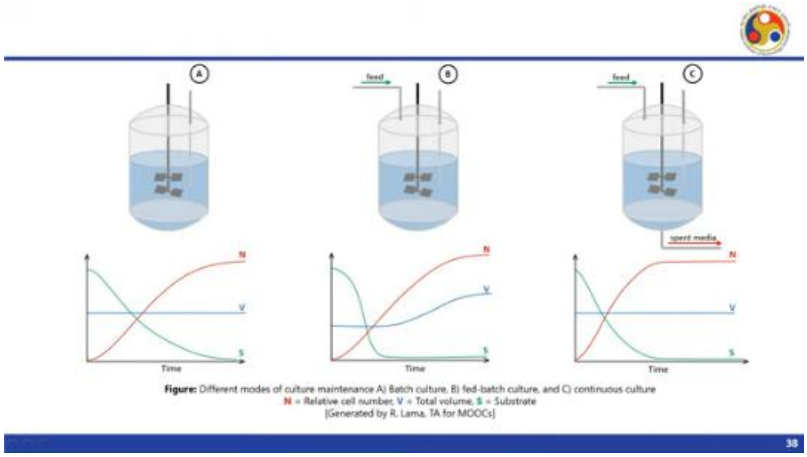


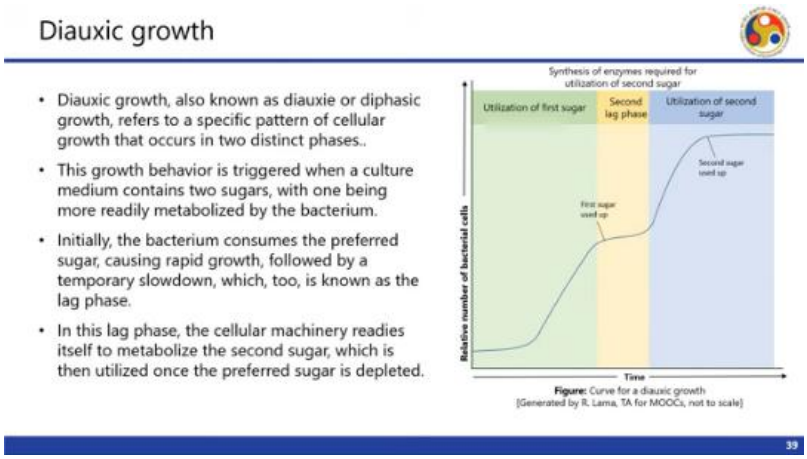
Figure: Different modes of culture maintenance A) Batch culture, B) fed-batch culture, and C) continuous culture
N = Relative cell number, V = Total volume, S = Substrate
[Generated by R. Lama, TA for MOOCs]

Now, let us discuss another important concept, which we call diauxic growth. This growth is also known as diauxic or diphasic growth, and it refers to a specific pattern of cellular growth that occurs in two distinct phases. And now, in this graph, if you look, we have some lag phase followed by an exponential phase, and then there is another lag phase over here, followed by another exponential phase and then another lag phase. Now, why has this typical type of growth pattern occurred?

It takes place when we utilize two different kinds of substrates subsequently or together. So, the first graph will be due to the consumption of one of the sugars, which is followed by a lag phase. And here, after this, the second sugar will be utilized, giving rise to the next exponential phase. So, basically, this growth behavior is triggered when a culture medium contains two sugars, with one being more readily metabolized by the bacterium, which will be the first choice. And initially, the bacteria consume the preferred sugar,

Causing rapid growth followed by a temporary slowdown, which is also known as the second leg phase. In this leg phase, the cellular machinery readies itself to metabolize the second sugar, which is then utilized once the preferred sugar is depleted. So here, the first sugar is completely used up, and here, the second sugar is completely used up. Diauxie happens because organisms have specialized genetic systems, like operons or multiple gene sets, that regulate the expression of enzymes necessary to process different nutrients they encounter. When an organism invests its energy and resources to produce enzymes for

metabolizing a sugar that allows only slower growth, it might not allocate these resources effectively.



Meaning it might not utilize most of its resources to produce enzymes for metabolizing a different sugar that supports faster growth. Certain organisms could face reproductive challenges compared to others that prioritize faster growth-supporting sugars. Over time, organisms have evolved to fine-tune their genetic control mechanisms. They now selectively express genes that lead to the fastest growth rate. For instance, when Lactococcus lactis is in an environment with both glucose and maltose, it gears its gene expression toward metabolizing glucose first.

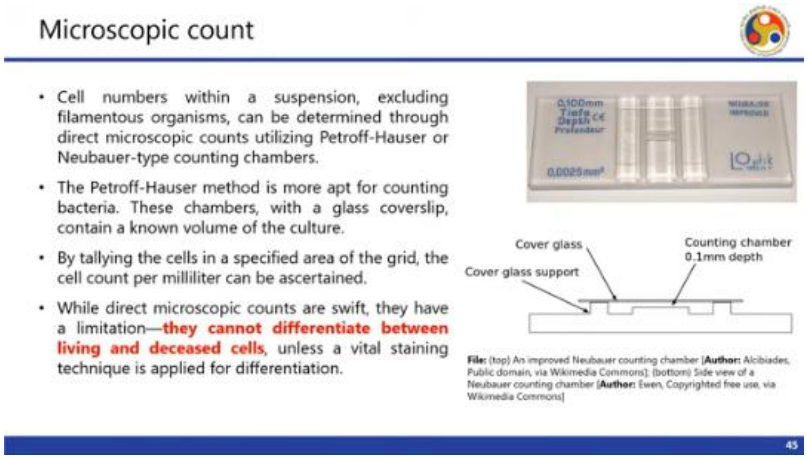
Only after the glucose supply is used up will it switch its gene expression to handle the maltose. So here, we can see the growth of E. coli in the presence of different carbohydrate pairs, serving as the only source of carbon in a synthetic medium. So here, the first carb sources are glucose-mannose, then glucose-fructose, glucose-galactose, glucose-xylose, glucose-arabinose, and glucose-rhamnose, and we can see the varying patterns of growth. Now, let us study the methods to estimate microbial growth. So here, we will be discussing microscopic count, the culture counter, flow cytometer, plate count technique, then the MPN test, turbidometric estimation, dry weight estimation, and ATP bioluminescence.

During fermentation, methods are required for the routine determination of microbial population, cell number, and/or biomass to monitor its progress. Numerous direct and indirect methods are available for this purpose. Direct procedures involve dry weight determination, cell counting by microscopy, and plate counting methods. Indirect methods include turbidometry, spectrophotometry, and estimation of cell components like protein, DNA, RNA, or ATP. Online monitoring of carbon dioxide production or oxygen utilization is also used.

Some estimation methods will be discussed in the next few slides. So, let's start with the microscopic count. Cell numbers within a suspension, excluding filamentous organisms, can be determined through direct microscopic counts utilizing Petroff-Hauser or Neubauer-type counting chambers. This is a Neubauer-type chamber, which is, of course, a modern and improved one.

Here, you have this diagram, which shows an improved Neubauer counting chamber, and this is a side view of this counting chamber. The Petroff-Hauser method is more apt for counting bacteria. These chambers, with a glass coverslip, contain a known volume of the culture. By counting the cells in a specified area of the grid, the cell count per milliliter can be ascertained. While direct microscopic counts are swift,

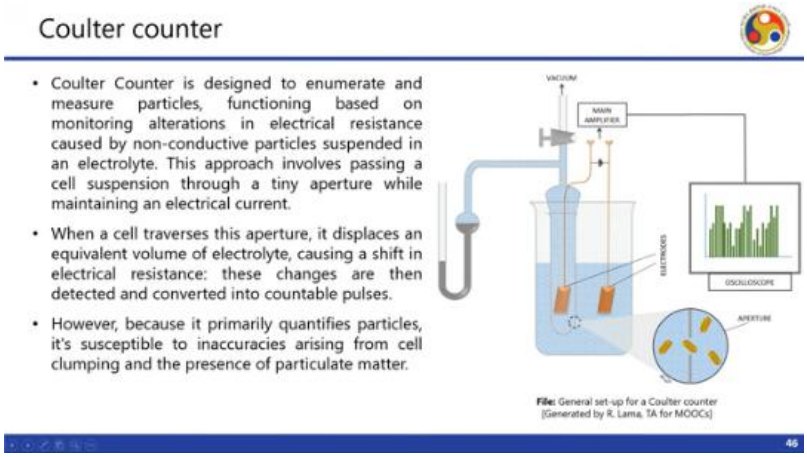
They have a limitation. They cannot differentiate between living and dead cells unless a vital staining technique is applied to distinguish between them. A culture counter is designed to enumerate and measure particles, functioning based on monitoring alterations in electrical resistance caused by non-conductive particles suspended in an electrolyte. This approach involves passing a cell suspension through a tiny aperture while maintaining an electrical current.



So here we have a general setup for a Coulter counter, and you can see here the electrodes and then there is the electrical signaling, which is being captured by this oscilloscope. And then, when a cell traverses this aperture, it displaces an equivalent volume of electrolyte, causing a shift in the electrical resistance. These changes are then detected and converted into countable pulses. So here we can see this diagram.

The cell is passing through this aperture, and then, for that particular moment, this space will be occupied by the bacteria, and the electrolyte is displaced, which is why there is a

shift in the electrical resistance. However, because it primarily quantifies particles, it is susceptible to inaccuracies arising from cell clumping and the presence of particulate matter. So, if this is not a bacterium but any other particle, that will also result in these electrical impulses. So, this method—or actually, no method—is 100% perfect, but we get some estimate of the particles by various measuring methods. So another method is flow cytometry.

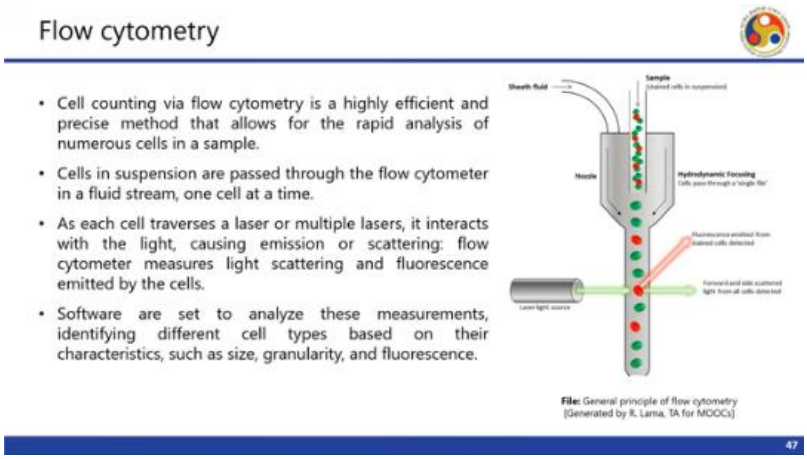


Here cell counting via flow cytometry is a highly efficient and precise method that allows for the rapid analysis of numerous cells in a sample. So you have a sample which are stained and these are in a suspension and then these are introduced into the flow cytometer. So there is hydrodynamic focusing as cells pass through as a single file. Then there is a laser source over here

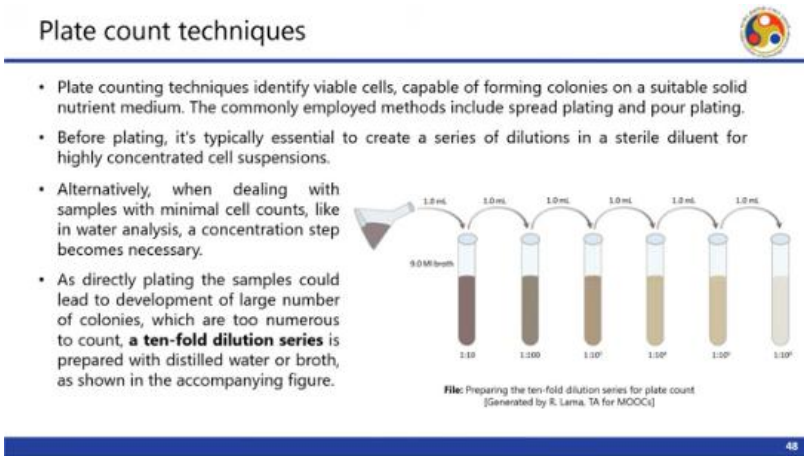
and then this will interact with the cell which is stained as it passes through and then this is the side area where the signal will be picked up. So, here the fluorescence that is emitted from the still cells are detected and here the forward and side scattered light from all the cells are detected. So, by these mechanisms the cell counting becomes quite highly efficient. So briefly cells in suspension are passed through the flow cytometer in a fluid stream one cell at a time as each cell traverses a laser or multiple lasers. It will interact with the light causing emission or scattering.

Flow cytometer measures light scattering and fluorescence emitted by the cells as we have discussed in the figure. And then we have softwares which will analyze these measurements, identifying different cell types based on their characteristics of the size, granularity, and fluorescence. Another method, less sophisticated, of course, than the flow cytometer are the plate count technique. These identify viable cells capable of forming

colonies on a suitable solid nutrient medium. The commonly employed methods include spread plating and power plating.

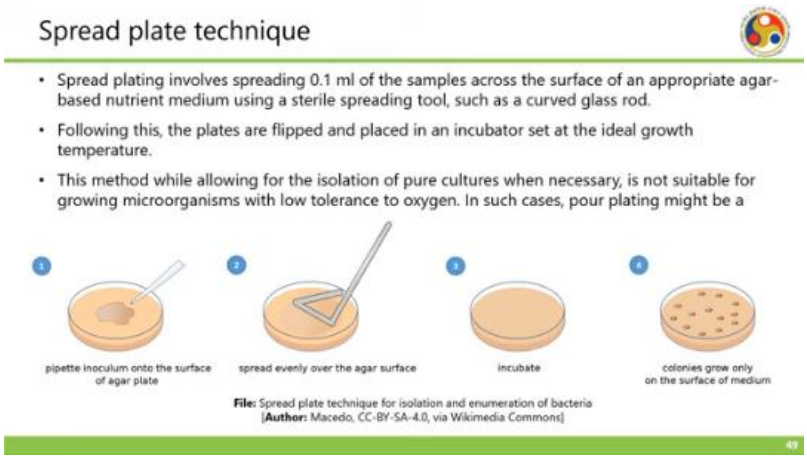


So, as you can see here, we are diluting the sample with every step. So, in the first step, we have a ratio of 1 to 10, and in the last, we have a ratio of 1 to 10 to the power of 6. Before plating, it is typically essential to create a series of dilutions in a serial diluent for highly concentrated cell suspensions. Alternatively, when dealing with samples with minimal cell counts, such as in water analysis, a concentration step becomes necessary. As directly plating the sample could lead to the development of a large number of colonies that are too numerous to count, a tenfold dilution is prepared with distilled water or broth, as shown in this figure and as already discussed.



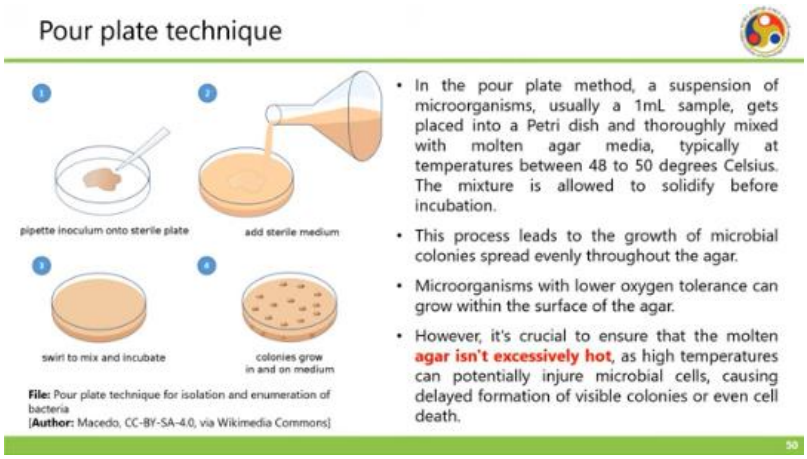
Another method is the spread plate technique. This involves spreading 0.1 ml of the sample across the surface of an appropriate agar-based nutrient medium using a sterile spreading tool, such as a curved glass rod. Following this, the plates are flipped and placed in an incubator set at the ideal growth temperature. This method allows for the isolation of pure

cultures when necessary. It is not suitable for growing microorganisms with low tolerance to oxygen.



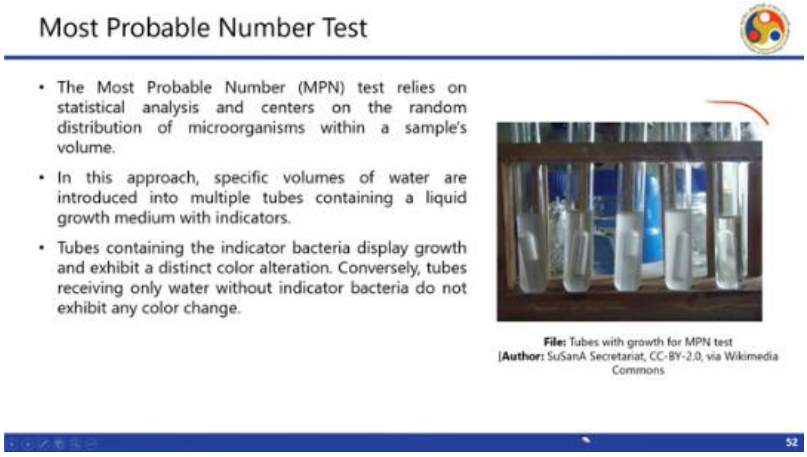
In such cases, pour plating might be a better method. In the pour plate method, the suspension of microorganisms—usually a 1 ml sample—gets placed into a petri dish, as we have shown here, and thoroughly mixed with molten agar medium. Here, we are mixing it typically at temperatures between 48 to 50 degrees Celsius. The mixture is allowed to solidify before incubation.

So here we do swilling to mix and incubate. And finally, the colonies will grow on this medium. This process leads to the growth of microbial colonies spread evenly throughout the agar medium. Microorganisms with lower oxygen tolerance can grow within the surface of the agar. However, it's crucial to ensure that the molten agar isn't excessively hot, as high temperatures can potentially injure microbial cells, causing delayed formation of visible colonies or even cell death.



How do we estimate the viable cells? Both techniques quantify colony-forming units, or CFU, which might not equate to the actual cell count due to clumping and microorganisms' cellular structure. Estimating the cell concentration in the initial sample involves considering the dilution factor and the plated volume. CFU per ml is given as below, where we have the CFU per ml as the number of colonies multiplied by the dilution factor and divided by the volume of sample added. To ensure statistical reliability, data is documented solely from plates hosting 30 to 300 colonies.

While these methods offer accuracy, a minimum incubation period of 1 to 2 days is typically required before colonies are countable. Let us now discuss another technique: the Ampion test, or the most probable number test. The Ampion test relies on statistical analysis and centers on the random distribution of microorganisms within a sample's volume. In this approach, specific volumes of water are introduced into multiple tubes containing a liquid growth medium with indicators, as shown in this figure. Tubes containing the indicator bacteria display growth and exhibit a distinct color alteration.



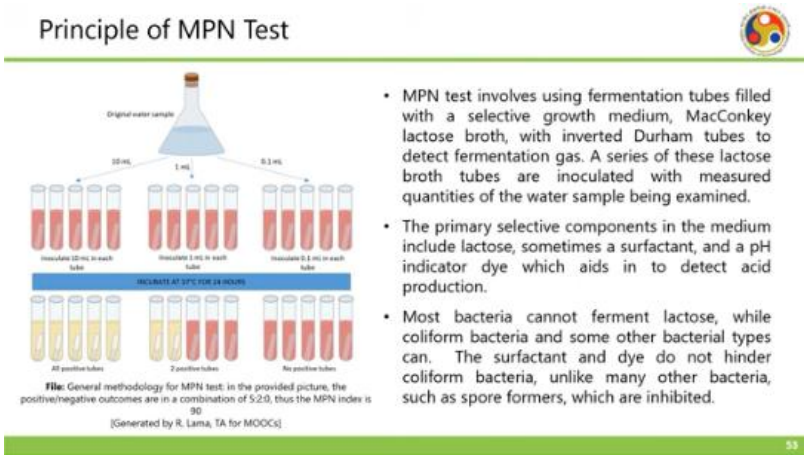
Conversely, tubes receiving only water without indicator bacteria do not exhibit any color change. By evaluating the quantity and pattern of positive and negative reactions, the MPN of indicator organisms within the sample can be estimated by consulting statistical tables. Now, let us look into the principle of the MPN test. Here, the MPN test involves using fermentation tubes filled with a selective growth medium. For example, McConkey lactose broth with inverted Durham tubes to detect fermentation gas.

A series of these lactose broth tubes are inoculated with measured quantities of the water sample being examined. So, this is the original water sample. So, here we are taking 10 ml. Then here we are taking 1 ml, and here we are taking 0.1 ml. So, we will inoculate 10 ml

in each of these tubes, then 1 ml in each of these tubes, and then 0.1 ml in each of these tubes, and incubate at 37 degrees centigrade for 24 hours.

So here, we have all positive tubes. Here we have, say, two positive tubes. And here we do not have, for example, any positive tubes. So, the positive-negative outcomes are in a combination of 5 to 2 to 0. Thus, the MPN index will be 90.

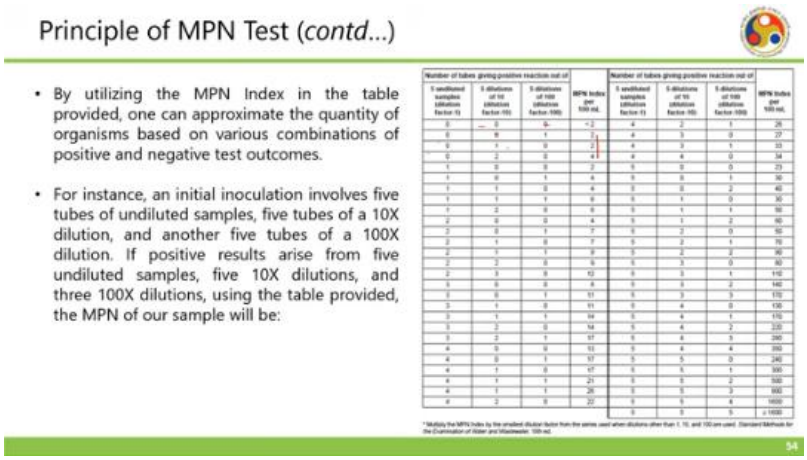
So, we will further see how this is being done. The primary selective components in the medium include lactose, sometimes a surfactant, and a pH indicator dye which aids in detecting acid production. Most bacteria cannot ferment lactose, while coliform bacteria and some other bacterial types can. The surfactant and dye do not hinder coliform bacteria, unlike many other bacteria such as spore formers, which are inhibited. So here you can see the number of tubes giving positive reactions out of five undiluted samples.



The dilution factor is 1. Here the dilution factor is 10. And here the dilution factor is 100. And this is the MPN index per 100 ml, less than 2. So in this case, it is 2.

So, for 000, it is less than 2. For 001, it is 2. For 010, it is 2, and so on. So, this is already calculated in this table. So, by utilizing the MPN index in the table provided, one can approximate the quantity of organisms based on various combinations of positive and negative test outcomes.

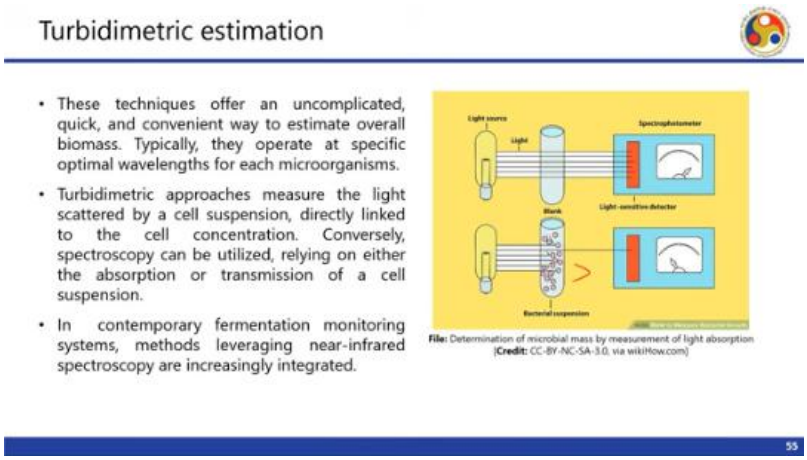
For instance, an initial inoculation involves 5 tubes of undiluted samples, 5 tubes of a 10x dilution, and another 5 tubes of a 100x dilution. If positive results arise from 5 undiluted samples, 5 10x dilutions, and 3 100x dilutions using the table provided, the amount of our sample will be an index of 100 per 100 ml. So, 900 into 100 per 100 ml, this will be 900 cells per ml. Here we can see these 900 ml.



Let us now discuss the turbidimetric estimation. These techniques offer an uncomplicated, quick, and convenient way to estimate overall biomass. Typically, they operate at specific optimal wavelengths for each microorganism. So here we have a light source, and then we have a sample here, and this is basically a glass vessel through which light can pass, and then there is a light-sensitive detector over here. And then we get some kind of reading due to the presence or absence of sample in this sample holder.

So, you can see here the transmission of the light is stopped if the sample holder has a lot of bacterial cells or any particulate matter. So, you can see the indicator representing the kind of signal that it has collected. In this case, the transmission of light is being stopped. So, turbidimetric approaches measure the light scattered by cell suspension, directly linked to the cell concentration. If we dilute this sample, actually some of the light will pass, and we will have a different kind of reading in that case.

Conversely, spectroscopy can be utilized relying on either the absorption or transmission of a cell suspension. In contemporary fermentation monitoring systems, methods leveraging near infrared spectroscopy are increasingly used. Turbidimetric and spectrophotometric methods rely on making calibration curves with standard cell suspensions of known concentrations. These curves can then be used for estimation of number of cells in an unknown sample or in monitoring the number of cells in a fermentation media at a given period of time. Care, however, must be taken when performing with fermentation broths that have particles or strong colours which could lead to faulty data interpretation.



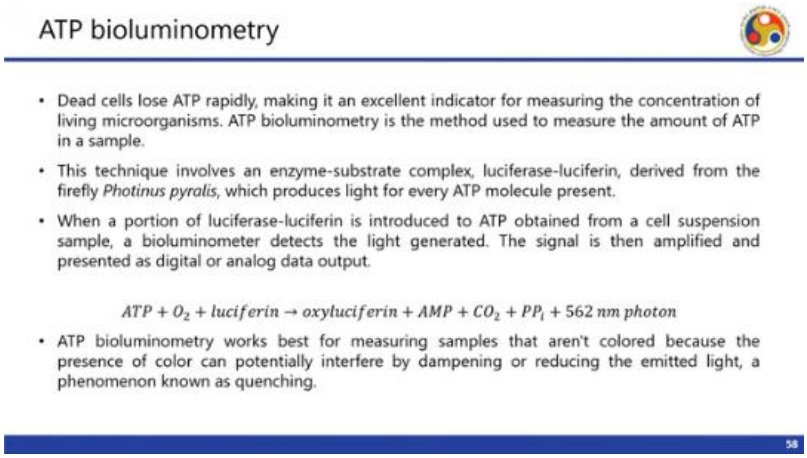
File: Determination of microbial mass by measurement of light absorption (Credit: CC-BY-NC-SA-3.0, via wikiHow.com)

Let us now discuss about the dry weight estimation. This technique measures the total weight of cells encompassing both living and deceased ones in liquid culture samples. The process involves isolating the biomass from a known volume of a uniform cell mixture typically achieved from vacuum filtration using a pre-weighed membrane filter with pore sizes of 0.2 mm or 0.45 mm. The collected cells on the filter are rinsed with water to eliminate residual growth medium and then dried in an oven at 105 degree centigrade until reaching a constant weight the results are usually presented as milligrams of cells per milliliter of culture it's important to note that on any other suspended materials larger than the filter pores can also accumulate potentially causing errors Moreover, obtaining results with this method takes time and a relatively substantial sample volume is required to gather enough biomass for precise weighing because the way of one bacterial cell will be infinitesimally small.

Another method that is used for estimation of cell numbers is ATP bioluminometry. Dead cells lose ATP rapidly, making it an excellent indicator for measuring the concentration of living microorganisms. All the other methods we discussed earlier actually many of them measure both the dead and the live bacterial cells together. But with these we can separate that reading very easily. ATP bioluminometry is the method used to measure the amount of ATP in a sample.

This technique involves an enzyme-substrate complex, luciferase-luciferin, derived from the firefly, which produces light for every ATP molecule present. When a solution of luciferase and luciferin is introduced to ATP obtained from a cell suspension sample, a bioluminometer detects the light generated; the signal is then amplified and presented as digital or analog data output. So here we can see ATP, in the presence of oxygen and luciferin, will get converted into oxyluciferin and bind to AMP, carbon dioxide, and

pyrophosphate, then emit light at 562 nanometers. So, this ATP bioluminescence works best for measuring samples that aren't colored because the presence of color can potentially interfere by dampening or reducing the emitted light, which we call quenching.



So, with this, we come to the end of this lecture. I thank you for your patient hearing. Amen.