Environmental Chemistry and Microbiology

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Module - 10
Lecture - 50
Microbial Growth - II

Welcome everyone. This is the second part of Microbial Growth. This is lecture 50 of module 10.

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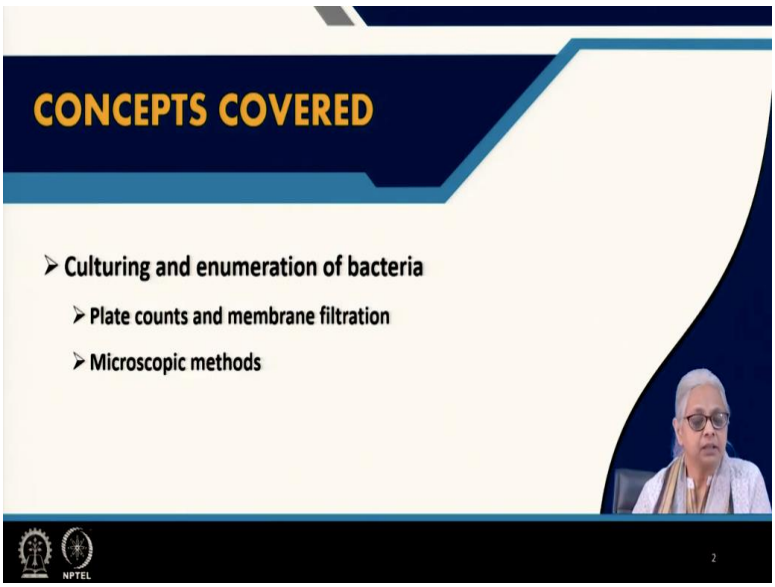

So, in this particular topic, we are going to look at how to culture bacteria and how to quantify them. So, we will take a look at plate counts and membrane filtration as well as microscopic methods.

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Now, when we talk about growing cultures of bacteria in the lab, how can it be done? So, the first thing is to understand what are the different types of cultures. The first thing is pure versus mixed. When I am growing a culture in the lab, it can be a single species in which case we call it a pure culture and if we take a bacterial consortium; for example, if I take a soil sample and use it to inoculate my media, then I will have a large number of organisms, not just bacteria, but fungi and so many other organisms. So, in that case, it becomes a mixed culture. So, that is the first difference.

The second difference is whether it is a solid-based media or a liquid media. I can grow bacteria on either a solid surface which is generally an agar-based surface or I can grow it in a liquid media; so I can have what is called a nutrient broth. That is the most common liquid media that is used in the lab for culturing bacteria. It is generally a multipurpose or a general-purpose kind of media, and it is always in liquid form.

So, you can have agar-based media or broth-based media. These culture media can be chemically fully defined. So, you can have very specific chemicals or salts added to the media, and these are called chemically defined culture media; or you can have undefined or complex media. So, I will show you examples of all of them.

And these chemically defined media may need the use of growth factors. Growth factors, we have seen, are micronutrients and vitamins that are required for culturing specific organisms. And there is another method that I am not going to go into, but you can also add certain inhibitor compounds which will inhibit the growth of all other compounds (correction: bacteria) except

the one that you are trying to culture. So, that is also possible. And we are not going to go into it, into any detail, but I will just show you examples of a chemically defined culture media.

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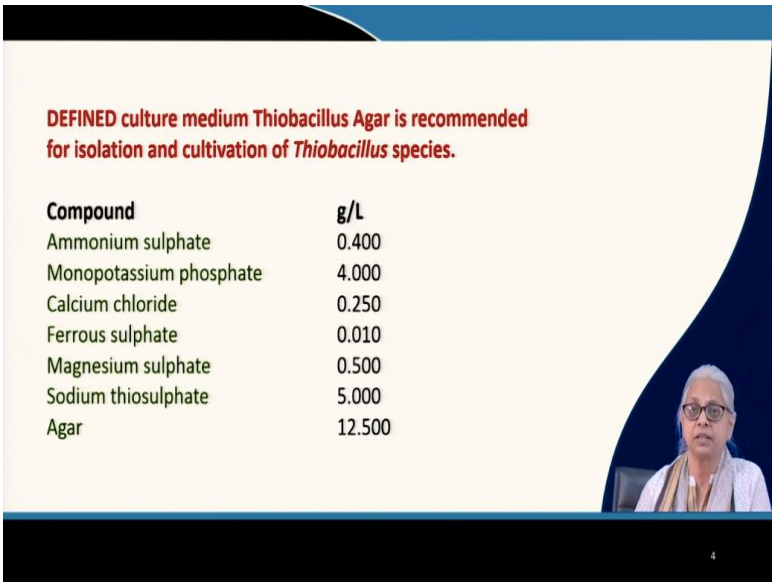

So, here you have a very simple culture media for cultivating Thiobacillus species. So, defined culture media which can be grown on Thiobacillus agar; and you can see the salts; ammonium sulfate, monopotassium phosphate, calcium chloride, ferrous sulfate, magnesium sulfate, sodium thiosulphate, and agar which is the solid base on which the cultures, the colonies of the bacteria will be formed.

So, it is a well-defined culture medium. And there are several examples. This is just one example. You can do this for any number of species, and there are well-defined culture media that are used for different species.

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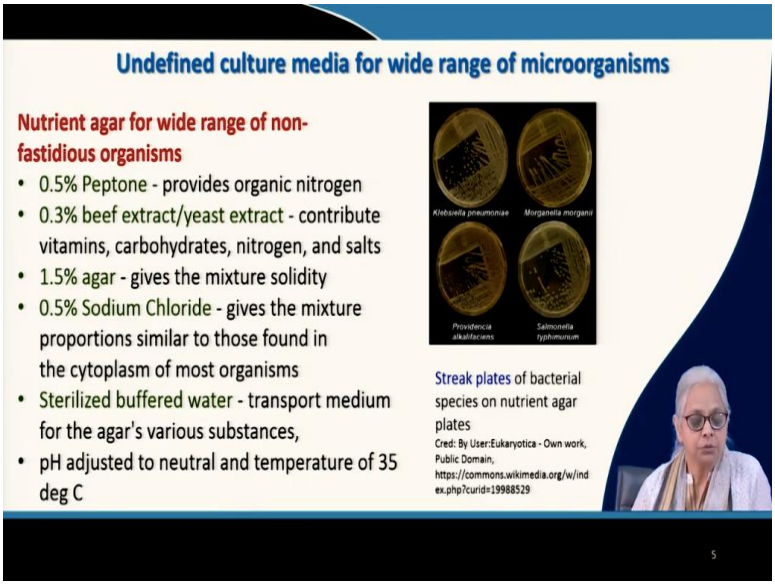

Here we have an undefined culture media which can be used for culturing a large number of microorganisms. So, nutrient agar, which is used very commonly, you can use it for any number of organisms. And you can see the kinds of materials that are used. You have 0.5% peptone, to provide not just organic nitrogen, but also other nutrients. Then you have 0.3% beef extract or yeast extract. It contributes vitamins, carbohydrates, nitrogens, salts, and so many other things. So, it is a fully mixed media. And you have 1.5% agar to give the mixture solidity when it is incubated. And some amount of salt is required. And this is, remember what I said, to maintain isotonic conditions similar to the ionic strength of the cytoplasm of most organisms. So, that will help to maintain the ionic strength inside and outside the cell, so that, that does not become a problem factor.

So, we can use sterilized buffered water. Now, sterilization and buffering of the water that is used is essential to ensure that the isotonic conditions are met, that the pH is near neutral. So, we try to keep it around 7. It can go to 6.5, 8 even, the bacteria will not die. But if it goes beyond that, they are likely to die. Generally, the incubation temperature is 35 °C.

And I say that because we are generally, especially in environmental microbiology, the kinds of experiments we do are either with pathogenic species that may be found in water or the normal environment, and we want to keep the temperature as close to environmental conditions as possible. So, we may want to keep it at 35°C, but depending on other objectives of the experiment, you may have any other temperature or pH even.

So, here you see streak plates of pure cultures of 4 different species. So, you have *Klebsiella pneumoniae*, *Morganella*, *Providencia*, *Salmonella*, and so on. So, these kinds of things can be done very easily in the lab.

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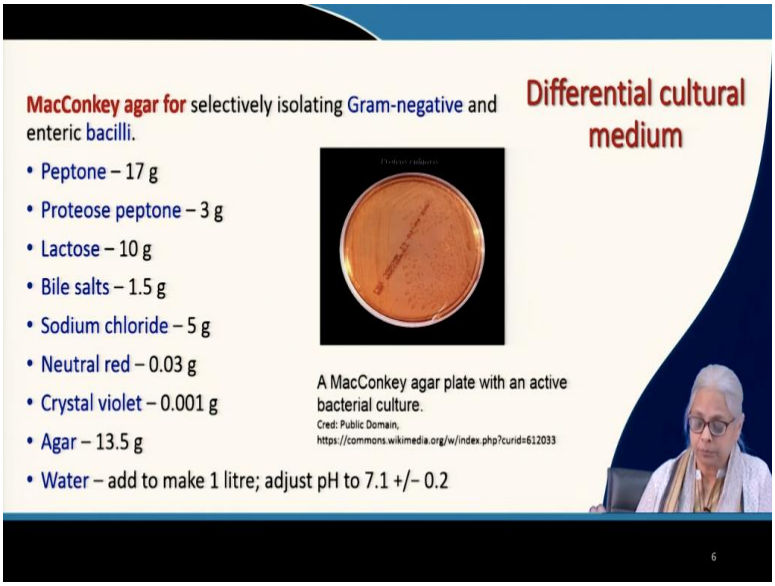

Then we have what is called a differential cultural medium. Now, you can use different types of culture media for isolating a large number of bacteria. So, for example, as I said, you want to look at gram-negative bacteria or you want to look at enteric bacteria or you want to look at some other type of bacteria, then you have certain types of agars that are designed for culturing those types of species.

So, here you have another. It is not entirely undefined. There are complex mixtures of peptone, lactose, bile salts, sodium chloride, and so on. So, this is for cultivating a particular type of bacterial species. So, many different types of bacterial species will come up on this type of media.

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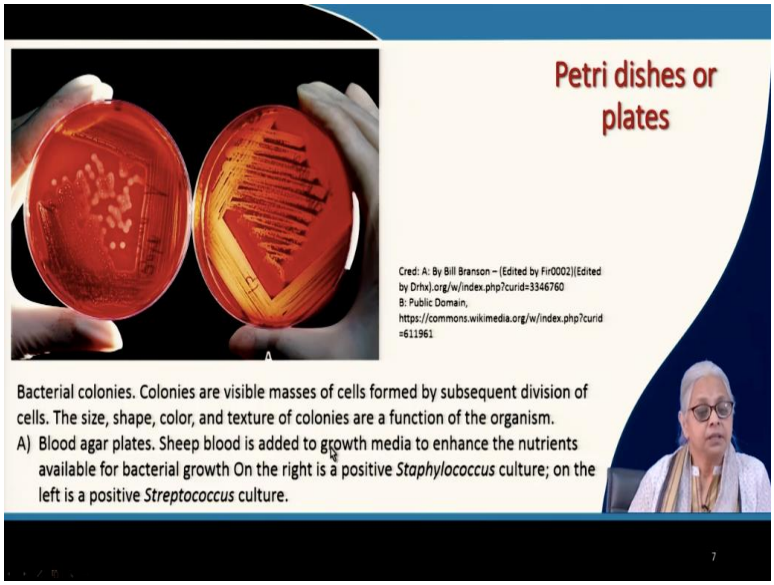

These are Petri dishes or Petri plates. These are the equipment that we use for culturing on solid media. So, these are the agar media that are added to the Petri dishes or Petri plates. These are the terms we use. And when you incubate them for anything from 24 hours to 7 days even; it depends on what you are trying to do; it can be anything. The temperature can change; the incubation period can change; it all depends on what you are trying to do.

So, these are bacterial colonies that will show up after the incubation period. So, if you are looking at the plate; initially when you plate it when you add your sample of water or any other sample that you are trying to look at; when you add it to the plate, initially nothing is visible, because these cells are invisible to the eye. But after a day, 2 days, or even more, you will find these kinds of colonies. So, each colony is assumed to represent a single cell.

It is not true. It is an assumption, just to simplify matters. It may be a cluster of cells that is the starting point, or it may be a single cell that is the starting point. And what we usually see in the lab is that the colony size varies. You will have very tiny pinprick-sized colonies, or you will have very large colonies. And that is because the starting point may be a single cell or clusters of cells.

So, all kinds of things are possible. So, these are examples of bacterial colonies. They will be different in terms of size, shape, color, texture; all these things will be different about different bacterial species. So, this is an example of what are called blood agar plates. Sheep blood was added to the growth media to enhance the availability of nutrients. In one case, on the right you have *Staphylococcus* and on the left, you have *Streptococcus* cultures. You can see how different the different species of bacteria are.

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More examples of plating and how you can use this method for creating pure cultures. So, these are streak plates. These are called streak plates. I will come to more details later about how to create streak plates. It is not just for bacteria. Very often what we get is yeast growth, yeast, or fungal growth on the surface of these solid agar media. So, the first 4 that you see over here are bacteria. This fifth one is yeast. This is fungi, and Candida is also a yeast. So, this is very common. And it happens in the lab very often that you get the growth of fungi or yeast on these plates if they get contaminated. So, these are some examples.

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Now, let us come to aseptic transfer. Now, whenever you are doing lab experiments, you have to ensure that the media, especially if you are working with pure cultures, you have to make it, the first objective is to ensure that it is an aseptic transfer from one media to another. So, in the

first case, you can see 2 tubes. One of them probably has the bacterial culture. And you take a metal loop. So, these are the metal loops. Sometimes they have wooden handles; sometimes they have metal handles. There is a loop at the end of this particular tool. And when it is dipped in the nutrient broth; so, this is the nutrient broth that is supporting the culture of the bacteria. This droplet of the nutrient broth which will stick to this tiny little loop is then going to be transferred to another loop (correction: media). Now, you can just do it without making sure that the loop is entirely sterile before you dip it into the pure culture.

So, sterilization is done by what we call flame sterilization. So, it is a metal loop; you hold it over a flame; everything is assumed to be dead when it is burned. And only then will you dip it into the growing culture, and then transfer it to another sterilized growth media. So, that is an aseptic transfer. The results are shown over here. There are more details for you to look at if you are interested. And this is how streaking is done.

Now, in this first case, this is an aseptic transfer from one liquid media to another liquid media. You can have an aseptic transfer from liquid to solid or solid to liquid, and the same process can be used. So, this part is flame sterilization where the loop is sterilized over a flame. Then it is transferred from the nutrient broth to the solid media. The media is the plate, the Petri plate. And you have a streak.

So, you just take the loop and run it lightly, very lightly over the solid surface. And that will allow the cells that are part of the loop; they will be transferred to the solid media. Remember, everything is sterilized except the loop which contains the pure culture or even a mixed culture. From this loop, at the end; it is a single streak that is used; at the end of the loop, you will have the most diluted concentration of cells.

That little concentration is then used to further streak it, and the direction changes. So, you can see how it is done. The heaviest growth is over here. That is the beginning of the streak. The last point on the streak plate is then used to transfer it to the next one. And you can see 1, 2, 3, 4, and 5 and finally the sixth streak. The sixth streak has individual cells. And you can see the sizes of the colonies, they are very small. And that shows that individual cells have now been separated from each other; otherwise, they are all overlapping each other.

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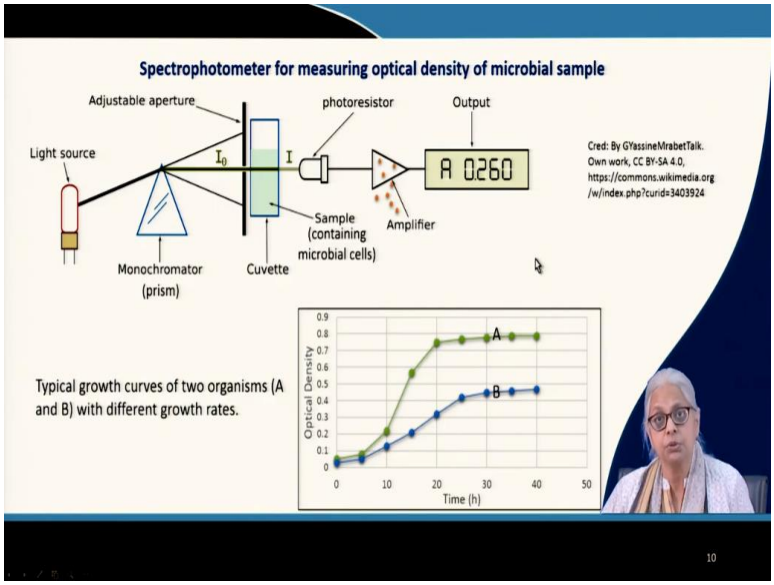

Then we come to another method which is called optical density measurement. So, you can grow your cells in clean nutrient media. When it has no cells in it, it will have a certain optical density which will be close to 0, because the cell concentration is 0 at the time of the sterilized nutrient broth or whatever media you are using. So, at t = 0, with 0 cells added, you will have a certain cell optical density when you use it when you take a sample of that and add it to a spectrophotometer or even a turbidity meter.

You can use anything. You can use a visible spectrophotometer or a turbidity meter for these kinds of measurements. So, here we have our spectrophotometer. You can use a turbidity meter for doing these measurements. And we have the cuvette which contains the sample. So, the light will pass through the cuvette. Now, this is the light intensity. I_0 is the light intensity that is incident on the cuvette, and I is what passes through the cuvette, and that is picked up by a sensor.

What you are actually measuring is the light that is transmitted through the nutrient media. Now, this light, when it is measured, is going to tell you something about the optical density of the media itself. So, the optical density when there are no cells is close to 0. As the cells begin to grow, depending on the species, you will get different types of growth curves. So, you can see the lag phase, the exponential phase, and the stationary phase, based on optical density only.

So, this is a very crude method. And I say it very clearly that it is a crude method. It does not work for low concentrations of substrates; it perhaps works for high concentrations of substrates. And getting calibrations between optical density and the numbers of cells or biomass can be difficult. It can work, but it can be difficult as well.

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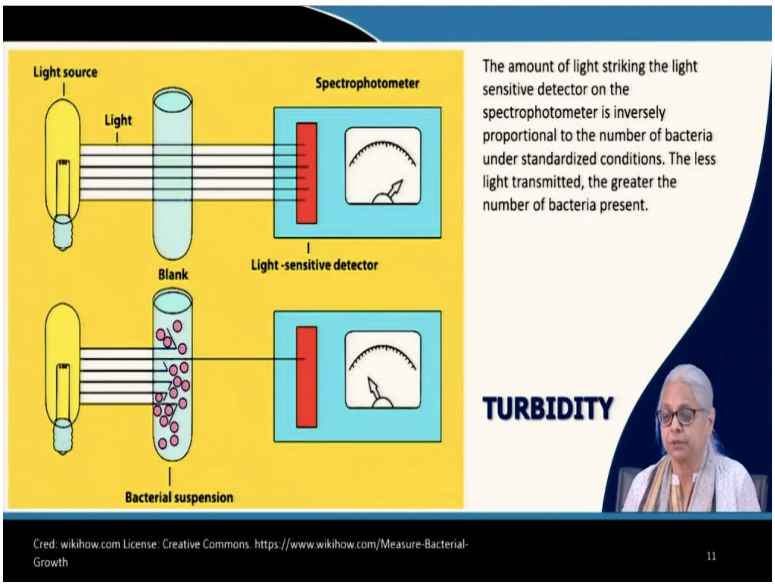

So, as I said, the same principle, whether it is a spectrophotometer or a turbidity meter can be used. And just remember that the light passing through the sample is inversely proportionate to the bacterial concentration. So, the lesser the amount of light transmitted, the greater the number of bacteria present in the sample.

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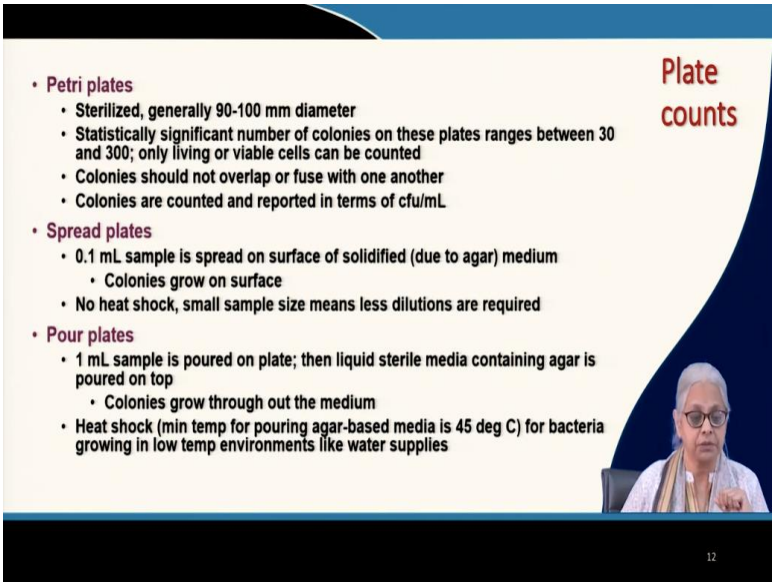

I have already mentioned something about Petri plates. As I said, Petri plates are standard methods for growing and culturing bacteria in solution. So, these have to be sterilized; they come in standard sizes of 90 to 100 millimeters. So, the bottom plate is 90 millimeters in diameter, and the top plate, the lid is 100 millimeters in diameter. So, what we call statistically significant numbers of colonies on these plates is anywhere between 30 to 300.

And the best part about plate counts is that it allows you to measure living or viable cells only. It does not tell you the total number of cells. And it tells you that only the cells that are visible on the plate in terms of colony-forming units; so, that is the unit that we use; CFU stands for colony-forming units. And those are indicative of the number of living or reproducing cells in your sample.

And these colonies should not overlap or fuse with one another. This also can be quite challenging, because you need just the right amount of dilution to be able to get this kind of result. So, it is tough work; but after a certain level of practice, most people get it right. There are 2 ways of doing enumeration using plating or plate counts. One is spread plate and the other is pour plates. So, spread plates; I will show you examples of that.

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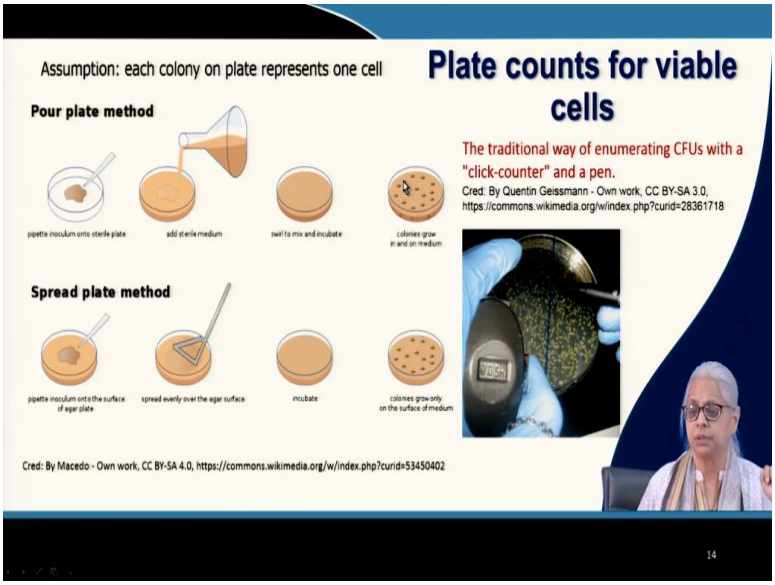

So, here we have a spread plate. Now, in the spread plate, the nutrient agar has already been solidified and a small amount of inoculum is added. We generally add either 0.1 ml or 1 ml of inoculum. So, this is added to the surface of the already solidified media. The spreader, the spreader can be plastic or glass. It is a hook-shaped object, and it is used to spread the sample over the agar surface.

It is then incubated always in an upside-down position, to prevent the condensate from falling and disturbing the colonies. So, this incubation is done upside down. And then the colonies that grow on the surface of the media can be counted after whatever the incubation period is. So, it can be 24 hours, 48 hours. I have done it as much as 5 days later. So, it all depends on what you are trying to do.

In the pour plate method, which is the other method, the inocula or the amount of sample that is required; you can add 1 ml because there is no limitation over here. I will come to this point again. You can add 1 ml of the sample, and then add the sterilized agar-containing medium to the plate. Now, the problem with this is that the temperature of the agar-containing media has to be fairly high.

So, it is about 45 to 55 degrees. And at that point, if your samples, the cells in the sample contain bacteria that have an optimum temperature of let us say 20 °C or 15 °C, they may die because of heat shock. So, that is the only problem with the pour plate method. If they are bacteria that are capable of surviving even 45 and 55°C temperatures, then it is not a problem. So, you combine it, swirl it to mix it, incubate it again in upside-down condition, and the colonies can be counted after the end of the incubation period.

So, the advantage of the spread plate method is that there is no heat shock, but the small sample size means fewer dilutions are required, but it also means that the sensitivity of the method is poorer compared to the larger sample sizes. So, when you have large sample sizes, you get better results. And then you have pour plates. So, you have 1 ml sample, as I said can be added; the colonies can be grown; I have mentioned all these points. And this can be quite challenging because if you are used to doing rich cultures and then you move to doing very poor nutrient environments like water supply systems, then it becomes very difficult to figure out what is going to work and what is not going to work. So, these are important factors to keep in mind and work out what are the best conditions.

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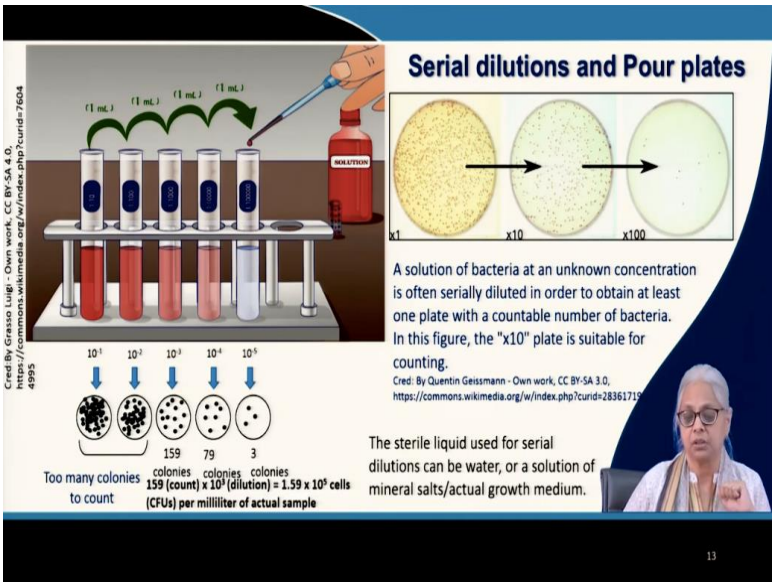

Then we come to what is essential for serial dilutions. Now, let us say you take a wastewater sample. And wastewater contains millions to billions of cells per ml. I obviously cannot take 1 ml of that wastewater and put it on a plate, because I will get a complete lawn of bacteria, and there will be nothing that is countable. So, to get a countable result, I must do what are called serial dilutions. So, for serial dilutions, we have what is called 1 in 10 dilution.

So, you take a tube; you take 1 ml of your sample; make it to 10 ml total volume. And so that is 1 ml of the sample and 9 ml of your mineral media, whatever you use to buffer the dilutions. All of it has to be sterilized including the test tube. So, all solid and media have to be sterilized. So, here you have 1 is to 10 dilution. You can start with the original sample. 1 is to 10, 1 is to 100, 1 is to 1000, and it keeps going on in multiples of 10. So, this is called serial dilution. Now, you take 1 ml (or 0.1 mL) from this after mixing it properly and plate it on Petri plates. You can use spread plates or pour plates, either way. You can see the first 2 results; they are too close. The colonies are overlapping, they are fused, so you will not get a clear result. In this case, we say too numerous to count. TNTC stands for too numerous to count.
Then we count to 10^{-3}, 10^{-4}, and 10^{-5}. You can see, in 10^{-3}, there are 159 colonies, clear and distinct from each other. There is no overlapping, there is no fusing. And this fits within our statistically significant window of 30 to 300. That is easier to count. Below 30, it is not very precise, because the amount of sample that you are adding to the plate is just 1 ml.
And when you get only 3; you can see the difference. You would want; if 159 is the most precise result, then after the next dilution, you should get about 16 colonies, but you are getting 79. And in the next dilution, you are getting 3. So, it should be one-tenth in each dilution, but it does not work that way. So, here you have 2 statistically significant results. But the higher number is generally considered better than the lower number. The lower the number, the greater the lack of precision in the count.

And after the plates are done, so we normally get plates like this. You put them under a magnifying glass; you have what are called colony counters. And the way to do it is, you have a clicker counter, so you have pens which have a clicker on them, and you can just put a dot against each colony; and each dot, when you make a dot, will click, and you have a digital readout of the number of colonies. So, this is the digital readout of the number of colonies. And if you have the ability to take photos, store them on your PC; makes it even more easy to count.
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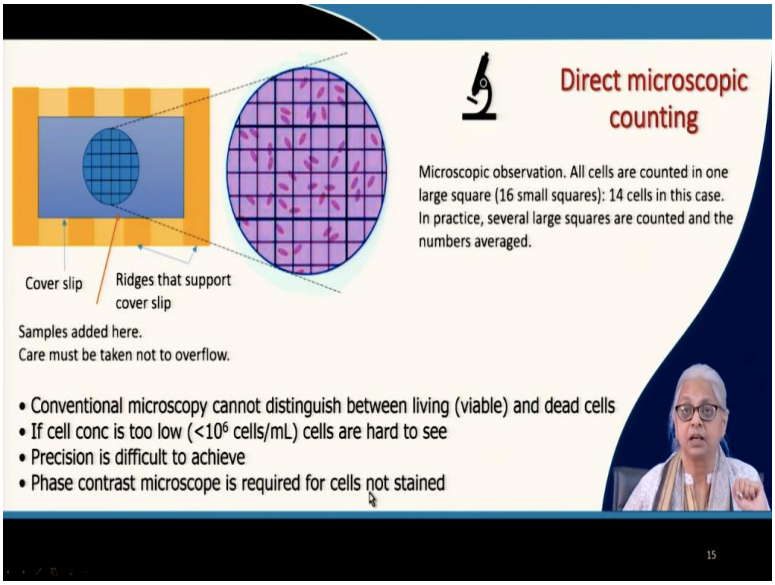

So, here is a glass slide. The yellow part is the glass slide. You put your sample on the glass slide, and the glass slide itself has ridges. So, the sample will collect in those ridges. And you have a coverslip; the blue part is the coverslip, and this coverslip may have a grid on it. So, when you put it under the microscope, all these cells will be observable through the grid. And you can count the number of cells.

So, if you get such a clean image, then you can count the number of cells in any of these squares. So, ideally, you would count these 16 squares within the central larger square. So, we have 16 small squares, and you count each one of them. And in this case, you have 14 cells. Now, in practice, we generally count this 3, 4, or even 10 times to get a good number of fields of vision. And then you take an average of those 10 fields of vision, to get a good average and a standard deviation. Because it is not often that you get uniformly distributed samples because bacteria have a tendency to cluster. Even though they are free-living and independent organisms, they do not break away. Even when they are dividing, they tend to remain stuck together. So, conventional microscopy cannot distinguish between living and dead cells.

However, we have already seen in fluorescence microscopy that you have specific dyes for enumerating living cells and separating them from dead cells. So, I have already covered that. Cell concentration can be too low, in which case, the number of cells in this window maybe 0, and it becomes very difficult to quantify. Precision, as I said, is very difficult to achieve. The standard deviation and the mean values can be very close to each other, and it becomes very difficult to count. And then you may need a phase-contrast microscope if the cells are not stained, and that again adds a level of complexity to the entire process.

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These are the results of membrane filtration. I have already mentioned membrane filtration where you take a water sample. So, for people like me who work in water samples, what do we need? Water unlike wastewater has very few cells. Cell concentration is in the range of about 1000 cells per ml or even less. Now, when you are dealing with very small samples, not like wastewater which has billions of cells; so, when you are dealing with water samples that have very low cell concentration, then you need large sample volumes to get a detectable number of cells or cell colonies.

So, we use membrane filters. So, you vacuum filter the sample; you can take as much as 1 liter, 10 liters whatever you want. And depending on the precision you want, you can take any volume of sample. Now, you can see what has happened over here. 100 ml of the sample was filtered and you get colonies that have fused and overlapped with each other. And you can see the size variation. So, this is what is called chromogenic agar media.

So, this membrane filter is resting on a nutrient pad. This nutrient pad has been, it comes in sterilized packages. The membrane and the nutrient pad come in sterilized packages. They are commercially available. All you need to do is add sterilized water to this. And when you filter the sample through this filter, you can count the number of colonies and you can see that the larger colonies represent a cluster of cells.

However, from the point of view of standard methods, we stick to the idea of colony-forming units, keeping in mind that each colony-forming unit may represent more than a single cell. In any case, it represents, at the minimum, a single cell. The others are SEM photos, which I have already shown you in the previous module.

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These are plate counts and these are colony-forming units. You can see, if the distribution and the spreading of the cells is good, then each of the colonies is fairly well-defined, and they are more or less of the same size. What you can also see over here is that the colonies are slightly different in color. So, there are greyish-colored colonies, there are bright yellow colonies and there are bright yellow-orange colonies.

Now, each (colour) colony represents a different species. Now, you need to isolate these species and look at them using biochemical test kits to decide or to decipher which one of the species it is. So, plate counts will not give you that information unless you are using species-specific media. This is the nutrient broth. This is a generic media that is used for cultivating all kinds of bacteria.

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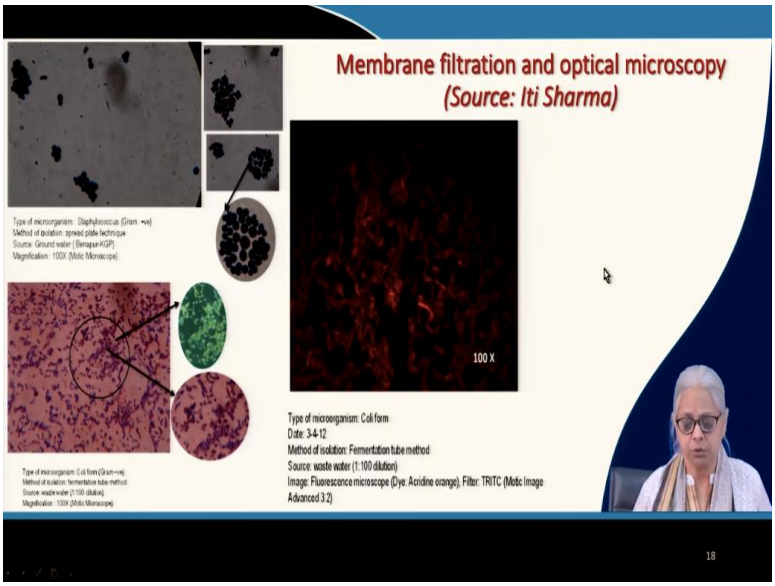

I have already shown you these examples as well, so I would not spend any time on it, but just to remind you that membrane filtration and microscopy can be used for enumerating bacteria. I will stop at this point. Thank you.