Applied Environmental Microbiology
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Lecture - 31
Techniques in Environmental Microbiology I

Dear students. In today's class I want to equip you with the Techniques that we use in Microbiology that will help you understand what is going on in your environmental samples and environmental situations.

Now, these techniques are that I am going to cover in this class and the lecture after this class, will be the techniques that are used across the globe and also including the techniques that are that have recently emerged, as high throughput cost effective and yet sophisticated incense that they require special specialized skill set.

So, we will be covering a wide range of techniques ranging from the older, but better established techniques to the newer and more promising ones. And I will be going through the basic in environmental laboratory setup to give you an idea of what it is like and what not to work in an environmental microbiology lab and what are the precautions that we need to take care of alright. So, let us start techniques in environmental microbiology.

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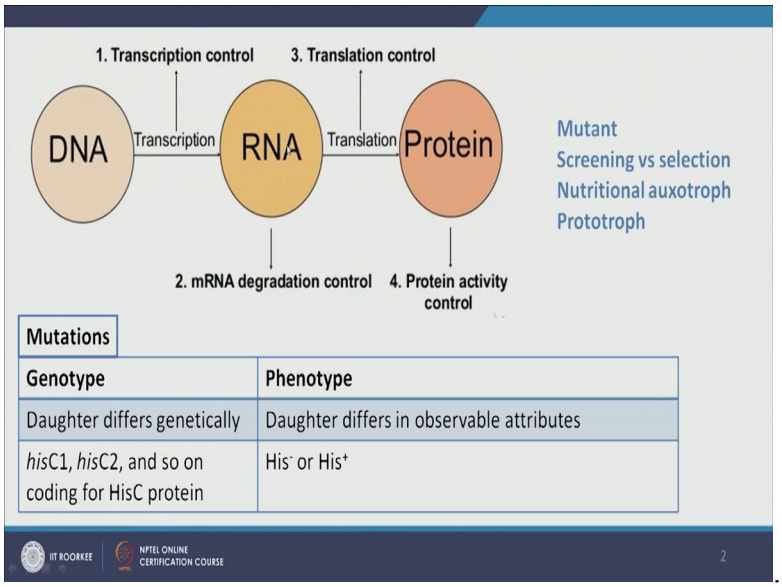

So, in the last class I told you I reminded you about the basic paradigm of microbiology which is DNA to RNA; RNA to protein RNA is a messenger protein is your actor. So, now, I want to understand what the genomic signature is what R N as have been recently transcribed and what proteins have been translated.

So, in order to get information about these I definitely need to extract the nucleic acids including DNA RNA or the proteins separately and then analyse them, sequence them in case of nucleic acids and characterize them in case of proteins. I might also look at the metabolites, which are affected by the protein activity and get metabolomics information.

Now, how do I go about this it is very interesting and easy to know in theory, but how do we do it in practice. So, let us together explore the key microbiological methods in bioremediations.

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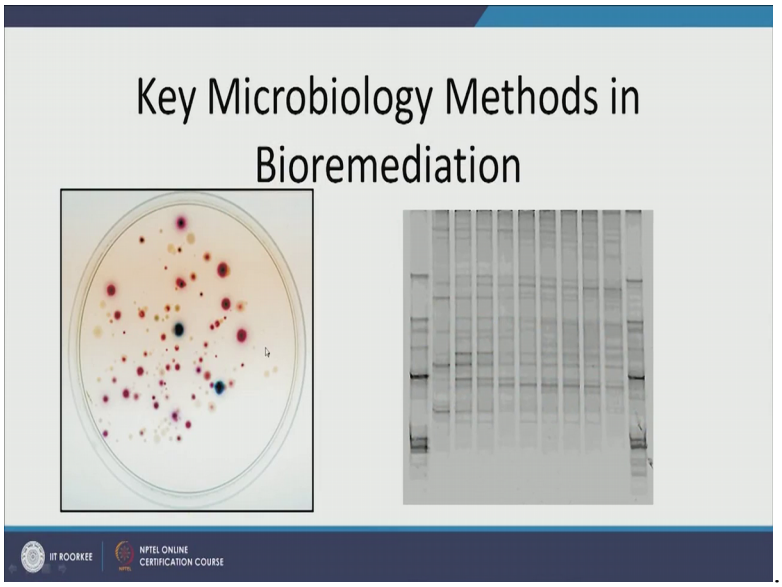

Now, the 2 pictures here the on the left panel you have a cell culture and you can notice that there are colonies of different morphologies different colours they are different sizes. So, the bigger ones perhaps are the faster growing, but colonies the smaller one are slow growing ones.

And then the different colours perhaps hint to different kinds of microbe different species and strains of microbes growing in this plate. On the right we have an out dated technology called D G G E I call it out dated because we have cost effective time

effective and easy to do sequencing techniques, now which make this the D G G E quite obsolete, but it has been a goal standard for quite some time until recent past actually I guess until 2011 when this was phased out.

So, let us explore these techniques. The first question is now you know your environmental problem. For example, let us take excess of chromium in groundwater and you want to find out the microbes that would sequester the chromium from groundwater, now the step to find out the right microbe how do you do it well?

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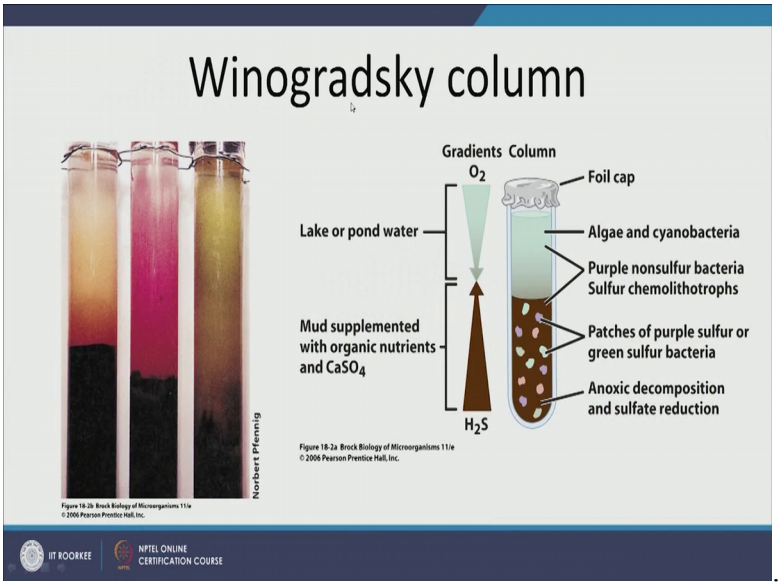

We have something called Winogradsky column which is basically a column that allows the water or your sediment in case of lake, in case of groundwater your ground water and sediment to form vertical gradient.

Such that on the top we have a foil a stopper that stops things from falling in and because it is in communication with air and when the air can freely move in and out on at the top we have higher oxygen concentration, but as you go below we have lower oxygen concentration. Also notice that in all the 3 of this winogradsky column we have black material or the bottom these are the sulfides.

So, as the oxygen gets depleted we move towards the anaerobic zone. So, at the top we have an aerobic which resembles at top of the lake. So, remember in lake we talked

about in previous lectures, how we have oxygen gradient? We have electron acceptor gradient, we have light gradient. So, all these gradients are ressembled in these columns.

Winogradsky use these columns to detect and to isolate different kinds of bacteria, such as purple non sulfur bacteria, purple sulfur bacteria and green sulfur bacteria all righty then. So, one option is to set up something like winogradsky column where you can simulate environmental conditions and then with depth you have different microbial communities right or the top will have algae in cyanobacteria, then you will have purple non sulfur bacteria, sulfur came only through drops and then you have patches of purple sulfur or green sulfur bacteria and then here you have suffered reducing bacteria.

So, if you want sulfate reducing bacteria sample them from the bottom not from the top you want algae sample it from the top.

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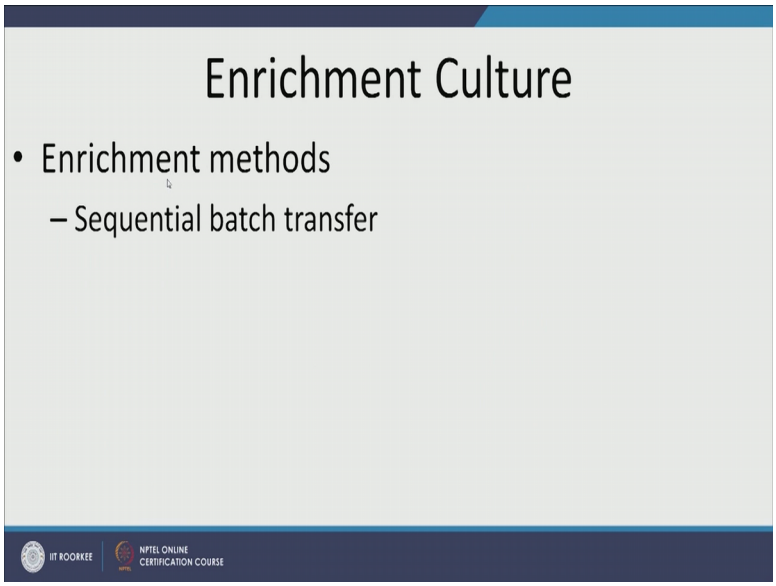

The other option is to do enrichment culture in enrichment culture I take my microbes from environment and I enrich them on solid conditions. For example, I want microbes that are tolerant to arsenic, they do not they can live in very high concentration of arsenic.

So, what I can do is I can take microbes that are already exposed to arsenic or you know just environmental microbes of interest. And then I can put them in arsenic rich

conditions and I can this enrich them in sense that the microbes that are tolerant to arsenic, they will grow they will flourish and rest of them would not.

Now, how do we enrich their 2 basic methods of enriching one is sequential batch transfer? So, in sequential batch transfer I have a batch. So, by the way if you did a let me go through briefly 2 different kinds of reactors batch reactor and C S T R. So, that you understand the Chemostat in sequential sequential batch reactor that we are going to talk about. So, in environmental science and engineering we talked about 3 different kinds of reactors which are very very important.

So, let us briefly go through them. So, that we can understand the 2 different methods of enriching microbes of interest.

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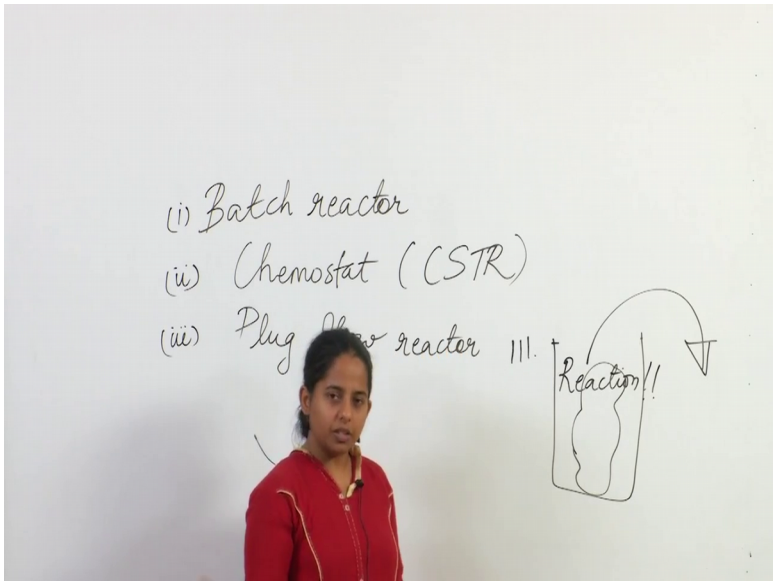

So, the first kind of reactor is called as Batch reactor the second kind of reactor we can call it Chemostat, the third kind of reactors which is more relevant for actual environmental conditions is plug flow reactor. So, let us try to briefly understand what these reactors are for enriching we use batch reactor and chemostat reactor. Chemostat comes under many different names, but the most popular one in environmental science is CSTR.

So, CSTR stands for continuously stirred tank reactor. So, starting with batch reactor it is more like your washing machine. So, you have a reactor you put things in for example,

in washing machine we put our dirty clothes, we put the soap, we put the water and then you close it. So, in steps one you have put things in in step 2 you have closed it. So, in step 2 you have closed it and you allow the reaction to occur here.

So, your reaction is happening here and once your reaction is over you open it again in third step you open this reactor again and you take your react product out. So, this kind of reactor is batch reactor we put reactants in we allow the reaction to happen when reaction is complete we take our products out. In continuously stirred tank reactor it is a very different scenario.

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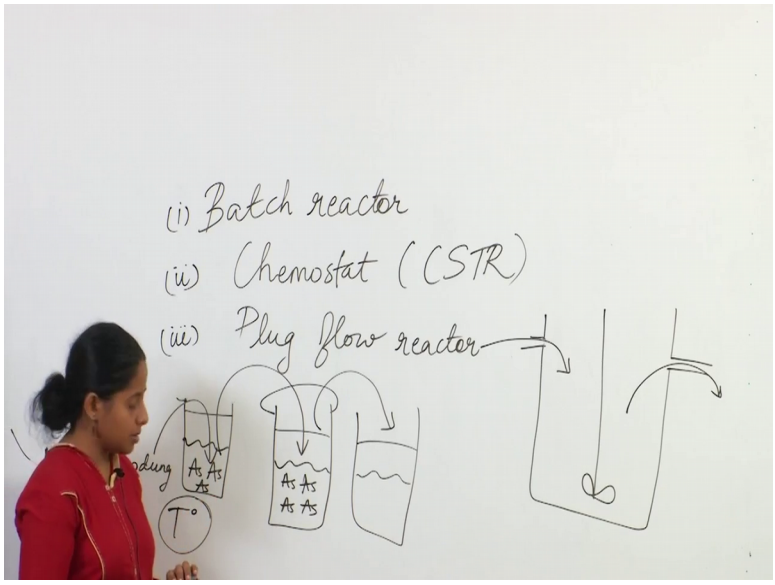

In continuously stirred tank reactor our reactor looks very different; it will typically have a port for entry of reactants continuous entry of reactants and another port for continuous exit of products. So, we have a infield and then usually if it is chemostat or continuously stirred tank reactor, it will go continuously, it will undergo continuous mixing.

So, continuously the reactants are coming in this is being mixed. So, constant concentration is being maintained inside the tank and then there is a continuous output. So, a good example for this would be a lake this continuously water coming in the lake and this continuously water going out of the lake and we can assume that the lake is completely mixed at least some lakes are completely mixed at any given tank.

So, if for enriching microbes we used the first 2 kinds of reactor batch reactor and chemostat reactor. In batch reactor what we will do is you will set up a batch reactor we will put let us say I want to enrich microbes that are arsenic. So, I will put medium that is rich in arsenic. So, now media is now rich in arsenic and I will put my environmental microbes here. So, let us say I am interested in isolating arsenic tolerant microbes from cow dung.

So, I will put cow dung here, I will put arsenic rich media. So, there is food and there is arsenic, I will give it the right temperature and other conditions that are required and then I will seal it. For some time I will let the microbes grow now because some microbes in the cow dung will not be resistant to arsenic tolerant to arsenic they will die off, and those populations that are resistant and tolerant of arsenic presence they will get enriched. So, their abundance will increase and the abundance of rest of them will decrease. So, after some time what I will do is I will open the lid away.

Now, I have a microbe set more microbes that are tolerant to arsenic and I will set another batch reactor again, I will give it food, that is rich in arsenic and I will take the microbes here that are already partially enriched in arsenic tolerance and I will put them here. And there I will seal this off and let it sit for some tank let the microbes grow using the food and again we are selecting for the ones that are arsenic tolerant or arsenic resistance and we can repeat this as many times as we want and so on and so forth.

Such kind of microbial enrichment is referred to a sequential batch transfer, because sequentially we are transferring from one batch reactor to another, to another, to another until we have a population that is resilient, then it comes in face of arsenic presence, which is arsenic tolerant or arsenic resistant.

This sequential batch transfer method can be slightly modified, to go to different to go for a dilution to extinction method to characterize our microbial communities and even isolate our microbes, that are of that have particular quality. The second way of enriching microbes from environment that meet our requirements, that meet certain conditions or have certain characteristics is to use a chemostat and now what happens in the chemostat let us take a look.

So, this is your sequential batch transfer, we are taking from one batch reactor to another; to another and this is your chemostat.

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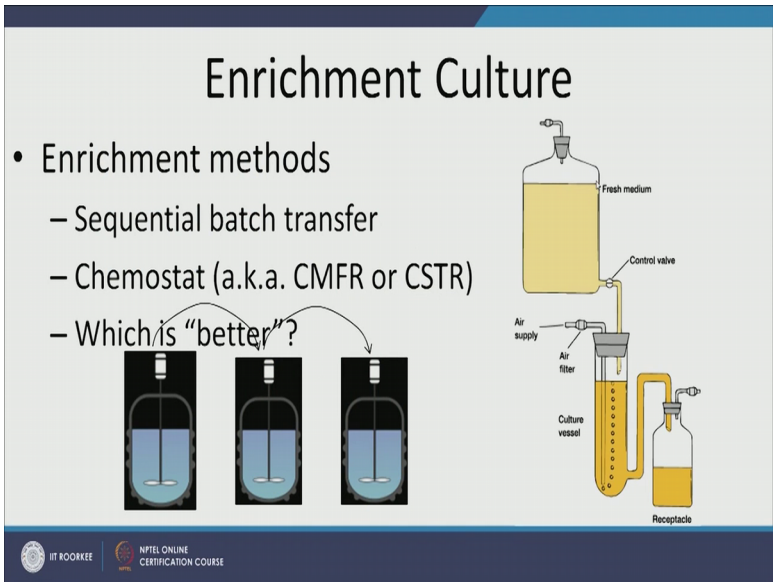

So, in chemostat this is your reactor, this special tube it has a continuous air supply, you can oxygenate it if you want to enrich aerobic microbes, or you can make it anaerobic by purging it with nitrogen.

You have a fresh medium here. So, this is your fresh inlet crust container and then this input media it will have food it will have microbes if you need to and will have a continuous or near continuous or regulars input of food. And here you have a receptacle where you have a continuous output of your product. So, continuous input of reactor and continuous output of product this is your CSTR it is also called CMFR or chemostat.

Now, which is better a sequential batch reactor better it is chemostat better, well it depends on conditions. Sometimes for example, sequential batch reactor would be better because it gives more time for microbes to grow and we can actually isolate select microbes, that are very resistant to arsenic, but sometimes for example, if you looking at cellulose degradation some of the degradation by products are toxic or inhibitory inhib inhibitory for the parents or pain for the in original cellulosic products.

So, the more we had degradation going on we have a negative feedback and the degradation stops. So, in that case a sequential batch reactor will not be nice, because after some time the microbial communities will not be able to degrade food because some of the daughter products are toxic or inhibitory. So, microbes will die out eventually.

So, the batch reactor would not work what would work in this case is chemostat, because we are continuously removing the product. We are continuously removing daughter product. So, we can as control the chemostat flow in a way that the concentration of the toxic daughter products or inhibitory daughter products, remains below the threshold value.

So, this is a very beautiful diagram that I want to highlight, in serial the batch sequential batch transfer method, this is how your substrate dynamics look like initially there is a lot of food.

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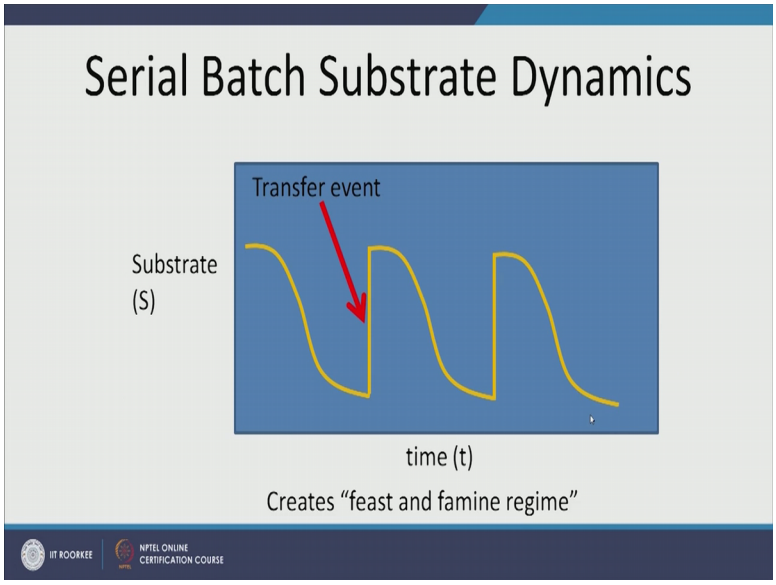

And then the food is consumed by microbes it drops and then there is an when you transfer to new bottle, new batch reactor, there is another spike in the amount of food because you added fresh food and this goes on and goes on so for.

In CSTR or in chemostat it looks very different. In chemostat initially you will have good amount of food, because you are adding fresh meal, but over time your amount of substrate and amount of microbes will stabilize in your chemostat.

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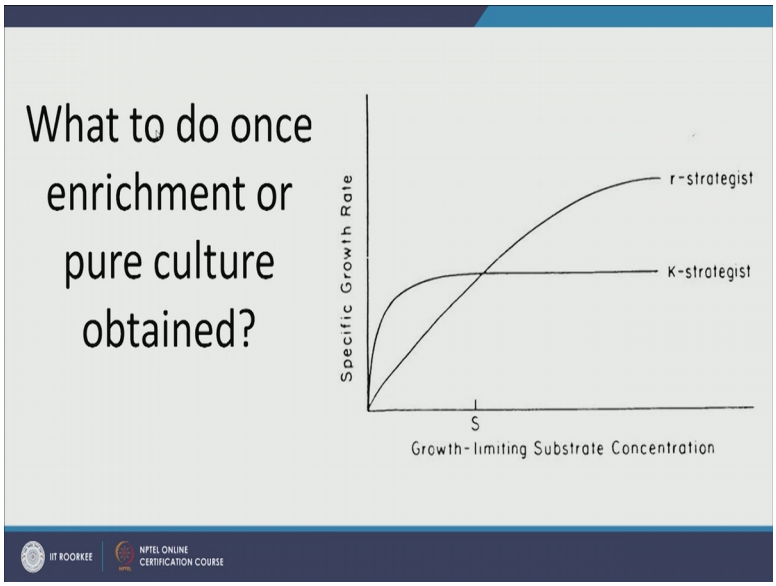

So, what to do once your enrichment of pure culture is obtained, what do you do now you have enriched either using sequential batch reactor or using chemostat.

Well you need to understand what kind of microbes you have grown r-strategist or K-strategist. So, let us see on y axis we have the growth rate and x axis we have the concentration of growth limiting substrate. So, when the growth limiting substrate substrate is very high. So, it means there are no limitations the food is implenting there is more food than, non-there is more food than that is required where microbes. So, it has high food to microbe ratio in this case as the r-strategist microbes they out compete. And they have a slightly different growth kinetics than microbes that make use of low food conditions low sub limiting substrate conditions. So, these are case strategies.

So, you need to understand what kind of microbes you have isolated. So, if we go back here notice here what kind of microbes will enrich and here, what kind of microbes will enrich now; obviously, here we have microbes that will out compete when food is sufficient they will enrich.

So, here we will have r strategist and at the bottom here when there is a famine regime will have k strategist. So, now, once you have enriched you need to find out for example, in case of sequentially batch sequentially batch transfer method that way do you want to sample your microbes from do you want r strategies to do in k strategist.

If you want r strategies then sample it right away after you do you next fresh transfer, if you want k strategies then do it prior to the transfer give plant transfer .

Now, the other question that arises whether when we are trying to enrich our microbes is what media to use. Do you use yeah if you are growing arobic microbes you need different kind of media, if you are going anaerobic microbes you need different kind of media. Now here are some tables that will tell you what kind of incubation requires what kind of microbe. So, if you are growing light phototrophic bacteria remember their main carbon sources carbon dioxide because the autotrophic their leaves light.

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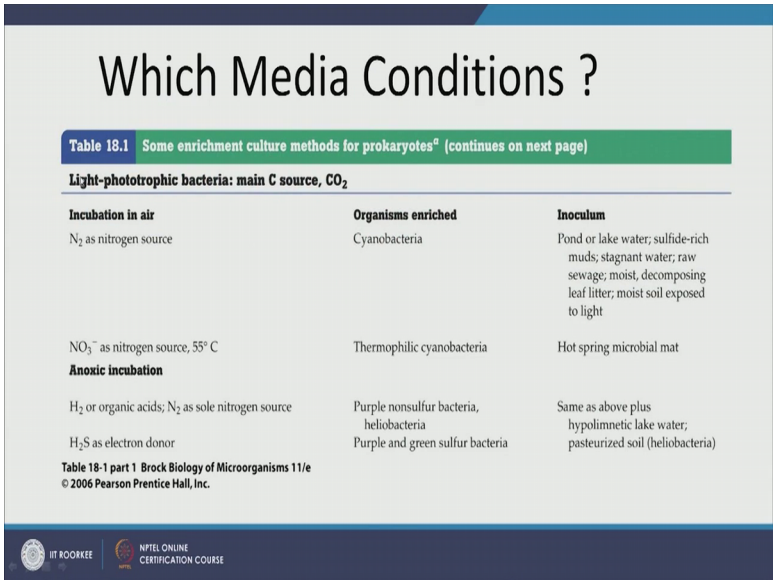

So, if you are growing them in air nitrogen is now a nitrogen gas with service nitrogen source, nitrate might also serve a nitrogen source then you would have high temperature. So, you need to supply the nitrogen gas or nitrate depending on the microbe we are enriching.

Now, if you are doing anoxic incubation of light phototrophic bacteria now because it is anoxic we do not want oxygen in there. So, we use hydrogen views organic acids and we supply nitrogen not nitrate, because the nitrate will oxidize say increase the oxidation potential. So, we use nitrogen as. So, nitrogen source and you can isolate some kind of bacteria or you can also H 2 as it is electron donor.

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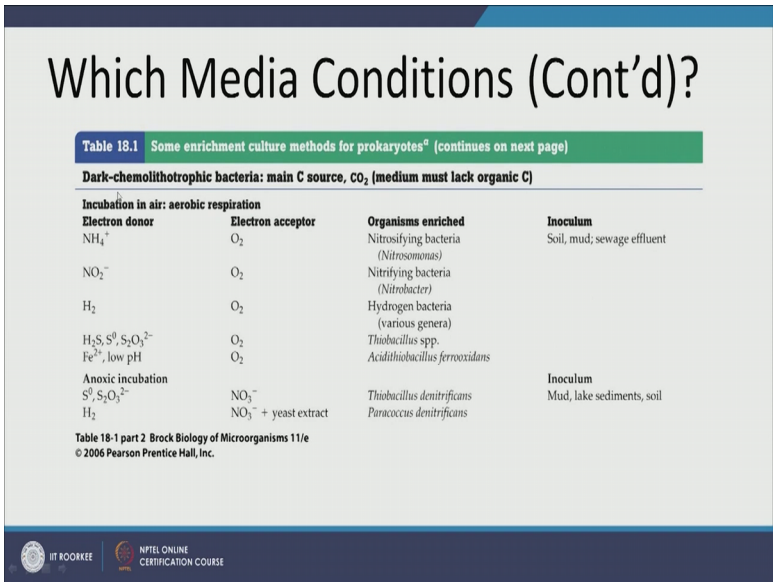

Now, if you want to do dark chemolithotrophic bacteria their main carbon source would be CO 2. So, you should make sure that apart from carbon dioxide there is no other source of carbon and you do not need light. So, you electron doing you can add certain electron donors and electron acceptors according to what you are trying to I enrich.

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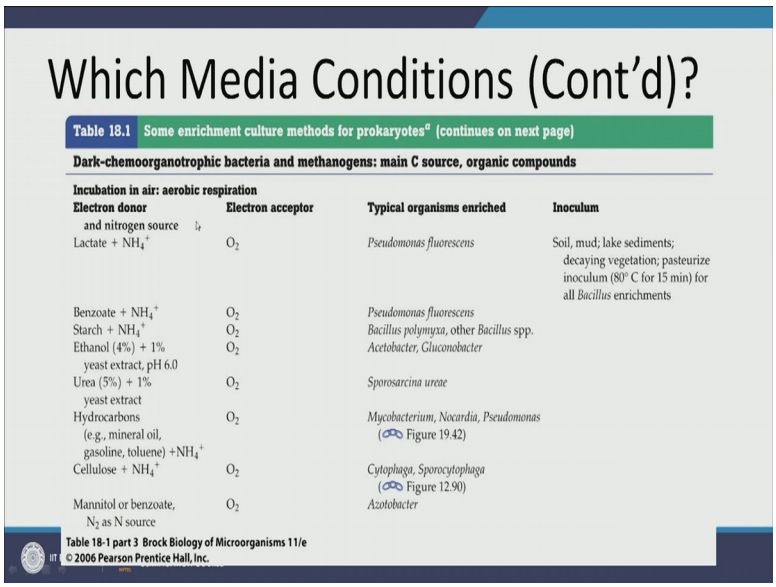

Similarly, here in dark chemo organic traffic bacteria and methanogens, you need to have a carbon donor a special carbon source and organic compound.

So, depending on again what kind of microbe we are trying to enrich, you need to add particular kind of electron donors and electron acceptors.

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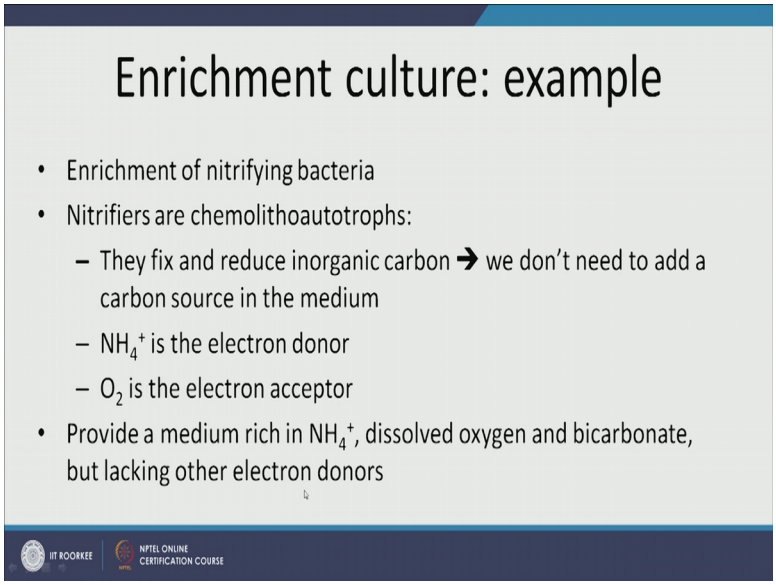

Take an example I want to enrich nitrifying bacteria. Now nitrifying bacteria are chemolithoautotrophs. So, chemolithoautotrophs suppose auto such as that they use carbon dioxide as carbon source we do not need to add a prime another source of carbon.

So, do not add any other source of carbon in the media, but make sure that there is plenty of carbon dioxide available. Now because there nitrifiers the oxygen will be the electron acceptors and ammonia will be the electron donor. So, add ammoniaauotopthation and make sure carbon dioxide is present and there is no other form of food available.

So, basically you have to provide a medium that rich in ammonium dissolved oxygen, because we need oxygen and bicarbonate for carbon dioxide source, but should not have other electron donors.

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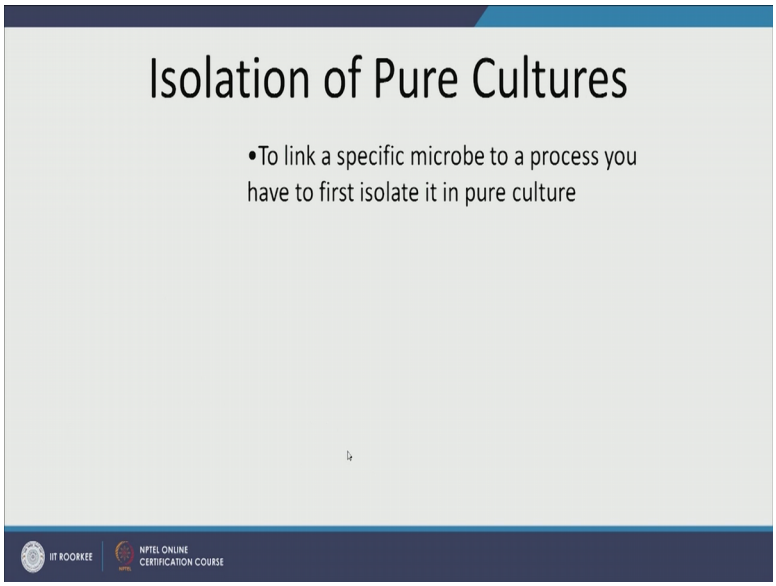

Now, how and why do we isolate pure cultures? So, when we are enriching from microbial communities, environmental microbial communities, even after undergoing chemostat or sequential batch transfer method. We might come up with microbes that serve our purpose for example, our arsenic tolerant or degrade cellulose, but it is very likely that it is not a pure culture, it is likely there are multiple populations that are existing and it is a community instead.

So, earlier there was a lot of emphasis in even now there is some emphasis in isolating pure cultures and for good reason because isolating pure cultures gave us insight into microbial activities that study of communities will not give us well. Now that we have metagenomics it is quite possible to understand population and community dynamics also.

But there are still benefits from isolating pure culture let us look at it. If you want to link a specific microbe to a process for example, I want to know, I have a disease because I am not feeling at ease, I have the symptoms, I want to know what disease what pathogen it is? So, now, I want to link my physical phenomena, my physical experience with a microbe with the pathogen.

Now, I want to identify it in that case it is very very important to isolate the pure culture. So, the first reason why you want to isolate the pure culture is to link a specific microbe to a process you have to first isolate it in pure culture. So, this is Robert Koch he was the

first one what he observed was he noticed that when potato peels or when cut sliced potato are left out and open eventually colonies grow on it, but he said that these are bacterial colonies and he assumed that each colony grows from one single bacterium himself.

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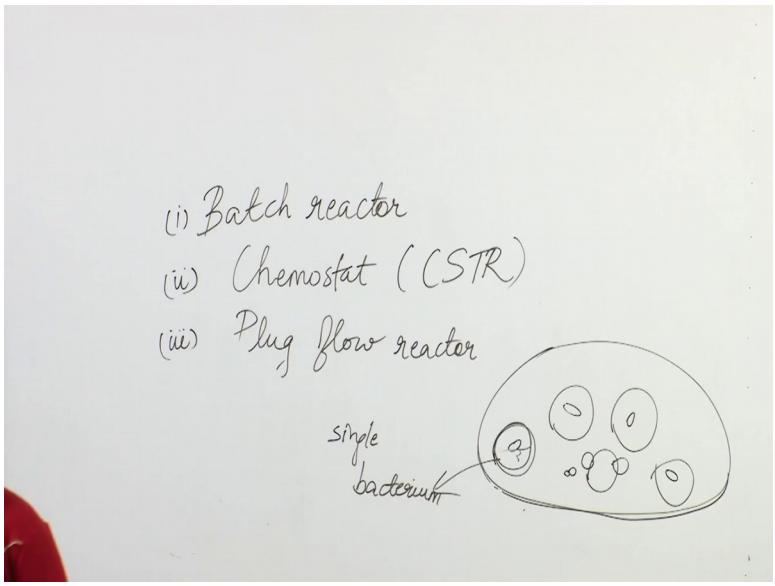

So, in the potato slice so let us see this is Koch Potato Slice and he said that he noticed after some times some kind of microbial growth on it. And he hypothesized that from air or from elsewhere single microbes single bacteria and these are invisible to eyes are very small. They fall on the potato slice and eventually they grow overnight or after some time and they form colonies which are visible to eyes. So, he said in this way it is possible for us to grow a pure culture.

Because each of these colonies each of these colonies was sourced from single bacterium and my dear students if you work in microbiology lab or environmental microbiology lab and you do culturing, you can tell when a culture when a colony is from multiple for more than one bacteria, because it will not be perfectly circular depending let us say equal I a particular equal I makes perfectly circular colonies. If you see colonies like this this shaped colonies then you know that they have there is mixture they are not all from the same microbe.

But because the Koch was able to sort of prove and give evidence that each of these colonies came from a single bacteria, if I take a swap of this colony and I picked this colony up and I do analysis I have, I am doing analysis of a pure culture.

So, let us say I did sequential batch transfer and now I have isolated microbes that are arsenic tolerant and I want to isolate their pure cultures what I can do is I can grow them on potato slices?

Now, they are not necessarily grow and potato slices, but something like a potato slice on which they can grow in a way that each colony comes from one particular microbe. And then I can pick them up, I can sequence them and you can have learned about sequencing techniques in previous lectures and once I have sequenced them, I will know how many different kinds of microbes, I have what are they and what are their characteristics.

So, eventually he decided he learned that not all microbes grow on potato slices. So, he practiced with different kinds of culture media and he tried Gelatin, because gelatin make solidifies and it is it serves as a very good surface for microbial growth, but eventually he agreed with auger and auger is still the primary cultural media for isolating pure cultures.

And let us say this is what I have received what this is let us say the mixed culture or tear that is actually arsenic tolerant.

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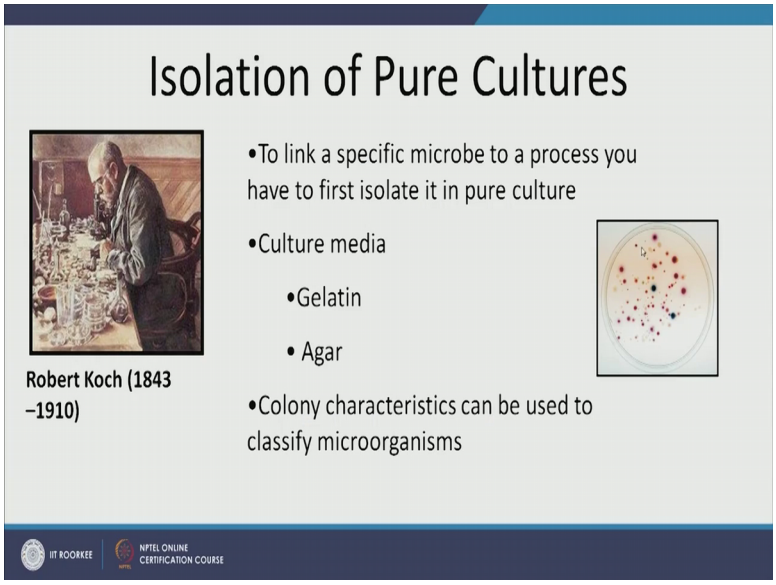

Let us say. So, I can to pick up the individual cells and then sequence them and get an idea of who they are. Now also I can look at this even before sequencing and see how many different kinds of colonies I have I can count, the yellow colonies, the small colonies, the small red ones, a small purple ones, the blue ones, the big red ones, a big purple ones. I can count them and I can get an idea of how many different kinds of microbes I have. So, even just looking at the colony can be used to classify microbes.

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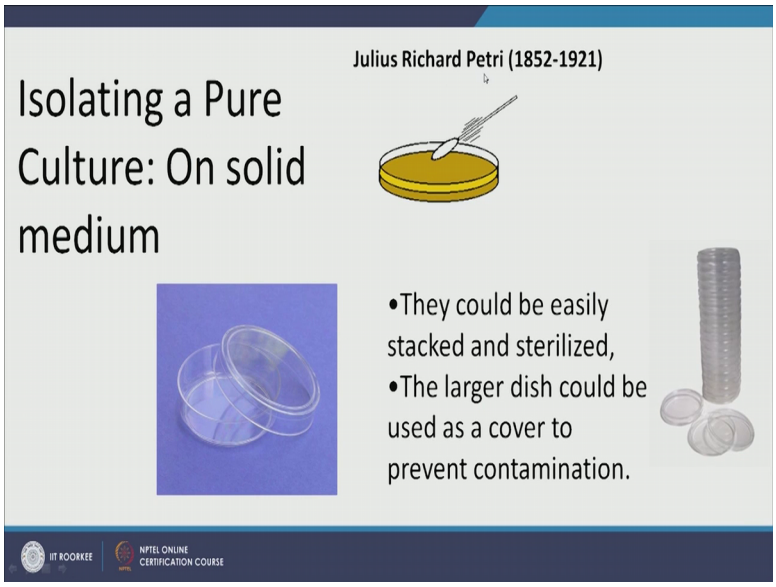

Now, came in petri Julius Richard Petri a long time ago what he discovered was that now nearly a century ago, that it is quite peter he discovered this beautiful plate system, which is quite an engineer's idea. Basically it is a smaller plate a dish and it has a lid, that covers it quite fully up throughout it is height and then inverted it does not allow any intrusion of microbes through air into the plate.

So, once I have poured my auger here, my medium here, I have strict it added the microbes that I want to grow and isolate, pure cultures from and then I invert it and I stack it like this, then it is nearly impossible form microbes to enter into the petri dish. These petri dishes are still used and widely used they very very helpful essentially later. In fact, I use them in my own laboratory and they are very very helpful, because the design engineers as it is very very applicable there easily stacked easily sterilized and the larger dish prevents contamination.

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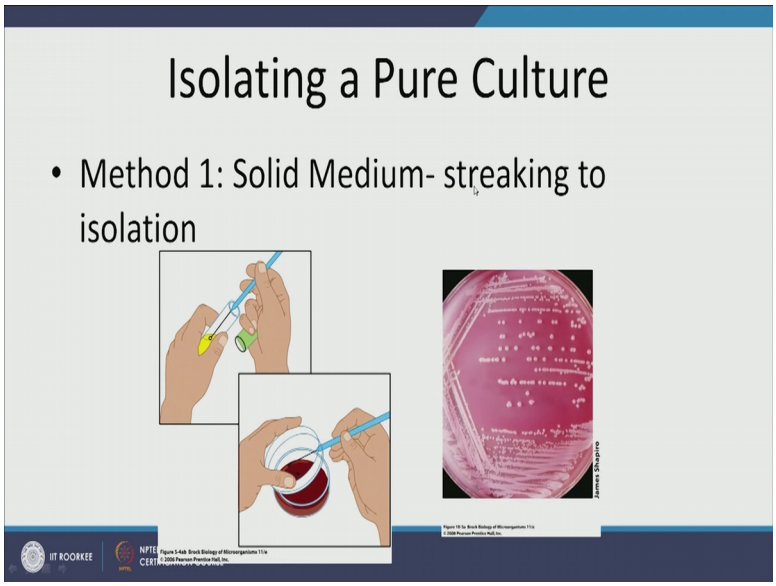

So, this is my petri dish I put my media here and this is a pink media, I pick up my consortio here and then I streak it. Now the way is this I have shown you I have shown you this picture before. So, take that take a look again the way I start streaking is I streak in one direction from one corner in this way and then assuming that I have. So, I started from here I streak like this and then assuming that I have a spent most of the microbial consortio on my tip I was make another streak here.

So, I am basically trying to del you trying to separate them away. So, that eventually I have less concentration of bacteria closer to each other and then I when I am done here streaking then I streak in this direction and then in this direction and then I slowly spread it across the plate. So, the idea is to spread the microbes in a way that sequential dilation happens and finally, we can have only one bacteria 2 bacteria separated with enough distance that individual independent colonies can grow.

So, here it is very hard to separate the colonies because there are. So, many microbes and instead of growing beautiful circular colonies they are growing streaks, but by the time I reach here most of the microbes on my tip here, this tip here are already spent. So, the few microbes that are still left here few bacteria that are still left on the tip will be separated by long distances and they can be easily picked up.

So, if you plate it plate to start streaking from a side streak like this and then when you think you have spread enough go in this direction, then this and then this, what I have

done in past is I would streak like this and then like this way this way and then another way and when there is no more way left only then, I will streak in middle just to spread them out better.

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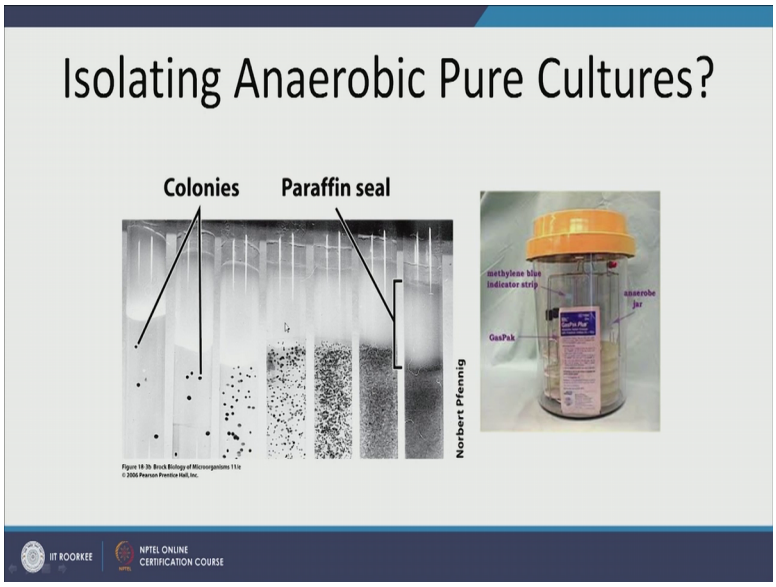

So, these were anaerobic cultures how do I did isolate an anaerobic pure cultures. Again this is a column column for isolating anaerobic pure cultures here I put my paraffin seals so that no air can enter and look here each of these are my colonies. So, I can seal that most free interaction. So, that no oxygen enters and the energy conditions maintain this is the more popular technique that I am familiar with.

We have an anaerobic jar; you gas it with my ultra-pure nitrogen or mix of nitrogen hydrogen. So, that there is no oxygen you remove the oxygen from here and then you put your plates here, the same petri dish the same old petri dish here these petri dishes are put here and then the microbes are allowed to grow here it works pretty well. So, I have grown by microbes now how do I enumerate them.

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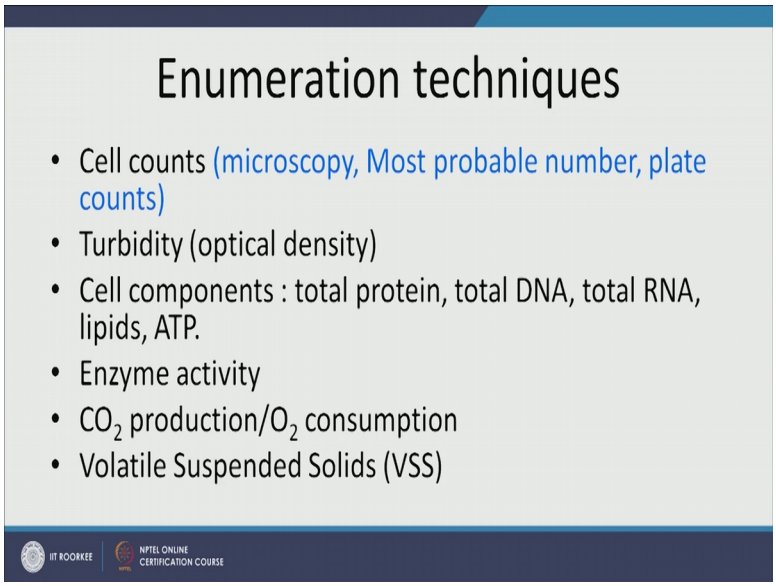

Now, enumeration techniques include cell counting. So, once my microbes have grown like this. I can count the cells; I can count how many cells are there, using microscopy so visually I can count this is still very much used when, we are looking at heterotrophic plate count. So, I want to know how many heterotrophic microorganisms are present.

It is also used for most when the another technique is most probable number. So, I can get an idea of what is the most probable number of coliforms or bacteria present in water or in a sample by counting it and I will go through most of all number a little bit more in detail, because this is very commonly used in environmental labs. And then I can do plate count.

So, basically I am growing them on my plates and I am counting how many colonies to remain? The other is turbidity I have some colleagues who have used, who used this technique a lot to understand, what phase of the growth microbes are?

So, if you go back to one of the early lectures where I am talking about microbial growth how they do undergo binary fission and how they start from lag phase? They hit an exponential phase and then they go to a stationary phase and then they have a decay phase. So, you can and in the diagram that I have shared you can see how turbidity of your media changes as the microbes shift from one phase to another.

So, turbidity is used a lot in the growing microbes and trying to capture them at exponential phase I can also look at the cell component. So, I can analyze the total protein. So, if I have proteomic data I can say oh these are the proteins it is from this bacteria if on this archaea all eukaryote. Similarly I can extract the total DNA analyze it. So, I can do matter genomics I can analyze the sixteens R R N A G or I can extract the total rabbo R N A. Now R N A also give me information about what microbes are.

I can also do lipids last class we talked about fame. So, which was a lipid based analysis of microbes. So, earlier we would just isolate the fatty acids from microbes and then characterize them through chromatography techniques. And then the signature of my fatty acid would tell me how many different microbes I have and now recently we also use ATP, next with enzyme activity I can actually look at protein activity.

So, if a microbe is for example, degrading some contaminant then I can this is this kind of microbe this one does not. So, just another kind of microbe, but now you can see that multiple different kinds of microbes can have same function for example; sulfate reduction there is not just one microbe that does suffer reduction. So, if the on basis of enzyme activity I might say well.

The 3 strains that I have all of them are sulphate reduces they have similar enzyme activities, when it comes to sulfate reduction, but they might be very different from each other. I can also look at carbon dioxide production and oxygen consumption in case of arobic microbes; I can also measure volatile suspended solids. Now VSS Volatile Suspended Solids are used very commonly for getting an indirect count of biomass in wastewater treatment plant.

So, those of you here who are waiting cannot wait enough for us to get into microbiology of wastewater treatment plant, understand that the VSS or ML VSS mixed liquor volatile suspended solids both of them give you an idea of how many bacteria? How much biomass is present in your sludge or in your water?

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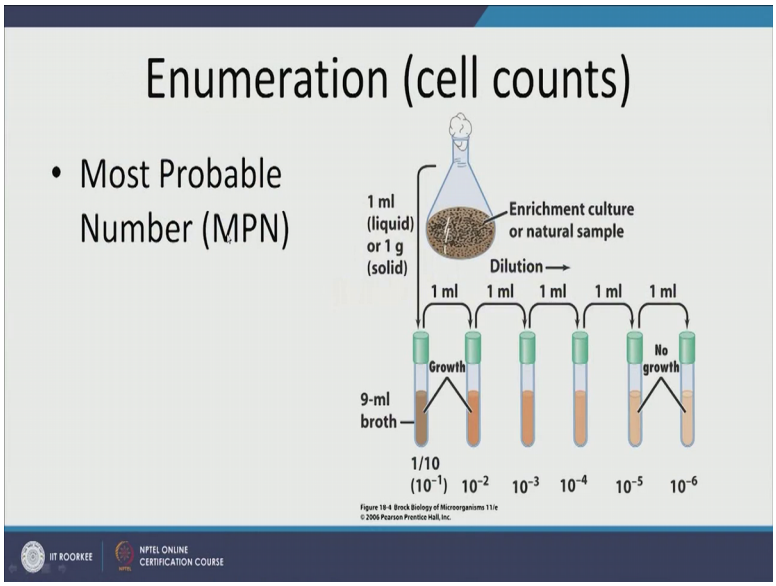

So, let us look at most probable number I promised you we will go through this in a little bit more detail. So, in most probable number what I do is I have a particular growth that has a particular die which changes colour when the PH falls below a particular number. And I take multiple tubes and in multiple tubes I put my broth and then I undergo serial dilution.

So, serial dilution looks like that this is my enrichment sample or naturals number it could be water, it could be fecal matter it could be river water it could be my enrichment sample and what I will do is I will put 1 10th dilution you know. So, if this is 1 m l this would be 9 m l.

So, I put it here and this 1 by 10 the concentration of this and then after mixing this I will put 1 m l here in this 9 m l, it is all these are 9 m l to begin with and then after mixing sulfide 1 m l, here 1 m l here.

So, eventually I will have 10 to by minus 1 concentration here minus 2 here, 1000 here 10 000 here 1 lakh fraction here and 1 millionth fraction here, 10 to a minus 6 fraction here. So, as I undergo dilution I what I am actually doing is I am doing serial dilution of initial microbes that we begin with; the theory behind most probable number based enumeration is that dilution to extinction.

So, finally, we will dilute the water the microbes. So, much that in 1 of the tubes last tube there will be no microbe left. So, here let us say I started with 100 microbes here we had 1000. So, we have 100 microbes here 10 1 0. So, this is a dilution to exact extinction. So, after 0 it will be just 0 0 clean. So, I will not see any growth and the way to see growth is that turbidity might change or the 1 ambient technique used a lot for measuring coliforms in water the PH falls down and the colour changes.

So, either colour or turbidity any optical measurement will give me an idea where the growth has happened, where the growth has not happened? And using this I can get an idea of what the most probable number is now most rural number is a name suggest it is a probable number it does not give me an exact count.

All righty dear students this is all for today in the next class we will go ahead and will explain more techniques in environmental microbiology. So, stay tuned for the next lecture and together they will hopefully comprise of the most common and useful techniques that microbiology has lent to us as environmental scientist engineers and students.

Thank you.