Environmental Biotechnology
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Lecture – 27
Methods in Microbial Ecology with Relevance to Environmental Biotechnology (Contd.,)

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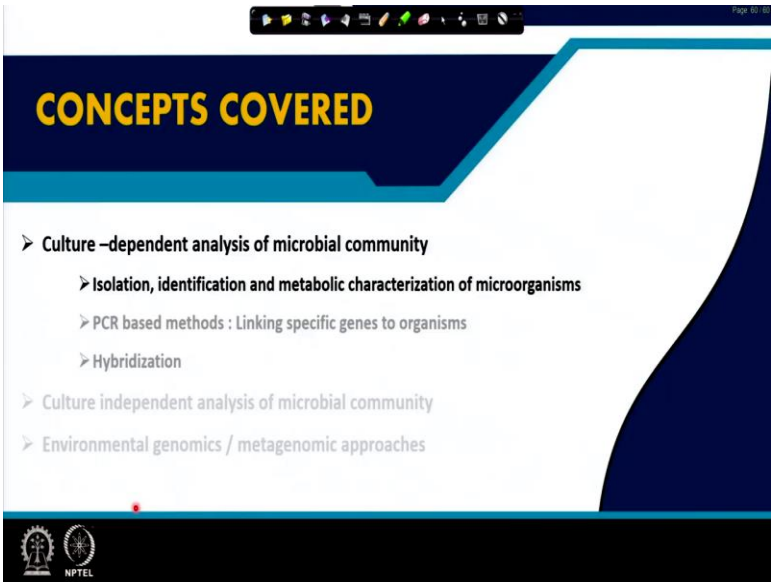

Welcome to the 27th lecture on methods in microbial ecology with relevance to environmental biotechnology. And in this particular lecture we would be discussing about the culture dependent analysis particularly the isolation identification and metabolic characterization of microbes from environmental settings.

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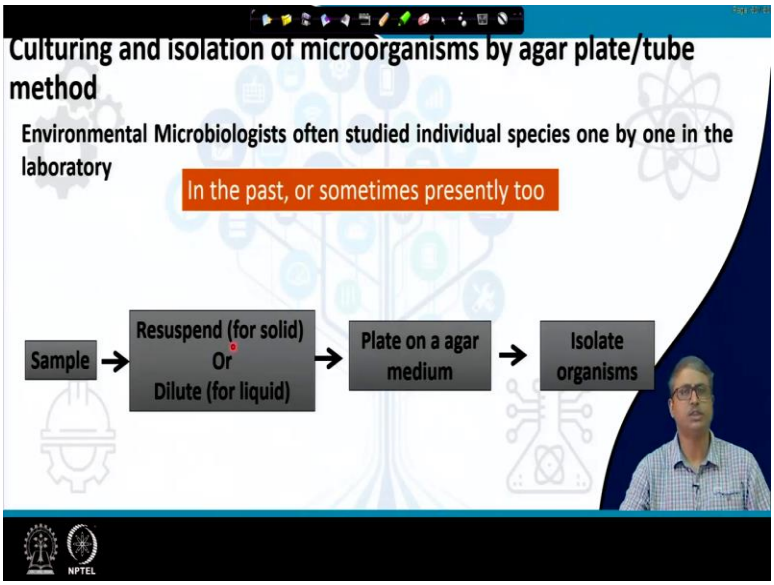

So, culturing and isolation of microorganisms by agar plate or tube method have been a very important step for environmental microbiologist as it is for a very important step for general microbiologist as well. So, the environmental microbiologist or biotechnologist often studied the individual species micro microbial species bacteria archaea from different type of environments one by one that means as I said earlier also that we can have a hundreds of different isolates or hundred strains as pure culture but easy each of these cultures will be tested and characterized individually.

And that is true for present also it is not that this type of techniques or approaches were used only in the past not today but today also in several cases we see or we do isolation of the individual species and then characterize them. And the general method or general methodological strategy is something like that the samples are taken samples are resuspended for solid or diluted for liquid sample.

And then they are subjected to isolation of the colonies pure culture colonies by agar plate method. So, we use a suitable method suitable agar medium and do a plating on the agar medium and the aggregate plates are incubated and following incubation the colonies appear and the colonies are subsequently purified and then purified colonies are tested and they are subjected to different type of analysis.

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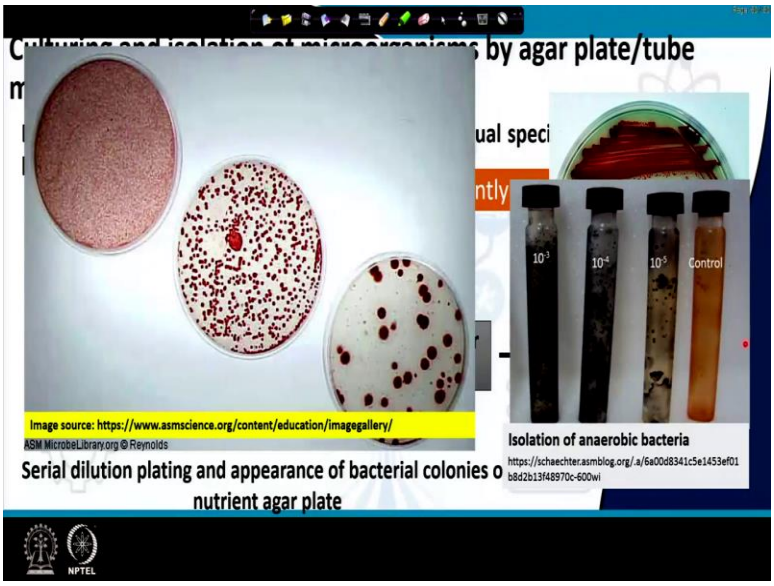

So, here is the picture of you can see that how the colonies appear from out of the serial dilution on a nutrient agar plate. So, these are the; colonies and as the dilution of the inoculum is reduced or increased rather sorry. So, we can see that in a more diluted sample we have the discrete colonies the colonies are well separated and in a close view of the colony will help us to understand the colony morphology and those are kind of a standard microbiological or environmental microbiological practices.

So, similarly we can have the anaerobic organisms also isolated as anaerobic colonies however in this case since they are anaerobic often they are embedded inside the egg agar. So, it is kind of molten agar which is used. So, maybe a roll tube method or some kind of similar methods are used where the colonies are allowed to grow while they are still embedded inside the agar. So, by some specific methods we can take out the colony and or colonies and then subculture them or characterize them as they are required.

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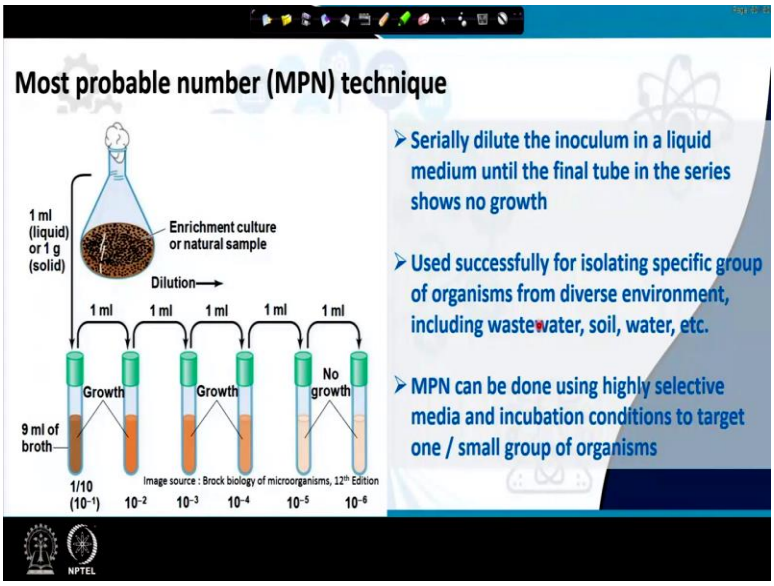

There is also another very useful method which is often used is called the most probable number technique or MPN technique. So, in case of MPN technique we may have a culture of enrichment culture or if natural sample from any environment and a small volume of that that is the alicort or the inoculum what you say. So, one ml for example or one gram of solid in case of solid samples are added to a broth medium now this medium is selected based on the scientist who is working on this and this should be suitable for allowing the growth of the particular organisms or particular types of organisms that he or she is trying to account.

So, MPN is a kind of count based method. So, it is a most probable number. So, it could be targeted towards all the organisms present there so all aerobic maybe or all anaerobic may be and as well as a specific group of organisms. So, suppose we want to know all aerobic organisms present there. So, we might use a medium which is which is used by a common soil bacterial strains like R2A or similar type of medium or we can have a nutrient agar or a minimal medium along with multiple carbon sources electron acceptor or sorry electron donors added and as electron acceptor oxygen is there for aerobic bacteria.

For anaerobic bacteria we can use similarly different types of medium media where we can change the electron acceptor or use kind of a mixture of electron acceptors because under anaerobic conditions oxygen is not going to be utilized. So, this broth is now severely diluted as you can see and each dilution we can have multiple replicates of the tubes and they are then

allowed for growth.

So, following incubation we will see that growth has happened in the highest concentrated cells or the concentrated tubes where the dilution was minimal like only one dilution happened like one ml of the culture or the soil or sample or rather liquid sample one ml or solid sample one gram was added to nine ml of the broth. So, it is a kind of a 10 time dilution. So, in the 10 time diluted sample growth has occurred in 100 times dilution also growths has occurred but in 1000 time dilution or more than that dilution or maybe these are one lakh time dilution there is no growth.

So, now here is one basic assumption that if at least one cell is there then there is a growth. So, if it 10 to the power -5 there is no growth that means not even a single cell has reached. So, that means the total number of cells are actually less than 10 to the power 5. So, because if the cell number had been 10 to the power 5 then after dilution at least one cell might have reached to this dilution tube and it has it it might have grown but it has not grown that means the dilution has allowed the cell not to reach to up to this particular tube.

So, it must be less than ten to the power five. So, that's some order of 10 to the power 4. So, in kind of approximation that approximately 10 to the power 4 number of cells possibly or 10 to the power similarly 10 to the power 5 number of cells because we may have one dilution over here. So, we if we use different media for example in this case we can discriminate based on the MPN counts that what are the relative abundance of individual group of organisms metabolic group of organisms.

So, it is relying on the principle that a serially diluted inoculum in a liquid medium will provide the results that until the final tube in the series shows no growth. So, final series will have no growth and based on that the tube where last growth has occurred that will be utilized to find out the approximate number of cells present in the original sample. And it is used or it has been used successfully for isolating different specific group of organisms from diverse environment including different wastewaters soil water and different other environments.

MPN can be done using highly selective media like iron reducing sulfate reducing hydrogen oxidizing petroleum oil or arsenic or any other substrate that you want to test that in a total community. How many cells are there approximately who are able to metabolize arsenic or how many cells are there possibly you can metabolize hydrogen and similarly other substrates or can withstand different conditions like pH, temperature, redox condition etcetera.

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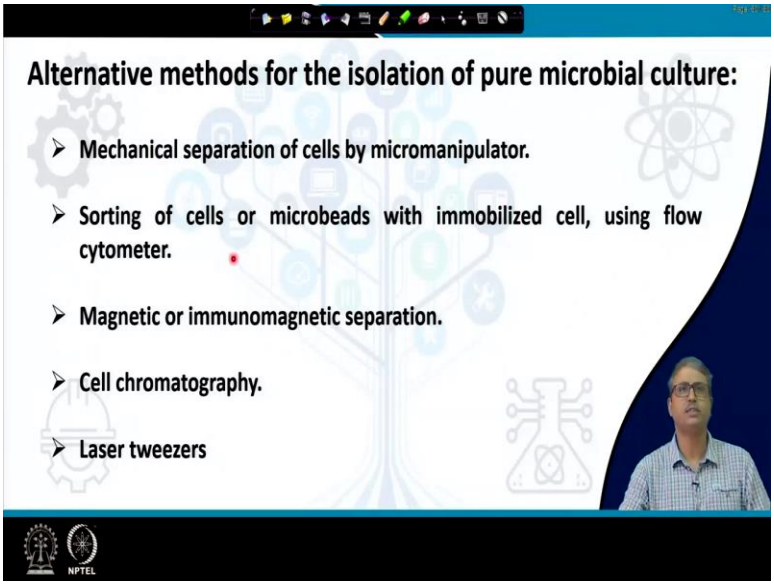

Now there are apart from this agar tube or tube based method or the MPN based method there are some alternative methods as well used for isolation of pure microbial cultures which are mechanical separation of cells by micro manipulator sorting of cells or micro beads with immobilized cells using flow cytometer, magnetic or immunomagnetic separation cell chromatography and laser tweezer.

So, for for the purpose of different purposes cells can be separated and then individually the cells can be isolated now with respect to the isolation of these organisms and the cells in as a pure culture there is a term called axonic culture or the cultural purity. So, the purity of the isolated culture bacterial culture archaeal culture or fungal culture etcetera or even alcohol cultures also is very important and the purity of the culture is made through a combination of microscopy colony characteristics and testing the culture for growth in other media.

So, we can always use molecular techniques but before we use the molecular techniques for

routine environmental biotechnology purposes this is very important to understand that the organism that we have isolated is a pure culture. Because some functional attributes some activity which are ascribed to a particular organism will be changed if we find that the actually the organism was not a single organism.

But it was a mixture of two or more organisms together. So, in order to decipher that the organism was actually pure culture these tests were done that is we see the organism under microscope we also see the colony and we also test the growth in other media. Now the qualifying criteria in this case would be the uniform straining characteristics. So, if we; just grow a single colony in a medium and then stain it.

So, all and see under the microscope. So, all the cells which are seen under the microscope will appear same color or with same straining properties uniform colony and cell morphology. So, colony morphology in subsequent subculturing of the pure culture if it is mixture of two or more organisms in subsequent culturing sub culturing those colonies will appear eventually maybe 1 or 2 out of 10 or out of 50 but they will appear.

So, we will be able to discriminate them looking at the colony morphology if we are examining them carefully often but it may be possible that the contaminants or the co-growing organism is also having the same colony morphology. However the cell morphology which can be seen under the microscope mo both the light microscope as well as the electron microscope can be used to discriminate that whether there are mixture of cells with respect to cell morphology and absence of contamination in growth test.

So, when we grow with different media conditions it is it is it is possible to actually find out that the organism is a pure culture or not.

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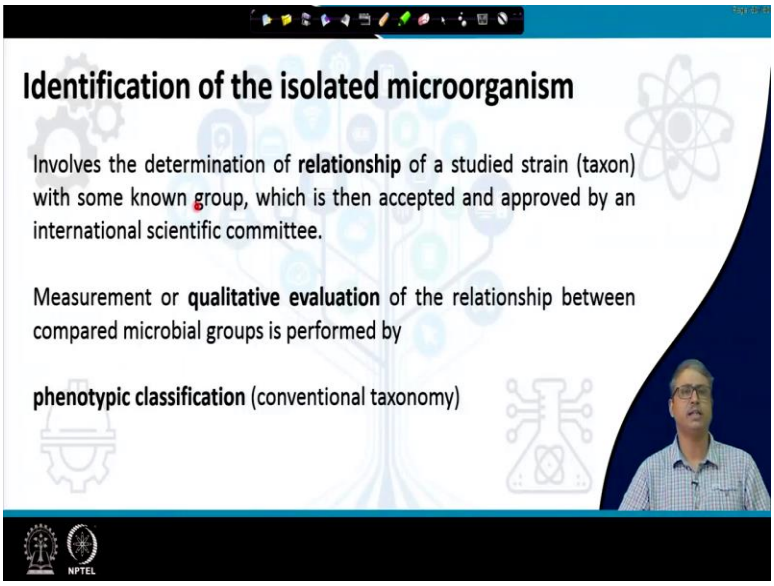

Now next is the identification of the isolated microorganisms. So, that is very important earlier I presented in another lecture that identification can be done through the chemical tests or the metabolic profiling or the biochemical assess assessment or assay but also through molecular characteristics assessing the molecular characteristics like the 16s ribosomal RNA gene or other genes which are used for phylogen as a phylogenetic marker.

So, during the identification process actually what we do we try to determine the relationship of a studied strain may be the organism that we are using we are isolating. We have isolated we want to establish a relationship between this organism with some known group there must be some group of organisms which are already isolated studied published and the informations are available in the database.

So, we want to establish a relationship exactly the way we establish the nucleic acid identity. For example the 16s ribosomal RNA gene sequence that we obtained from our isolate we put it in NCBI server and then go for a particular search is equal blast analysis which allows us to and find out the closest matches. The sequences which are having the the highest identity this is basically a relationship between the characters obtained from the isolates that we have with the isolates or the organisms already been studied and present in the database or the other resources and which is then accepted and approved by an international scientific committee.

So there are international committee for reviewing the relationship status and then we can attribute suppose I isolate a bacteria and just give a name of like pseudosynthemas or a bacillus. So, it has to be approved by international committee that the characters that I have assessed and I have tested with similar bacterial groups which are published and deposited as a kind of valid organisms in different database and then when we compare and submit the reports the international committee they review and then finally they grant that yes this can be identified as a bacillus strain or as a pseudo zentamona strain that one example I will show you.

Measurement of the qualitative evaluation of the relationship between compared microbial group is performed by two methods. One is the phenotypic classification which is the conventional taxonomy where the biochemical test and ecophysiological tests etcetera are used. Similarly the genotypic classifications are also used.

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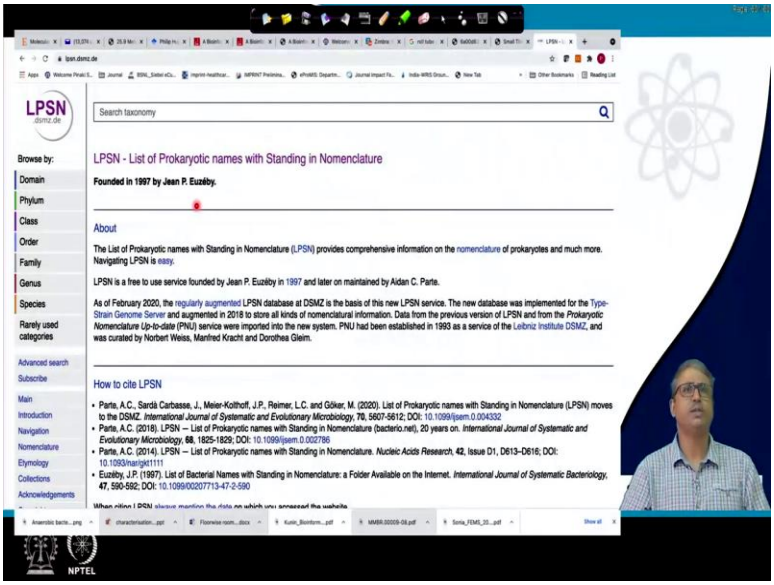

So, with this respect I would like to introduce you to this particular resource which is called the LPSN a List of Prokaryotic Names with Standing Nomenclature. This is a huge resource where we can see that the comprehensive information on the nomenclature of prokaryotes and much more in details are available. And one of the very purposes of this list of prokaryotic names is that names of the organisms which we isolate must be provided very carefully.

Because names when they appear in scientific literature for technical literature or or maybe other

laws and regulations and enforcing agencies these become very important to know understand that the organism which has been attributed with some function is is a very valid information. Like if I have isolated an organism and just claim that this is bacillus it is not going to be a bacillus I have to be validate I need to validate this.

And for this the validation process is done by international code of nomenclature of bacteria that is called bacteriological code which is provided through this LPCN website ah. So, I am showing you as a snapshot of the website itself and through which we can find out that the code states that the name of the tracking is validly published. So, we cannot just give a name just like that we cannot give a name.

So, the name has to be validly published. So, we have to communicate to the committee the bacteriological code has to be followed and then the name has to be certified or validated or approved by this competent committee and then only the name can be assigned. So, it is very important that whenever we work on environmental biotechnology isolate different organisms and then just by a 16s RNA gene sequencing or some similar methods.

We find a similarity and give a name to this and then claim that this organism is doing this or will be involved in this process this is not not a very fair practice or fair procedure. It is the naming it may be possible that the organisms taxonomy is same as what we are claiming but it has to be validated through the competent authority and that is the international code of nomenclature for prokaryotes and through that we can have actually comparison of this validly published organism and provide a correct name. So, that scientifically and technically also it is it is a credible identification process.

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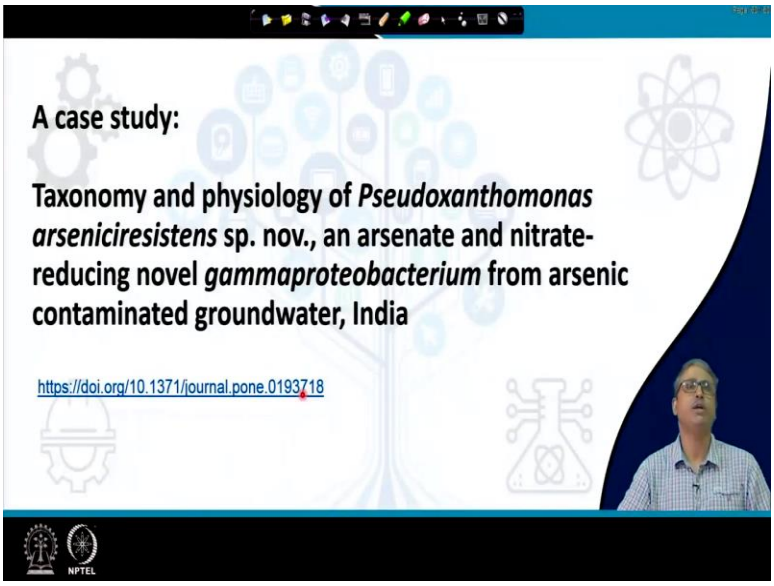

So, now I will take you to a case study where a particular bacterial strain was enriched with respect to its ability to reduce arsenic in the form of arsenate. So, arsenate is a form of as5 plus which is very abundant in ground water system arsenic is present as I mentioned is one of the major contaminants of the ground waters across many parts of India. So, this is a bacterial strain of called pseudo xenthomonas strain.

We have isolated this bacterial strain through enrichment procedure where arsenic as arsenic five that is arsenate was provided and then we tried to characterize and identify the organism following the procedure that we discussed in our lecture.

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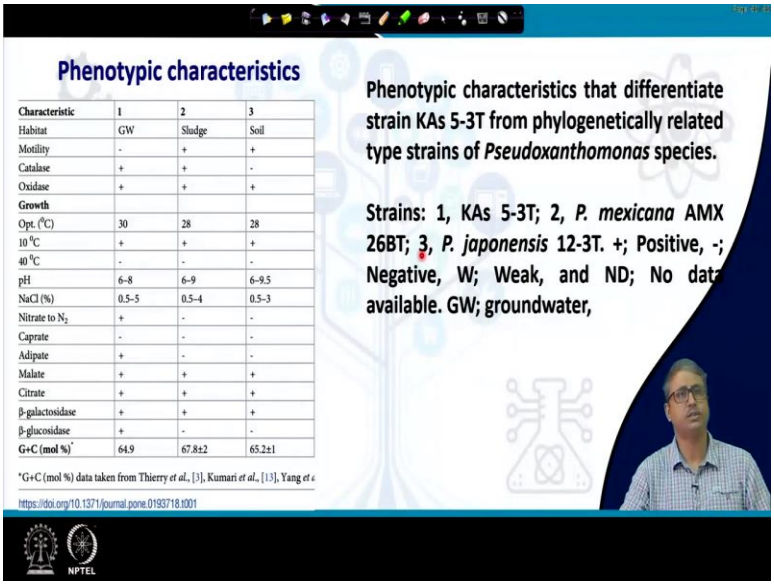

Characteristic	1	2	3
Habitat	GW	Sludge	Soil
Motility	-	+	+
Catalase	+	+	-
Oxidase	+	+	+
Growth			
Opt. (°C)	30	28	28
10 °C	+	+	+
40 °C	-	-	-
pH	6–8	6–9	6–9.5
NaCl (%)	0.5–5	0.5–4	0.5–3
Nitrate to N_2	+	-	-
Caprate	-	-	-
Adipate	+	-	-
Malate	+	+	+
Citrate	+	+	+
β-galactosidase	+	+	+
β-glucosidase	+	-	-
G+C (mol %)*	64.9	67.8±2	65.2±1

*G+C (mol %) data taken from Thierry *et al.*, [3], Kumari *et al.*, [13], Yang *et a*

So, as I mentioned earlier that a number of tests which are metabolic or the phenotypic tests can be done. So, the; phenotypic test of the organisms that of our organism that we have isolated is shown in a column one whereas in column two and column three these are the nearest neighbour organism of this organism. Why do we need to study these organism as well because in order to compare and when we submit this to international committee that our organism is actually this like pseudosynthemas in this case.

So, we need to compare the performance in terms of the phenotypic characters of our strain with the closest reference strains. And the closest reference strains can be obtained from different culture collections and there are a number of characteristics like including the motility enzyme as a growth at different temperature pH how they withstand different salinity whether they reduce nitrate etcetera and some enzymatic assay as well as their genomic GC content.

Because GC content is very important information in order to establish their taxonomic similarity.

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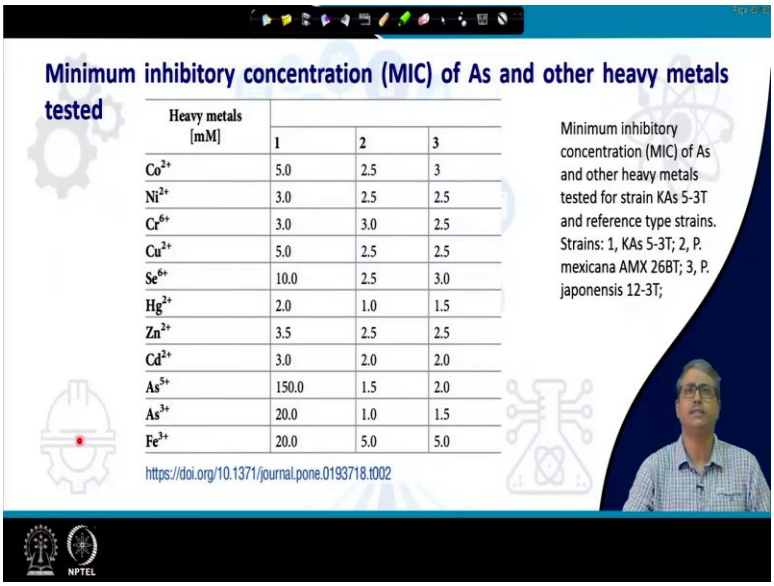

And followed by this since this is relevant to arsenic and maybe some other heavy metals are also relevant. So, the test with respect to the metal resistance is performed again with the reference strains are also performed with the same test.

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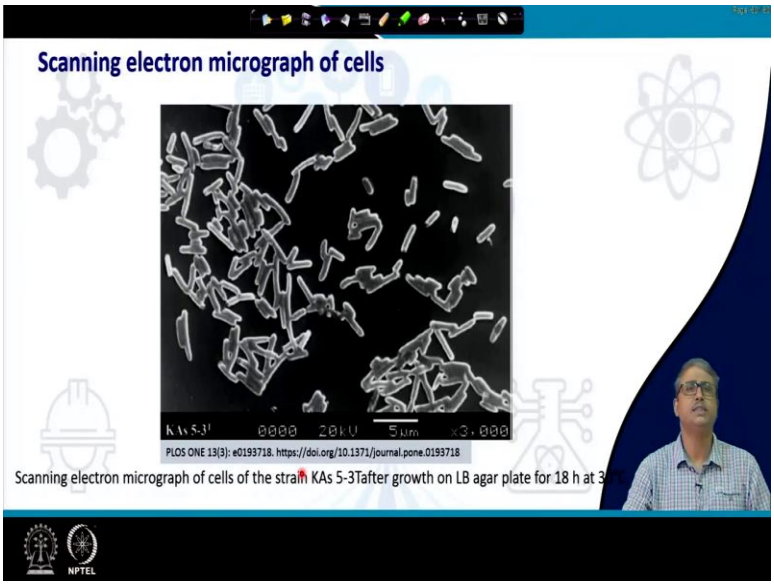

And here is the scanning electron microscopy of the organism to see the morphology. So, cell morphology. So, you can see the cell morphology is very clear these are mostly the bacillus type or rod separate structure the cell length etcetera can be seen very clearly.

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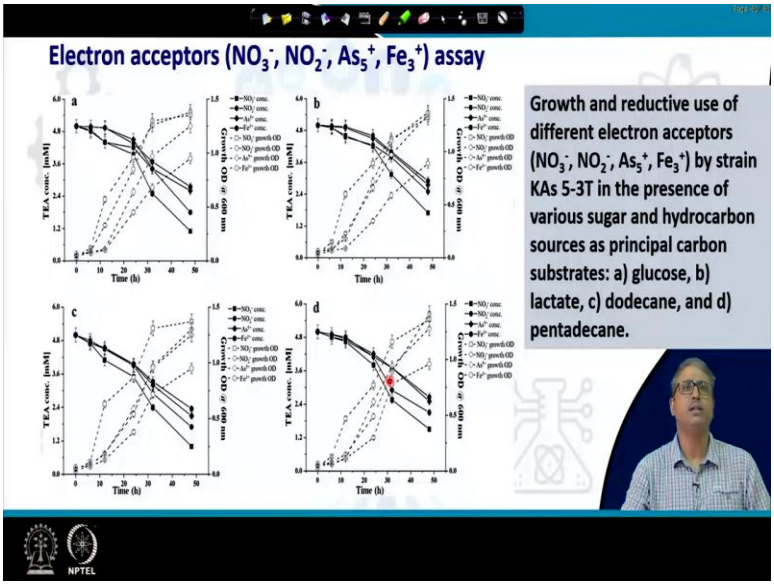

And then finally the metabolic performance of the cells with respect to different electron acceptors like nitrate nitride arsenide and Fe 3. We can see that how the electron acceptors are utilized the cells how the cells have grown with respect to these.

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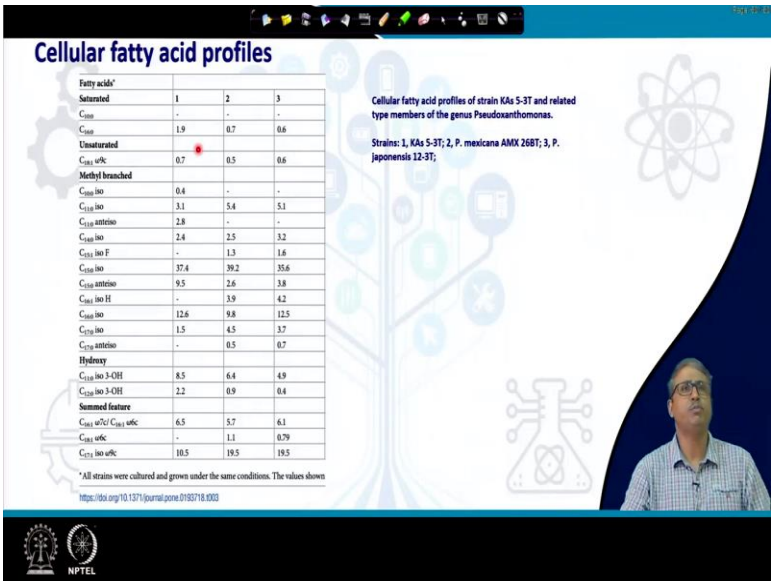

And then a very important parameter for taxonomic purpose which is called fatty acid analysis. So, I will elaborate fatty acid analysis in some of the other lectures because this is found to be a very important method to understand the community composition as well as individual species identification. So, different fatty acids are measured quantified through gas chromatographic technique.

And again the reference strains are also used in order to compare and to find out what are the fatty acids which are not present in our organism or present in our organism as well as in the reference strains.

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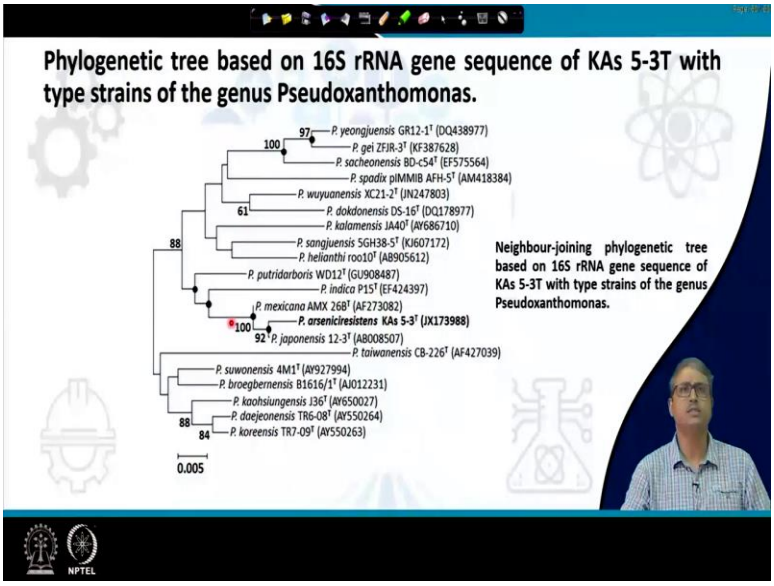

The lipid profiles and then finally this 16s ribosomal RNA gene sequence based phylogenetic analysis. Again I will discuss about the detail of the procedure but the procedural net outcome is this phylogenetic tree where you can see that this is our organism pseudogenetic strain and it shows maximum phylogenetic resemblance to another pseudogenetic bonus which is called japonensis. And finally we could actually detect the arsenic metabolizing gene.

In my earlier lecture I refer to functional genes. So, these are the functional gene like arsenic with reference to arsenic arsenic reductase is a functional gene. So, we could PCR amplify this gene from this genome and then try to look into the phylogenetic analysis of this and then finally we comprehend that this is a pseudomonas strain.

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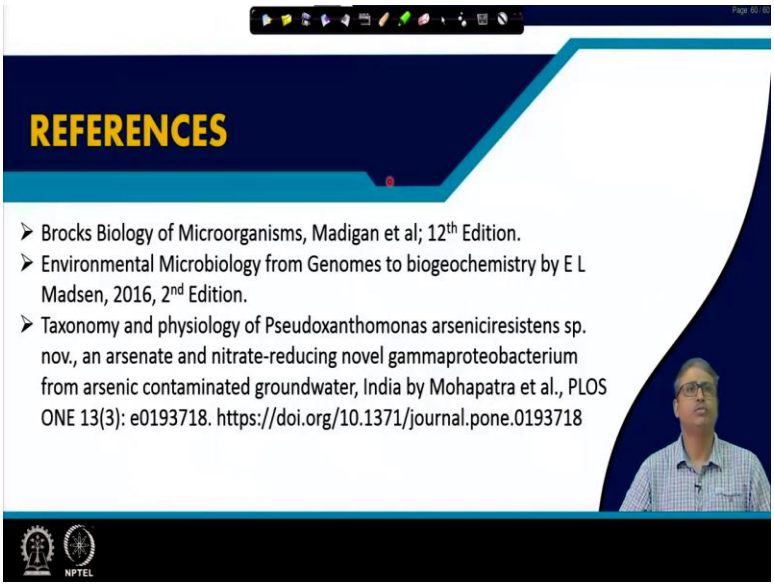

So, for reference Brock's biology of microorganisms and environmental microbiology from genomes to biogeochemistry would be useful along with this particular paper published by us may be consulted in order to understand some of the intricacies or details if you are interested.

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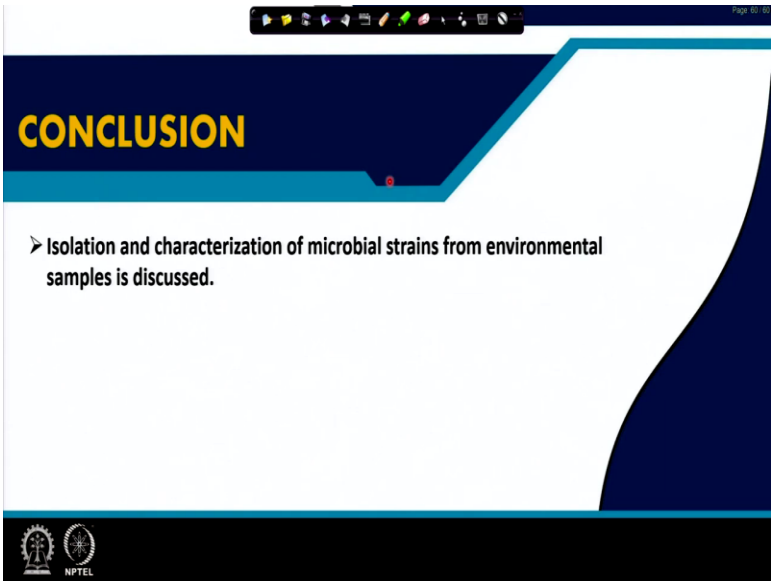

In conclusion the isolation methods including the plate and the anaerobic isolation the MPN based method the MPN kind of techniques are discussed. And how do we characterize the microbial strains particularly the isolates some glimpse or some examples are cited in this particular lecture, thank you very much.