Biomaterials for Bone Tissue Engineering Applications
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Week- 03
Lecture- 14

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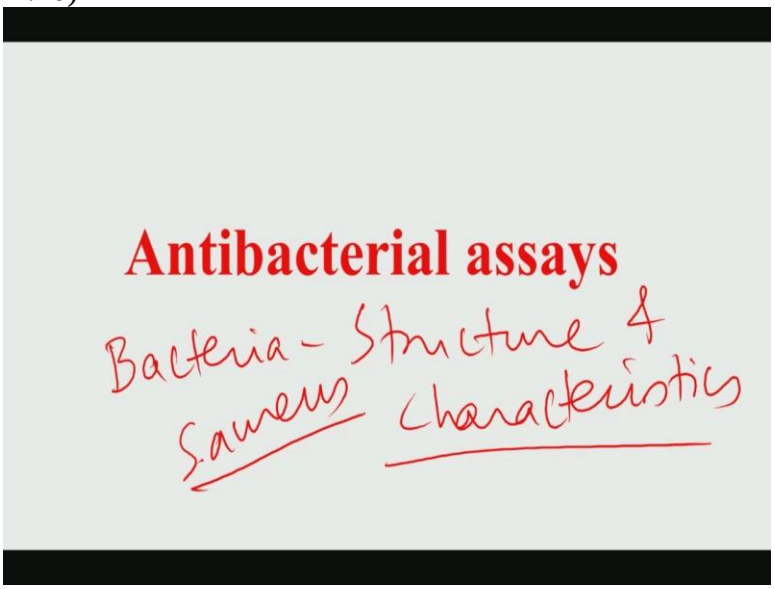

So in this module we will discuss the various anti-bacterial assays. Let is recall that in 1 of the earlier modules, we have discussed, the bacteria structure and characteristics. So just to quickly refresh our minds, there are 2 types of bacteria, gram positive and gram negative bacteria and out of that, you know gram positive particularly staphylococcus or staphylococcus aureus, S aureus and S epidermidis, they are like much (ma ya) they are known for the pathogenicity or they are like more pathogenic bacteria. So to cause any bactericidal effect or to kill these bacteria, that which are pathogenic in nature, that is extremely difficult.

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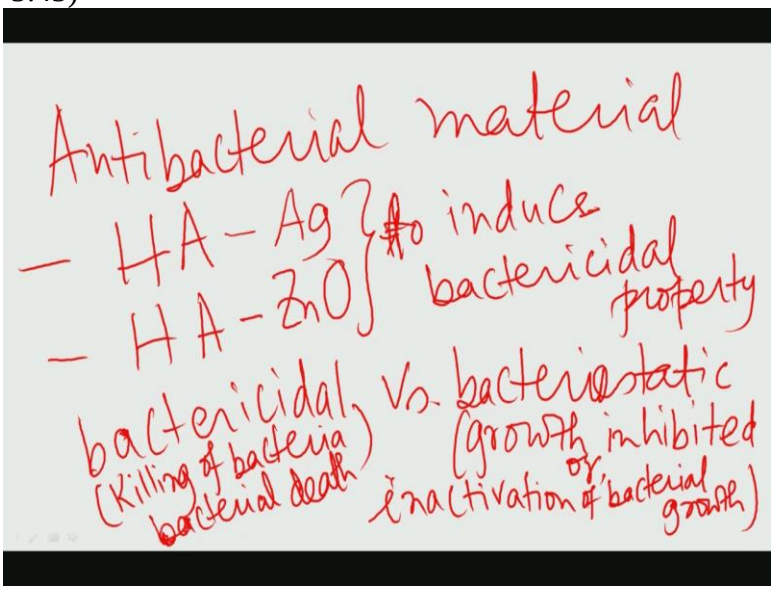

So there are 2 ways that 1 can deal these bacteria, this anti-bacterial and to (in) induce anti-bacterial property. 1 is that there is something called anti-bacterial material. So, just to show you the, or just to illustrate that how to develop this kind of material, is it like hydroxyapatite is widely investigated because it is the inorganic constituent of the human natural bone. But hydroxyapatite as such does not have any anti-bacterial property. Like if you grow E coli bacteria which is very well know gram negative bacteria then E coli bacteria can happily grow on hydroxyapatite as such.

So what people do, people use certain anti-bacterial phase like silver for example. So they add silver to hydroxyapatite to induce that anti-bacterial property. Like the presence of silver, will facilitate bactericidal property. Another material that people use like Zinc oxide, so people use, add zinc oxide. The researchers kind of (ba ba) develop that zinc oxide reinforced hydroxyapatite or zinc oxide added hydroxyapatite; now the role of silver zinc oxide to induce bactericidal property.

So, so (the) in this context there are certain terminologies which is important to induce bactericidal property. So there is 2 terminologies, 1 is bactericidal versus bacteriostatic. Bactericidal means that the materials with the capability or certain antibiotics with a capability of killing bacteria in growth medium. Bacteriostatic means, the materials with the capability, of inactivating the growth of the bacteria. That means if you grow the bacteria or if you want to grow the bacteria on this particular materials, those bacteria may not be killed, but at the same time, those bacteria may not undergo binary fission process so that 1 to 2, 2 to 4 like that, that, their growth would be inhibited.

So in other words let me summarise this, so it is growth inhibited in case of bacteriostatic property (bact) or inactivation of bacterial growth and bactericidal means killing of bacteria or bacterial death. Ok, so, so these 2 things should be clear, so let me summarise these 2 terms; (what a what is) what is, I have mentioned bactericidal means bacterial death or killing of bacteria. Bacteriostatic means growth inhibited or inactivation of bacterial growth. That does not necessarily mean if you have a bacteriostatic property it will kill all the bacteria. So,1 of the examples of that bactericidal property that are, materials exhibiting bactericidal properties, hydrocele silver, hydrocele zinc oxide, so these kind of materials.

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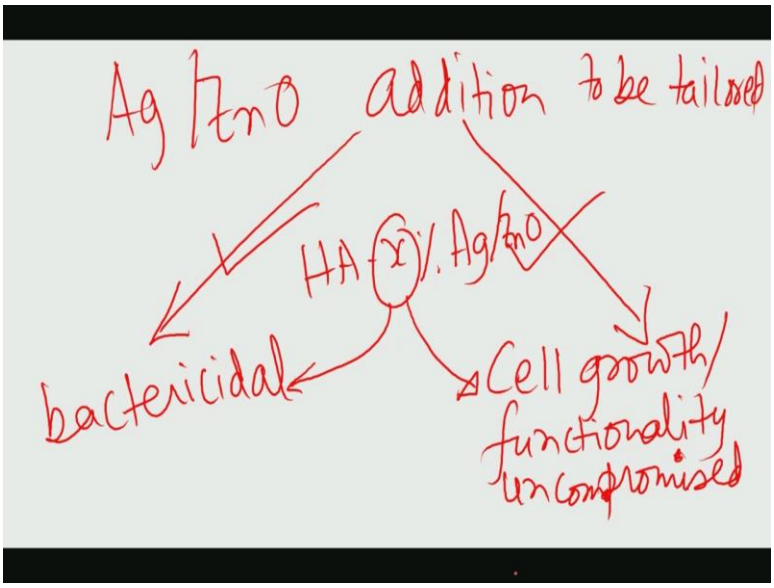

Now other things, that silver or zinc oxide, although they have anti-bacterial property, now these addition to be tailored in the context that; so what I said for example, hydroxyapatite you add these materials X percent silver or X percent zinc oxide. So this X should be such that, it will cause bactericidal property without compromising or without inhibiting cell growth or functionality.

Here growth and functionality in the context of eukaryotic cells, truly nucleated cells. So that means this addition of silver or zinc oxide to induce bactericidal property should not in any way inhibit the cell growth or functionality behaviour on that particular substrate. So these things needs to be very carefully tailored or very carefully kept in mind while developing these kind of anti-bacterial materials.

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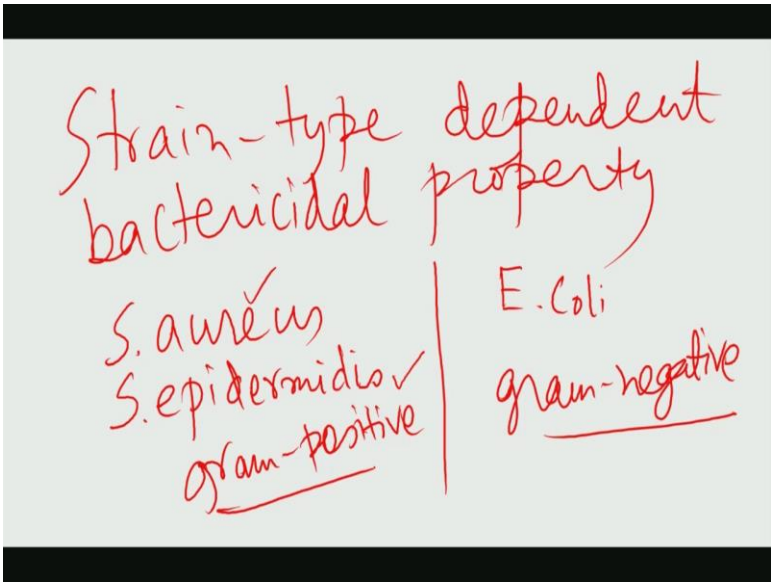

Now once you develop these anti-bacterial materials next thing that 1 should understand that how to quantify or how to (des) how to assess both qualitatively and quantitatively this bactericidal property. Now for that 1 is the strain type dependent bactericidal property needs to be assessed. What it means; for example gram positive if you take staphylococcus species. Now staphylococcus they have number of strains like, staphylococcus aureus, staphylococcus epidermidis (for a) and this the gram positive, in gram negative mostly people use Eshchiria coli. This is the gram negative bacteria.

Now all these bacteria (neb) so if you use just only 1 type of bacteria, it is not sufficient to establish bactericidal property. But instead, 1 as to 2 more (num) more cell types or more strains like S aureus after that S epidermidis so that (you have) the bactericidal property not only only 1 bacterial strains but also multiple bacterial strains. Not only1 bacterial type, let us say gram (positive) gram positive but also gram negative as well.

So these things must be mentioned, or these things must be carefully considered so that the cell type or strain type dependent property can be established, so 1 single material may be (goo) may have good bactericidal property against E coli but that does not mean the same material will have equally good bactericidal property against S aureus. So it may not (it bit) it may not be able to kill all the S aureus bacteria when this S aureus bacteria that will grow on that material substrate.

Now to confirm this, you know in, typically in biology it is very common to use not only 1 cell (try wo wo) only 1 cell type, or only 1 strain type but multiple of those to confirm certain evidence or to confirm certain mechanisms whatsoever it is.

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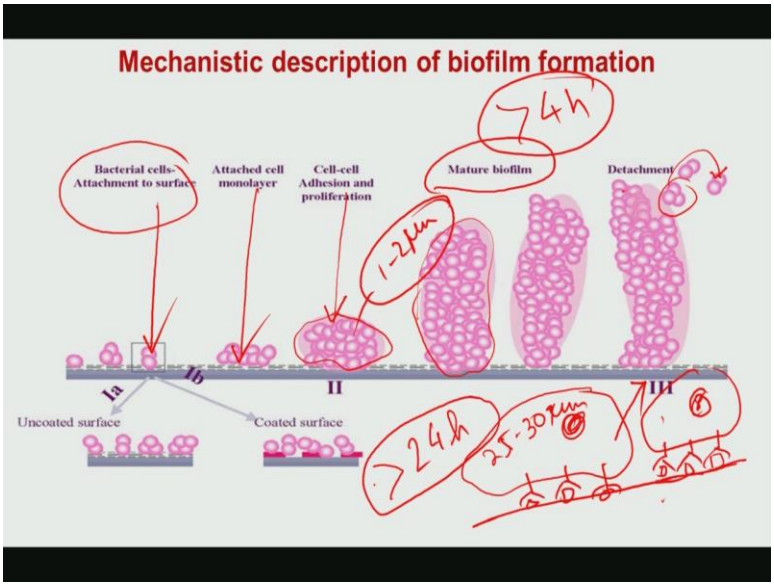

Now, just to refresh your mind on that biofilm formation and mechanistic description of the same, so initial stage is that 1 or 2 bacteria will come and adhere in this material substrate. Now if you remember, the bacteria they grow much much faster than eukaryotic cells. Like if the typical cell division types (t) cell division time, of eukaryotic cell or human cell is 11 to 12 hours, bacteria, the doubling time of bacteria is 30 to 40 minutes most of this bacterial strains.

So, so therefore, the moment, it it it, it adheres or it or it gets attached to a material substrate, then these, it (go) it, it, it undergoes binary fission from 1 to 2 bacteria and so on and there are 2 things that has been mentioned here. That is, that in 1 case it is shown that if you put some surface coating on the material or (surf) substrate coating and another case uncoated substrate. Now this colonisation would be there, but this colonisation would be more specific to the part where certain specific coating is given to the (bacterial) to the biomaterial substrate. Now once this cell monolayer forms then there is polysaccharides secretions and these polysaccharides would would act like a glue and then that will essentially help in the formation of some bacteria clusters.

Now these clusters will grow and then it will form biofilm and this biofilm, then once it matures biofilm, then any more bacteria which will come from the growth medium, that bacteria cannot get attached to this biofilm and as a result (did) those would be detached and these these actually tells you that bacterial biofilm grows to a kind of a critical size and therefore this biofilm cannot grow any further. So all these things needs to be kept in mind while assessing the, or while using different anti-bacterial assays.

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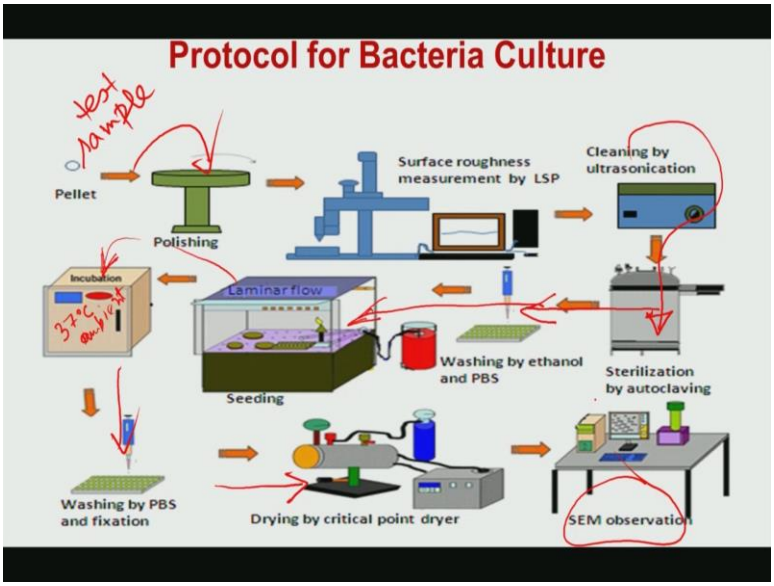

So this is a typical protocol for the bacteria culture. Like you know that you start with these sample preparations, then you grow the bacteria and then before that you have to do sterilisation then you grow the bacteria in an incubator, this incubator is 37 degrees Celsius, there is no requirement for the CO2 flow, unless eukaryotic cell culture because bacteria can grow in normal ambient environment and then after that you fix the cells by PBS phosphate buffered saline. After that you can do either critical point dryer or you can do HMDS hexamethyl disaline HMDS chemical. Then you fix it then you can see either HCM or live dead straining using a fluorescence microscope.

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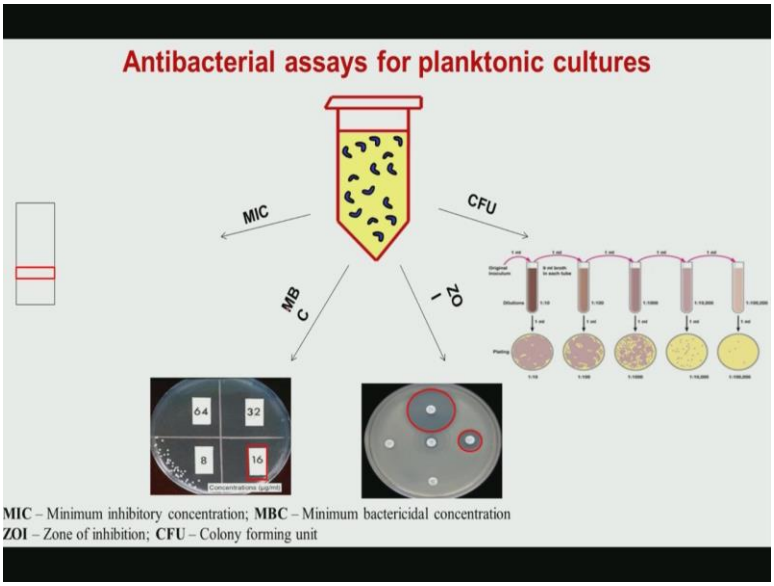

Now these are difference essays that that is important and these essays are MIC, MBC. MIC stands for minimum inhibitory concentration and in a planktonic culture like when the bacteria is kind of suspended in a append of tube not actually on the, growing on the substrate. Now this is, there is zone of inhibition ZOI and there is CFU that is colony forming unit. So there is 4 things, MIC, MBC, ZOI and CFU, these 4 things these 4 aspects need to be assessed qualitatively and quantitatively.

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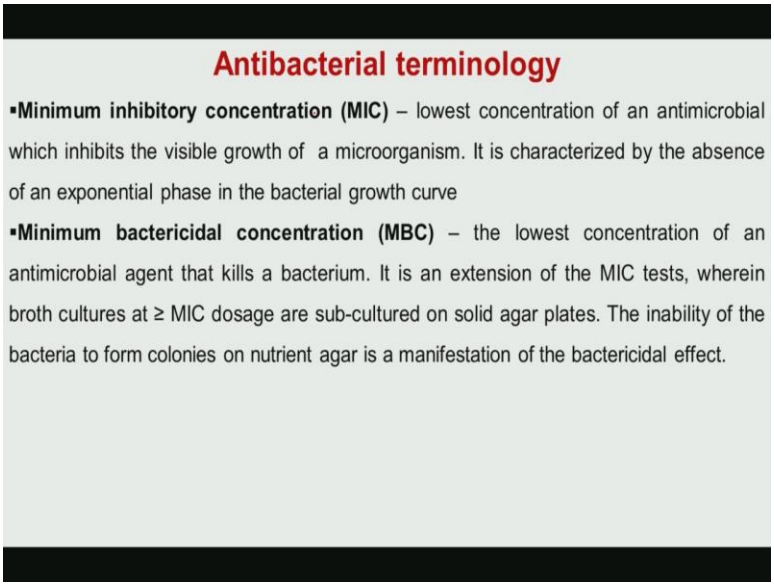

So let us see that what is the meaning of this kind of, what is the meaning of the stance, let us define that. So minimum inhibitory concentration means lowest concentration of an any anti-bacterial, (any) and any antimicrobial. Now here in the context of the bulk materials you can say

that HA x percent of silver or zinc oxide. Now x to be optimised that is what is the minimum percentage of A or minimum value of x which will give you that inhibitory concentration.

Now if it is, let us say, take the example of the gold nano particle, so Au and p what is the doses of this gold nano particle in solution which will cause this MIC that needs to be identified which inhibits the visible growth of a micro-organism, it is characterised by the (exp) absence of any exponential phase in the bacterial growth curve. If you remember the bacterial growth curve goes like this, it goes to stationary phase and then that needs to be, that, that, so essentially MIC is characterised by the absence of the exponential phase in this 1.

Second 1 is the minimum bactericidal concentration, MBC that is the lowest concentration of an antimicrobial agent that kills the bacteria. So it is an extension of the MIC test but wherein the, both the cultures are, contain greater than MIC doses which are sub cultured on the solid agar plates. So what it means that, so you do that MIC test, you extend this test further and if these doses of, greater than MIC and to see that what is the minimum bactericidal concentration.

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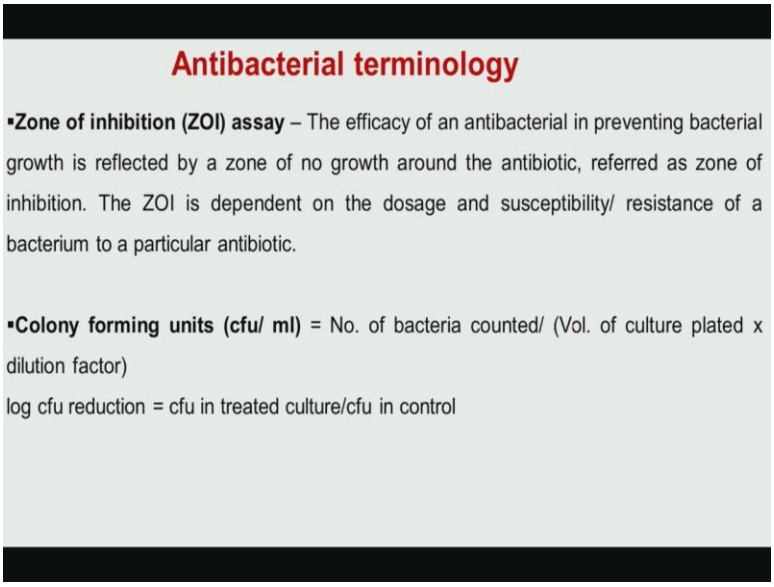

Third 1 zone of the inhibition assay, that efficacy of any (antibacteria) any (antimicrobial) any antibacterial in preventing bacterial growth and this is the zone zone of inhibition and 1 has to see that where is the no growth, or no growth of the bacteria around the anti-biotic and this is referred to as zone of inhibition. As you see that most of this definitions are essentially centred around the qualitative or quantitative effect of an anti-biotic which is being traditionally used as a drug or anti-bacterial drug.

Now in the context of the biomaterials the drug doses has to be converted, or has to be understood with respect to the anti-bacterial phase content in a particular biomaterial substrate. So let me (rembe) repeat what I said. I said let us say, there is a antibiotic drug A, you want to understand that what is the doses x or doses y, which is like minimum inhibitory concentration or which (has a zo) which can be assessed for the ZOI, ZOI or for the MBC.

Now in the context of the (microbial) anti-bacterial material let's say hydroxyapatite and I have given multiple times this example x percent silver composites. So now you are interested instead of antibiotic, you are interested what is the value of x percent silver which will give you the similar description (for the zo) MIC or MBC or ZOI. So antibiotic here is equivalent to the antibacterial phase or antibacterial material or antibacterial compound like Silver or zinc oxide which is added to another material to induce the bactericidal properties.

I hope I have explained you sufficiently so that you understand that, this definition. Then colony forming unit, that is the number of bacteria counted over the volume of cultured plate, volume of cultured is plated multiplied by dilution factor. This is also 1 of the quantitative aspects of this ant bacterial property evaluation.

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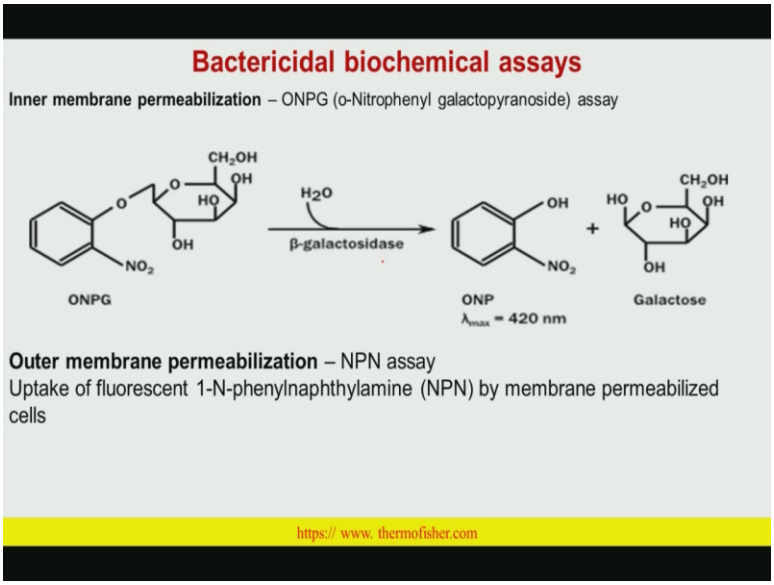

Ok, now while analysing that why a bacteria is not able to grow or why a bacteria is killed in growth medium certain assays typically is used to see the effect on the bacterial membrane itself and this is called inner membrane permeabilization, and this is 1 of the assay that is done, it is called ONPG assay. So in the ONPG assay as I described, as has been described in the slide, so ONPG is a particular biochemical or these ONPG when it is added to this 1, into the bacterial

growth medium, so you have a beta-galactosidase and this ONP is ONP react with that 1, and then the ONPG react with this beta-galactosidase and then you get ONP which can be detected at 420 nano metre wavelength and then 1 of the product is galactose.

The second 1 is that outer membrane permeabilization that also (show) that is also used to quantify whether there is any instability in the membrane integrity. And that is uptake of a certain florescent this is NPN is 1 of the fluorescent by membrane permeabilized cells. That means if the membrane becomes more permeable or if the bacterial membrane their integrity is disturbed or integrity is lost then what happens it will undergo it will undergo death phase.

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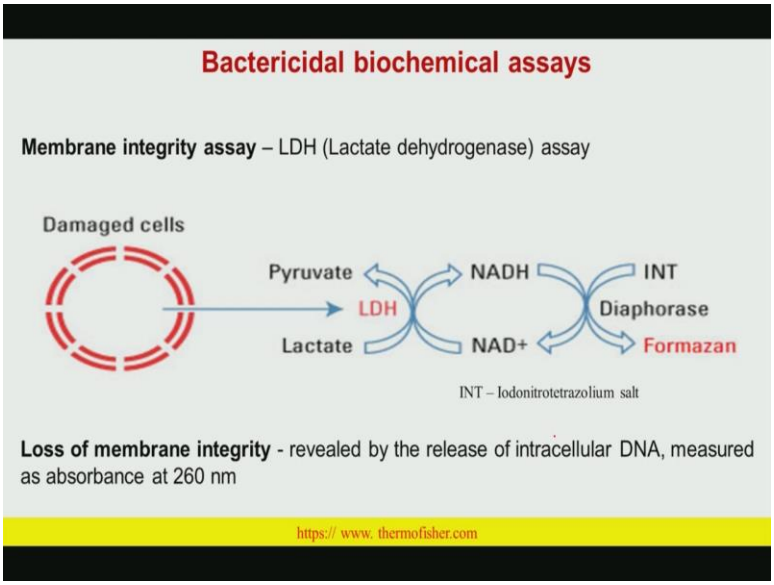

So (other) so this comes to the next description of the membrane integrity assay like LDH assay which I have described earlier in the context of the cell viability cell death, and if you recall, there I have categorically mentioned that for the cell viability assay or for the cell cell death quantification you need to use these assays in a complimentary manner. For example MTT assay will tell you that how much fraction of the cells are viable; LDH assay will tell you how much cells are dead. So these MTT and LDH assays needs to be done in a complimentary manner to quantify what is the fraction of the cell which are growing on a material substrate is alive, what is the fraction of the cells on a material substrate is dead.

Now LDH is an enzyme as mentioned and then LDH when it a, bacteria is damaged or the membrane integrity is lost or disturbed then what will happen, LHD will come from the (cyto) cytoplasm to outside the culture medium. And (then) LDH can be detected at an absorbance of

260 nano metre. And all these bio- chemical reagents or (this) any enzymes they are essentially detected at different wave lengths, that has been (measure) that has been mentioned earlier also.

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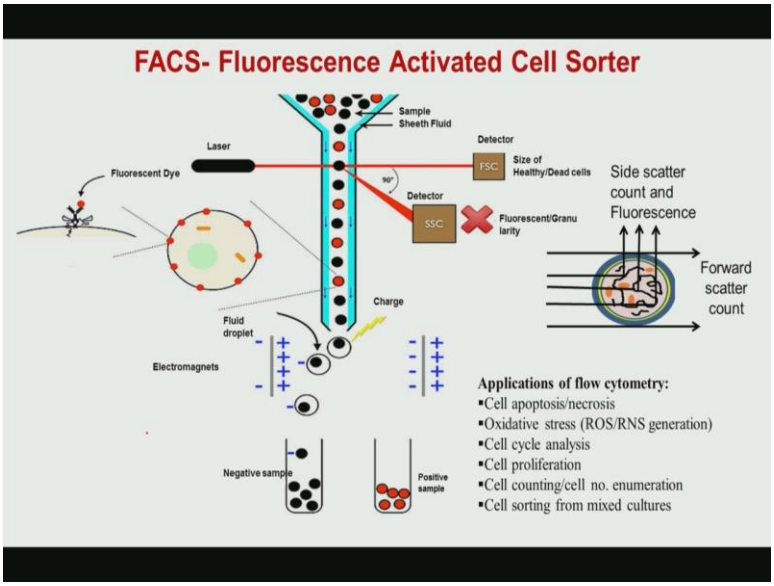

Ok, like in eukaryotic cells, 1 can also do precise cell level cell fed determination for bacteria also and in case, in case like eukaryotic cells 1 can use fluorescent activated cell sorter which I have described significantly when I discussed the cell fed processes. So you have 2 things there, 1 is side scatter and 1 is forward scatter, side scatter is SSC, forward scatter is FSC. Now what is the application of the flow cytometry; that is 1 is the cell apoptosis or necrosis in the context of eukaryotic cells; in case of bacteria it will be simply cell death or (cell) what is the cell viable or (say) what is the cell death. Oxidative stress ROS generation that can quantify cell cycle analysis which is very appropriate for the eukaryotic cells. Cell, counting cell number and cell sorting from mixed cultures.

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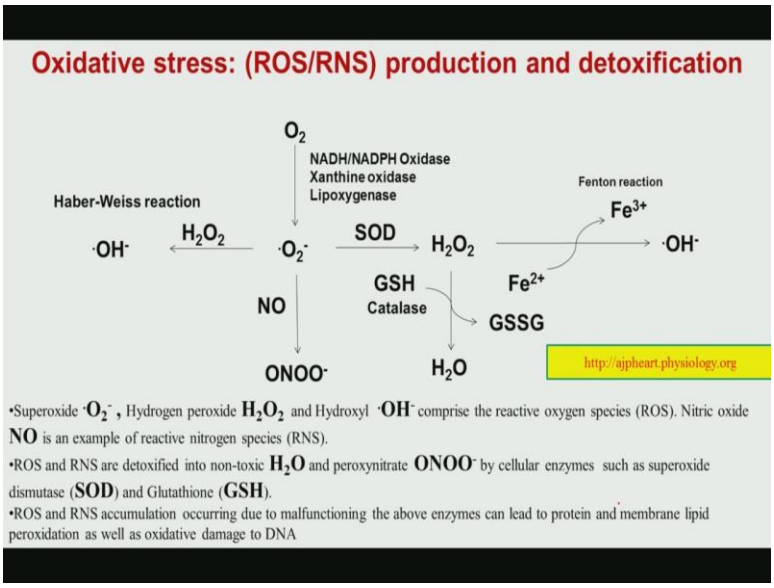

Now out of that, in the context of bactericidal property often the presence of ROS is (attribu) ROS is shown as a reason, for the bacterial death. So therefore oxidative stress needs to be quantified and this oxidative stress as per definition it is present either as super oxide or hydrogen peroxide or hydroxyl ion, this comprise that reactive oxygen species. And this nitric oxide is an example of the reactive nitrogen species. So you have ROS, reactive oxygen species, you have RNS that is reactive nitrogen species.

So reactive nitrogen species is this nitric oxide and ROS is essentially H2O2 or (O2) super oxide and so on. So this is the fundamental (equation) reaction that is Haber-Weiss reaction that is essentially, that essentially shows that how ROS is generated in the intra cellular region of a bacteria and then it finally gives rise to hydroxyl ion, reactive hydroxyl ion, which is 1 of the constituent of the reactive oxygen species.

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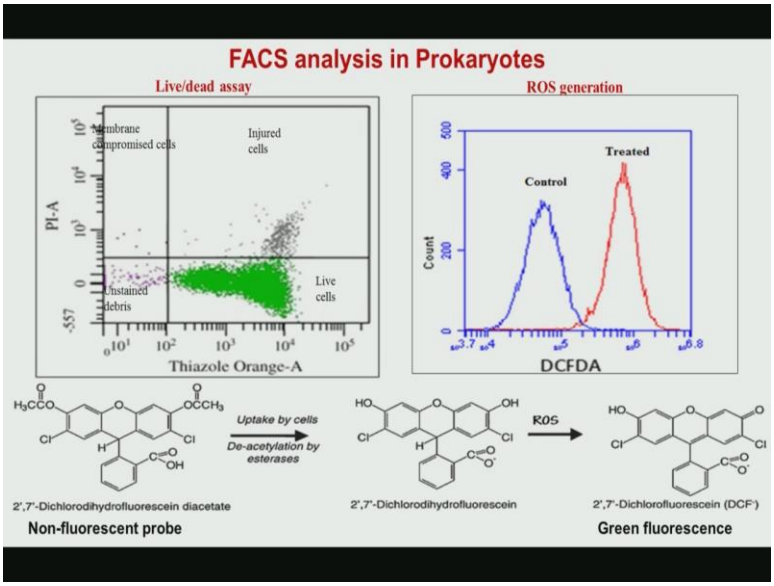

So once you get this reactive oxygen species, then the next stage is that, that next stage is that, that you have to quantify this reactive oxygen species and then you can do that using the fluorescent activated cell sorter and this fluorescent activated cell sorter analysis, this fluorescent activated cell sorter what, what has been shown here in the case of the live dead assay you use the propidium iodide and in case of bacteria you use the different reagent is called Thiazole Orange A. So these use of these 2 reagents will essentially stain the cells differently. In 1 case you have the unstained debris and green dots are like live cells.

If you recall the fundamental principles in these facts is that each dot in the facts plot essentially comes from 1 individual cell, okay? So therefore the multiple of these dots which are stained green that means these (many) so many numbers is the live cells, okay. That this particular quadrant coordinate you have, this particular quadrant you have these injured cells, and this is kind of membrane compromised cells. So from these particular examples here, if so many fractions of these white, green dots are there, that means close to 90 percent or more cells are essentially live cells, live bacteria, rest of them are either injured or unstained debris or membrane compromised cells.

Now coming to ROS quantification using fluorescent activated cell sorter, this is also alternatively known as DCFDA assay, so you use this DCFDA and as a reagent, bio chemical reagent and then what happens, if these particular reaction is taking place at the inter cellular region whose end product is 1 of the constituent of the reactive oxygen species, then these constituent if it is there then what will give, it will give some green fluorescence and this is your (trit) controlled cell population and this is your treated cell population which is shifted towards

the right hand side here in the DCFDA and from there you can see that what is the fraction of the cells where the ROS quantification is significant enough and then you (see) then you can quantify this what is ROS that is generated.

So to go back to this all these summarised, summarised what I have essentially taught you so far, you have to quantify what is MIC, MBC that minimum inhibitory concentration, minimum bactericidal concentration, zone of inhibition, colony forming unit which will give you the quantitative aspect of the antibacterial ability of any material and those, these parameters were defined in the context of the use of antibiotic but now it has been defined in the context of antibacterial material.

Now once you do these things, the reason for any antibacterial effect or bactericidal effect is to be analysed with respect to some additional assays like inner membrane permeabilization, outer membrane permeabilization or LHD assay which will give you the membrane integrity assay. So the rationale is that first to quantify whether any material has sufficient antibacterial property then you try to propose certain reasons to explain the origin of this antibacterial property of that particular material.

Now to support your rationale or support your logic you have to do some additional assays to confirm both qualitatively and quantitatively that it is indeed, that it is because of the membrane permeabilization or membrane integrity, disintegrity that this bacteria is being killed when in contact with the materials or the bacteria is not able to grow once it is coming in contact with these materials. And also more biological origin may be or biophysical origin can be reactive oxygen species, reactive oxygen species and then you need to be quantified that ROS using facts. Now up to this part it is done, then you confirm the anti-bacterial.

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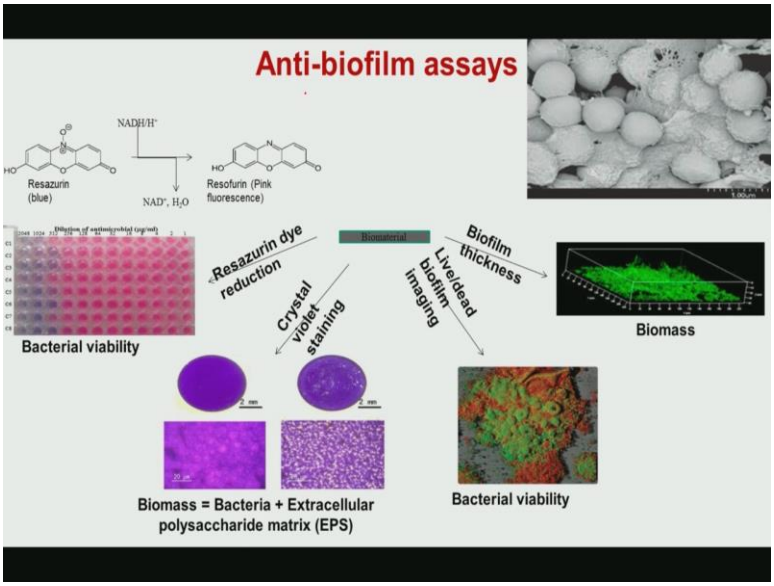

Next stage is that if a bacteria does, if a (bac) if a material has some indication that is has very strong antibacterial effect, but still detectable number of bacteria has the possibility to grow when this bacteria cultured for extended time period in the growth medium and they can potentially form the biofilm. Now this aspect of the biofilm formation I have mentioned very briefly in this module but I have mentioned more extensively in an earlier module where I have covered bacterial growth and bacterial, bacteria structure and characteristics. Now this is the sorry, this is the scanning electron (mic) images.

This is the SEM image of the biofilm as you can see that these are like spherical shaped staphylococci species either S aureus or S epidermidis and there is some SEM like features which is not very dense but it is more like a porous kind of surface layer which is forming and which kind of connects that this spherical species, and this is like polysaccharide rich biofilm which is formed on this material substrate. Now this biofilm also 1 has to quantify this (what) by different assays and several assays is been mentioned here 1 is called Resazurin dye Reduction, 1 is called crystal violet staining, third 1 is called live dead biofilm imaging, so you can do confocal microscopy to do that. And 1 is called biofilm thickness that what is the typical thickness of the biofilm that is to quantify.

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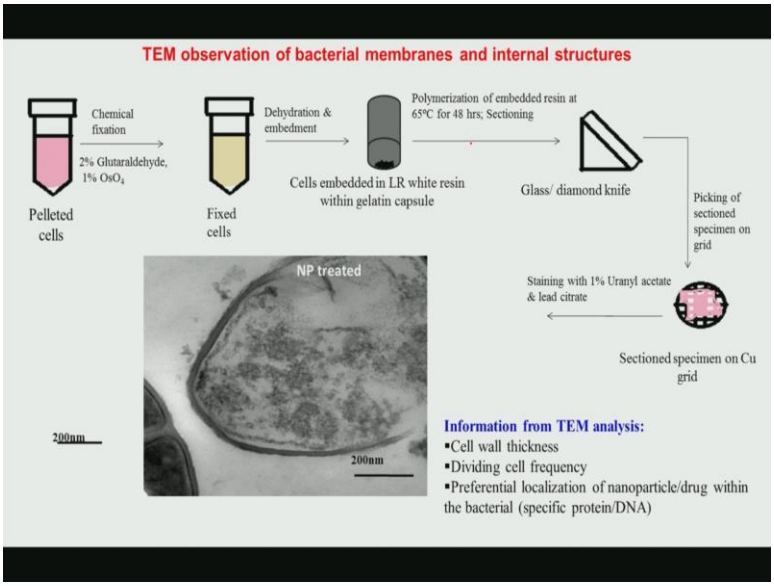

Now once you do all these assays, the last stage of this last stage of characterisation can be, which is, which may be little more difficult is called transmission electron microscopy observation of the bacterial cells. Now this what this, transmission electron microscopy which itself is a 1 of the higher level microscopy techniques to see that ultra-structural features of the bacteria, which you cannot probe using scanning electron microscope or fluorescence microscope where the magnification, allowable magnification is much less than transmission electron microscope.

The first hurdle in the using transmission electron microscope for cells and bacteria is to appropriately prepare the sample. The sample preparation is the most difficult stage in TEM investigation particularly for the biological samples, like cells or tissue sections. So there is a technique called ultra-microtome, so this ultra-microtome if you do this so there you can essentially measure very thin sections. So what you do, you first grow the cells in certain growth medium and then you allow the bacteria to grow and then 1 of the technique in the TEM sample preparation that 1 has to be careful while using this biological sample, 1 has to use osmium tetroxide OSO4, or glutaraldihide. Now osmium tetroxide, the use of osmium tetroxide will give sufficient contrast in the electron micrograph of the cells or the bacterial sample.

In the normal materials and samples 1 never uses this kind of additional supplement like osmium tetroxide and so on while preparing the TEM sample. So you need to know how to prepare this TEM samples before you can go even to the transmission to microscope to see the ultra-structural feature. Now to come back to the discussion again in this slide what I am showing that, so first you have to grow the cells, then you have to fix the cells on certain substrate. After (the)

you fix it then you do dehydration and all these things and in cells embedded in white resin or within gelatin capsule, then you can, so essentially you should use certain substrate to hold or to further attach the bacteria or cells or tissue before you can, very (thin) you can prepare very thin sections and for which you have to use ultramicrotome.

So ultramicrotome once you use, you get very thin sample. You can use glass or diamond knife then (pick) picking of the section specimens of the grid and once you grid it then again you do some additional treatment called, you can do staining using uranyl acetate or lead acetate. Only after doing all these things if you go to TEM and you put biological TEM I mean in the sample chamber, then this is the example of a staphylococcus aureus species and it is treated with gold nano particles and after it is treated in gold nano particles, you can see certain features like, whether these gold nano particle are internalised inside the cytoplasm of these bacteria and if it is internalised you can then correlate that observation with the reactive oxygen species than that you might have quantified before using facts.

So I am trying to give you some indication that how these TEM observations can be helpful to correlate with some of the other features which you have quantified as part of your total investigation. Now just few last things, what are the information that you can get or you can extract from transmission microscopy, 1 is the cell wall thickness, what is the cell wall thickness and there you can use certain control samples.

So in the context of gold nano particle treatment you can grow bacterial population without gold nano particles, with gold nano particles. With gold nano particle will give you the normal cell wall thickness or equilibrium cell wall thickness. In the are gold nano particle treated bacterial morphology you can see whether there is any thickness reduction because of the membrane permeabilization or because of the disintegrity of the cell membrane.

What is the dividing cell frequency and is there any preferential localisation of the nano particle or drug within the bacterial, for example specific protein or DNA, whether the gold nano particle gets attached to this DNA or not; those kind of things you can very well describe both qualitatively and quantitatively using transmission electron microscopy of the bacteria cell specimen. Thank you.