Genetic Engineering: Theory and Applications
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Lecture 34
Spectroscopy (Part 2)

Hello everybody, this is Dr. Vishal Trivedi from Department of Biosciences and Bioengineering IIT, Guwahati and what we were discussing, we were discussing about the characterization of product what you are getting from the host either it is the macro molecules such as DNA or protein or the small molecule such as the different types of metabolic byproducts or the secondary metabolites which you are producing by over expressing or modulating the environmental conditions or optimizing the production of secondary motive dies.

So one of the crucial factor is that you should be able to characterize the product and you should be able to know that the product what you are getting from these host cells is of good quality and it is up to the high standards. So for this purpose so far what we have discussed, we have discussed about the protein sequencing to know that the protein what you are getting from the host is of the right protein and in addition to that in the previous lecture, we have discussed about how the UV visible or the UV visible spectroscopy or in general the spectroscopic techniques can be used to characterize the product.

And we have discussed that how the different spectroscopic techniques will allow the probing of different types of molecular processes within the molecule and how that can help you in getting the different types of information from the same molecules and that information collectively can be used to characterize the product. Continuing our discussion about the UV visible spectroscopy in today's lecture, we are initially going to discuss about the UV visible spectroscopy, how the UV spectroscopy can be used to know whether there is a structural changes happening in the molecule or not.

And subsequently, we are also going to discuss about the IR spectroscopy to characterize the functional group present on the molecule. So, let us start with the UV visible spectroscopy and how UV visible spectroscopy, the absorption spectra is being modulated when you have different types of modulations or different types of changes in the molecule.

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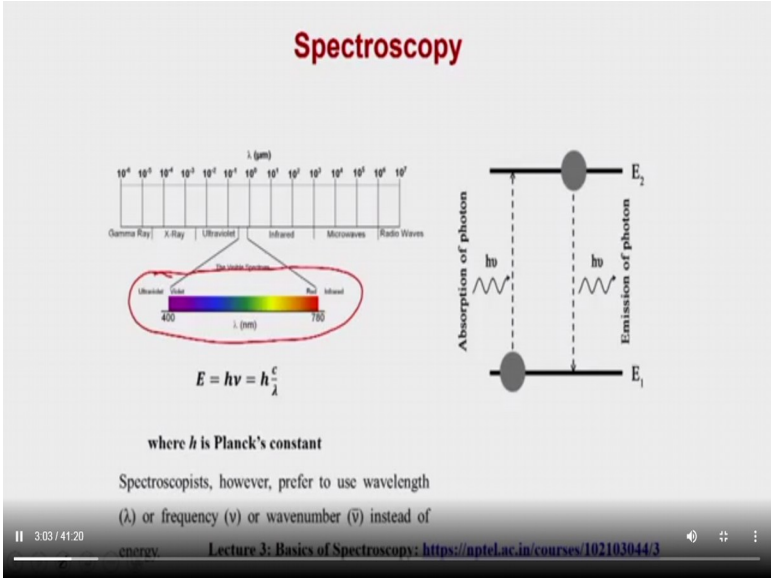

So this is what we were discussing about the spectroscopy. So we started with the UV visible spectroscopy in the previous lecture and what we have said is that UV visible spectroscopy is sensitive to the electronic transitions within the molecules. And now in today's lecture, we will see how the electronic transitions can be used even to map the perturbation or how to map the structural changes in the molecule.

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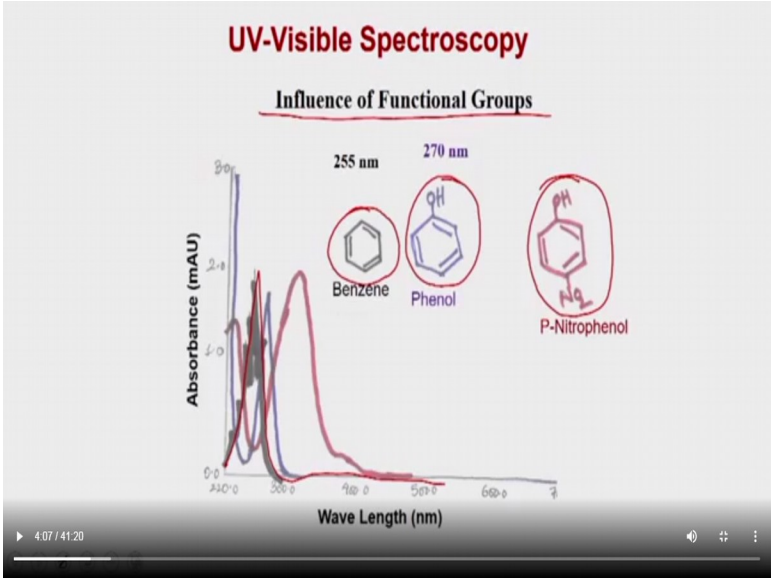

So you can imagine that if I am going to see the influence of functional groups, so if you remember we were talking about the three molecules that is the benzene where there is no functional group present and then you have the phenol which actually contains the OH as a functional group and then you have the para-nitrophenol where you have the two functional groups. So if you see the absorption spectra, what you can see is that the black colored spectra is actually belonging to the benzene.

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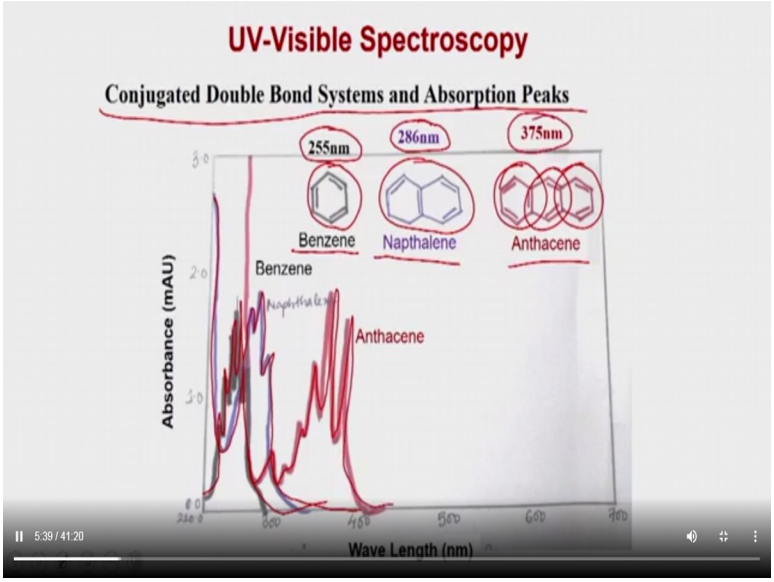

So and so benzene is absorbing very close to the thing what we have studied is how the conjugated double bonds system will going to affect the absorption peaks. So what you can see is we have taken the three molecule the benzene which is actually containing the single ring, single conjugated double bond system then we have the naphthalene which actually contains the 2 rings and then you have the anthacene where you have the 3 rings attached to each other.

So you have the 3 conjugated rings and what you can see is that the black colored spectra is actually corresponding to the benzene which is actually absorbing in the UV range and it is giving a lambda max at 255 nanometer whereas the naphthalene which is containing the two conjugated double bonds system actually is absorbing differently from the benzene.

So what you can see is this (yellow color) this blue color spectra and that is corresponding to the naphthalene and that is how it is actually giving a lambda max at 286 nanometer compared to that when you bring the 3 benzene ring or 3 conjugated double bonds system rings, you will see that there is a clear cut very large shift compared to the benzene or the naphthalene and it is actually giving a lambda max at 375 nanometer.

So what you can see is that apart from the appearance of the functional group, if you have the conjugated double bonds system, if you have the single ring the benzene you are going to see the lambda max at 255 nanometer. If you have the double ring which is actually present in the

naphthalene then you are going to have the lambda max at 286 nanometer which means you are actually shifting towards the left.

And then if you have the anthacene, you are also going to have further shift into the absorption spectra. What that mean is that as you are actually increasing the number of rings the conjugated double bond in system. You are actually providing the more freedom to the electrons to move around throughout this molecule and as a result, the electrons are going to absorb at a higher wavelength because it does not require that much high energy.

Because the electron that is present in the benzene ring is associated with higher energy because the electrons are not freely moving or freely moving within the system because they are present in a conjugated double bond system. Whereas compared to that when you are bringing the two rings it you are actually bringing some relaxation, so the electrons can move from this ring to this ring and so on. So as a result, the electrons are going to show the electronic transitions, even at the lower energy.

This means you are going to see the lambda max at a higher wavelength and when you move to the (third) 3 ring systems you are again bringing some more relaxation. So as a result, the electrons are going to show the electronic transitions at even a lower energy status and as a result it is going to show you the electronic transition at a large wavelength.

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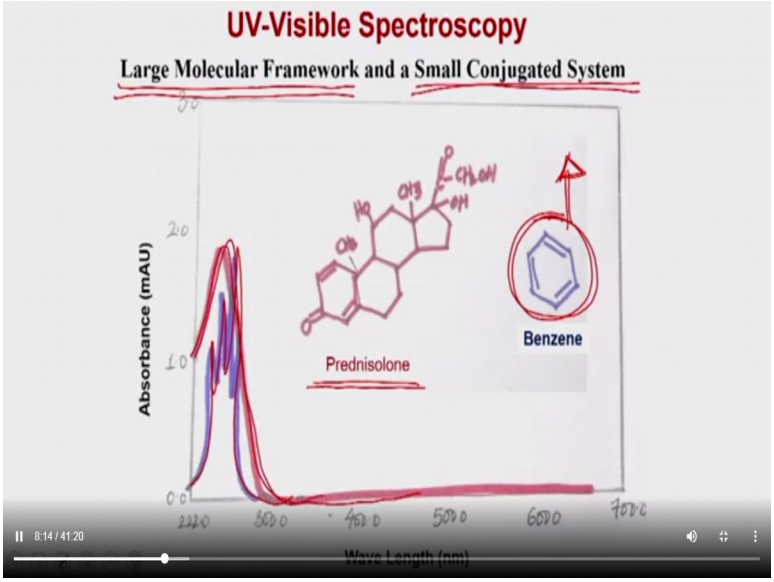

Apart from that we have also studied that how the large molecular framework is also going to give you a change compared to the small conjugated double bond system. So we have taken an example of the benzene which is actually a small conjugated double bond system whereas we have taken an example of the molecule which is called as prednisolone which actually contains the large molecular framework.

And as you can see the benzene is actually giving the clear cut the absorption spectra within the UV range, whereas the prednisolone is giving absorption spectra which is very-very different from the benzene. So with this we would like to emphasize the point that if you collect the absorption spectra of the molecule and if you are expecting a change in the structure or change in the presence of functional groups or the change in structure in terms of the single conjugated ring versus the double conjugated ring or triple conjugated ring, you are definitely going to see a change in the absorption spectra.

And that absorption spectra could be an indication that the molecule is been derivatized, molecule is been changed and it might be the right molecule what you are looking for. So with this we would like to move on and we would like to discuss about the next spectroscopy technique that is called as the IR spectroscopy.

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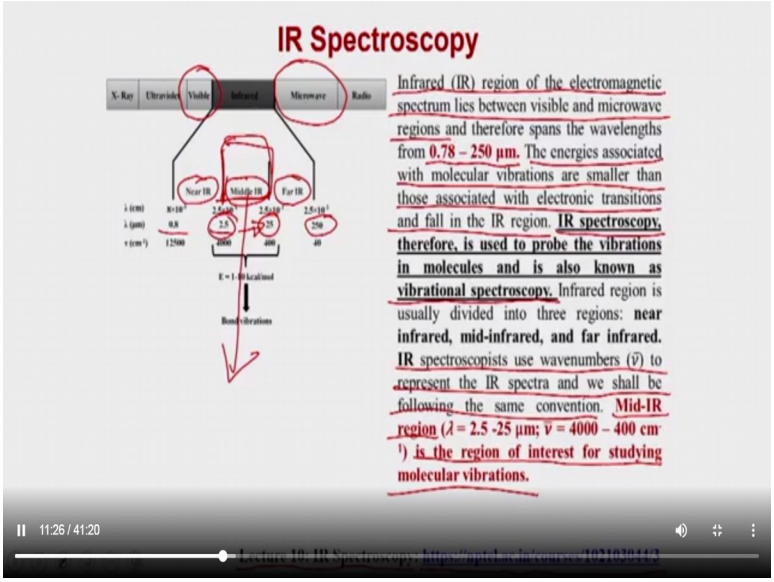

So the IR spectroscopy as the is lies within the region of the electromagnetic radiations between the visible and the microwave regions which means it lies within the visible as well as the microwave regions and the IR region is spanning from the 0.78 to 250 micron meter. The energy is associated with the molecular vibrations are smaller than those associated with the electronic transitions and those are falling into the IR regions.

That is why the IR spectroscopy is also called as the vibrational spectroscopy. IR region can be further divided into three regions, one is called the near IR then you have the middle IR and then you have the far IR. The middle IR (is start) is ranging from the 0.8 to 2.5 micron meter whereas the middle IR is ranging from the 2.5 to 25 micron meter and the far IR is ranging from the 25 to 250 micron meter.

The IR spectroscopy normally uses the wave number to represent the IR spectra and we shall be following the same conventions, the mid IR region, which is actually this region the mid IR region that is from the 2.5 to 25 micron meter is actually the region which is actually is interest for studying the molecular vibration. So the middle IR region is actually the region which actually been extensively been used to study the molecular vibrations. And that is the region which is actually been interested.

If you are interested that is the region which you can very much exploit to study the presence of functional groups. Regarding the IR spectroscopy, we have a lecture which is lecture 10 of the IR spectroscopy and you can download this lecture from one of our NPTEL course.

And so you if you would like to get the literature material you can actually browse through what I am showing you a very small overview of the IR spectroscopy and how that can be used to understand the potential of this particular technique so that if you are interested to use the IR spectroscopy to characterize the functional group you can be able to do that. But if you are interested to learn more about the IR spectroscopy, I will suggest that you can go through with the lecture 10 of one of our NPTEL lectures and that will give you the more detailed information about the IR spectroscopy.

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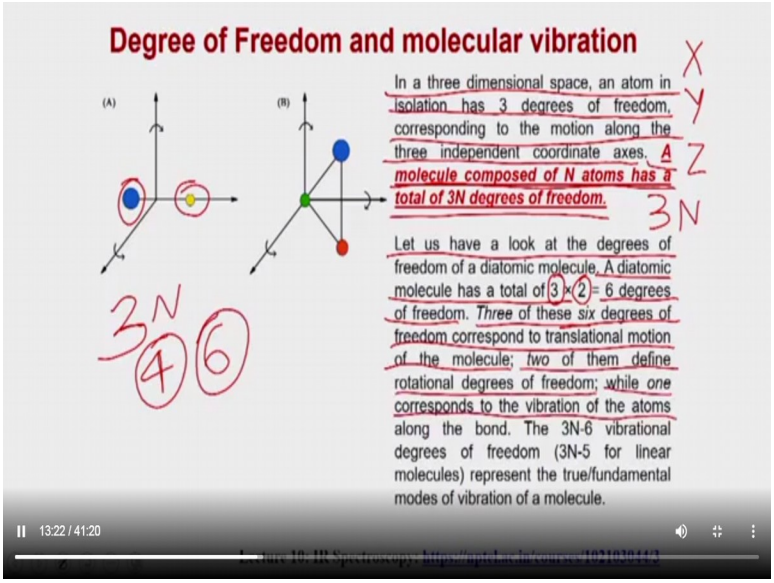

So IR spectroscopy depends on the two phenomena one is the degree of freedom and the molecular vibrations. So in a 3-dimensional space an atom in isolation has 3 degree of freedom corresponding to the motion along the 3 independent corresponding coordinate axis which means the molecule can move into the X, Y, and Z a molecule. whereas if you have a molecule the degree of freedom can be calculated simply by a formula which is called as a molecule.

If you molecule is composed of 3 atoms or n atoms then the number of degrees of freedom is equivalent to the 3n. Let us take an example, so if you suppose you have a diatomic molecule

such as in this case, so how many number of degree of freedom? You are going to have 3 into 2, which means the 6 degree of freedom. 3 of this so out of 6, 3 of these 6 degree of freedoms corresponds to the translational motion of the molecules then an additional two of them define the rotational degree of freedom while the one corresponds to the vibration of the molecule.

So this molecule can have the degree of freedom in terms of the vibration also because this molecule can vibrate because they are attached to one another by a single bond. So, let us see how in how many ways the two molecules or the molecules can have the by motions within the plane or out of the plane.

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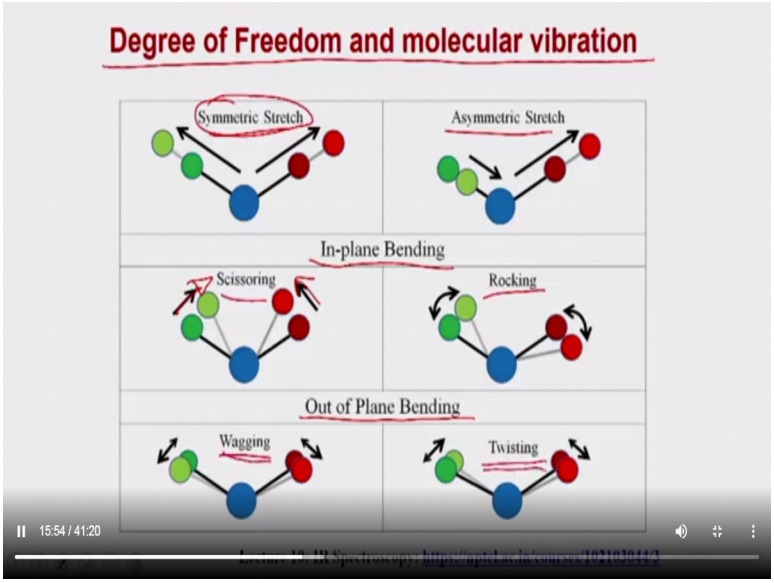

So, degree of freedom can be of different types and you can have the symmetrical stretching which means (if the 2 atoms) if the 3 atoms are present in a molecule and you can imagine that I am the central atom. Then the stretching of the 2 atoms could be of symmetrical in nature which means at the same time the 2 atoms are going up, going far away and coming down. This is called as the symmetrical stretching.

Whereas it could be asymmetrical which means one molecule is going while the other one is sitting, the other one is coming and the third one second one is going this means if the one is approaching the other one is going or one is approaching the other one is going so that is called as the asymmetrical stretching. So you can have the stretching which can be symmetrical

stretching which means the two atoms can move from the central atom at the same rate which means they will be moving like this. If it will be asymmetrical stretching, so one will be approaching the other one is going, the one is approaching the other one is going, so that will be asymmetrical stretching.

Then you can have the in plane banding which means it could be it could be the scissoring which means the molecule will be moving like this okay and it will be like moving towards a particular so it will be moving this direction, this one is moving in this direction that is called scissoring effect. It could be rocking so it will be rocking like this. So that will be also another kind of the rotations and then you can have the out of the plane rotation which means so this is the in plane rotation which means it is actually happening in the same plane.

You can have the out of the plane rotation which means like one is in the upward direction so other one is in the lower direction, so that is how it will be actually out of the rotation, out of the plane also. In that you have the two different types of motions, one is called as the wagging motions, the other one is called as the twisting motions. So because of these different types of motions the molecule is also having the different types of vibrational energies.

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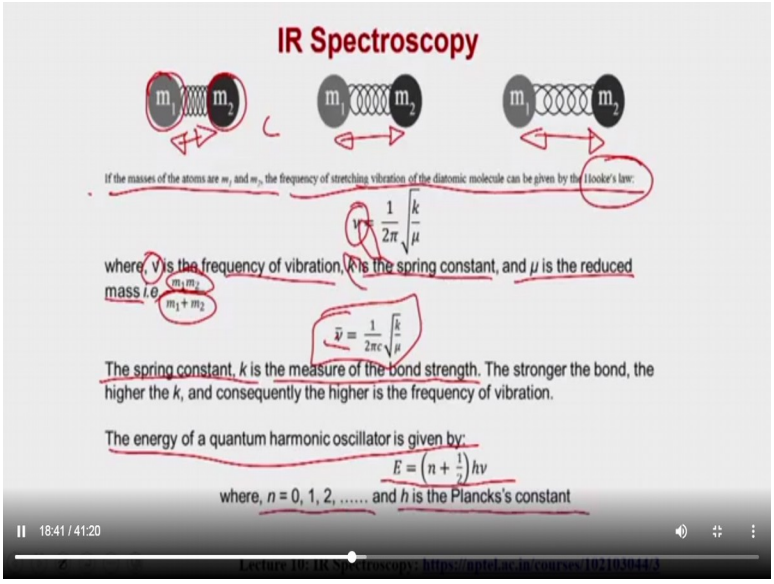

So you can imagine that if the two molecules are connected to each other with a spring so you can imagine that I have 2 atoms, one is called m 1 the other one is called as m 2 and they both

are having the molecular mass of m 1 and m 2 and they are associated with a particular spring so they can have the 3 different types of the motions.

One there when they are very close by okay, then they can go far away. So you can see that a string is getting stretched okay and it can get stretched further and then they can come together. So this means like the if the if you have the 2 atoms which are connected through a spring it can actually go far away and then because of the tensile strength they will come together. So because of this motion it is actually going to have some vibrational energy which is associated with this movement.

So if you would like to calculate you can see that if the masses of the two atoms m1 and m2 have the frequency of stretching vibrations that can be calculated with the help of the Hooke's law. So the vibrational frequency would be 1 by 2 Pi underscore k by mu whereas where the v is the frequency of the vibration, k is the spring constant and mu is the reduced masses that can be calculated by the m1, m2 divided by m plus 2.

If you divide this whole number by the lambda and if you simplify you will get the formula that is called as the mu equivalent to 1 by 2 Pi c underscore k by mu whereas the k is the spring constant and the k the spring constant is going to be a measure of the bond strength which means the higher the bond strength, the higher is going to be the value of k.

And that actually is going to decide at what frequency the molecule is going to do the vibrations. And if you would like to calculate the energy which is associated with this particular type of motion, you can calculate the energy by using this formula E is equal to n plus half multiplied by h nu where n is 0, 1, 2 and h is the Plancks constant. So with this Hookes Law, you can be able to calculate the vibrational frequency and as well as you can be able to calculate the energy associated with the vibration of this particular molecule.

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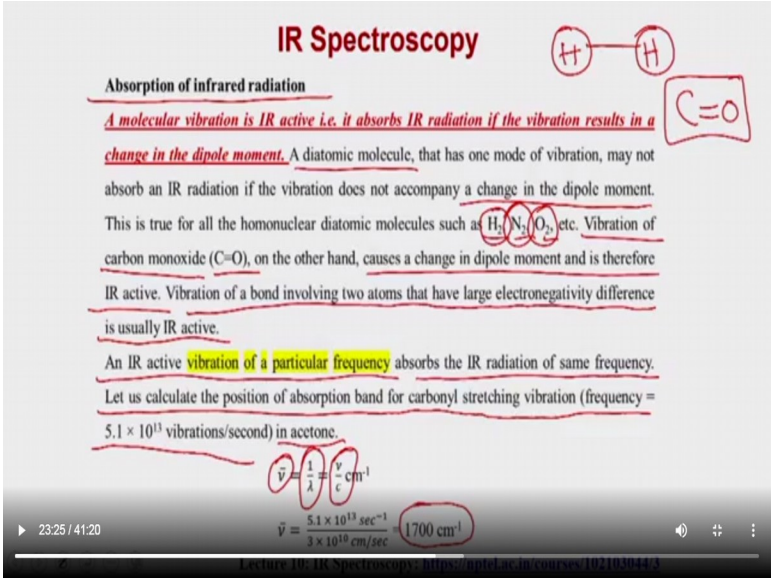

The absorption of so, what are the molecules are going to show the absorption of the IR radiations? So what is the pre-requisite? The pre-requisite is that you have to have a molecule where you can have the two atoms which are bound to one bond. So if you do not have the atom which is not non-bonding atoms like if you have a molecules like sodium chloride for example, NaCl is also a molecule, but NaCl is not going to give you the IR radiation or IR absorption because the sodium and the chloride which are coming together are not been forming a molecule because there is no covalent bond associated with that.

So because of that you the prime requirement of the IR spectra is or IR absorption spectra is that the molecule is going to be active for IR absorption only or IR spectroscopy only if the two atoms are associated with each other by a bond. The other condition is that even if you have a diatomic molecule the both of the molecules should show vibrational energy in such a way so that there will be a change in the dipole moment.

For example, if you have the hydrogen and hydrogen which means the two molecules which are abounding to each other by a single bond but both the atoms are identical in those cases, even if they are actually going to show a vibration there will be no change in the dipole moment and that is why all the molecules even if they are diatomic, even if they are connected through a bond, they are not going to be IR active because they are not showing any change in dipole moment.

So because of that the H2, N2, O2 all these kind of molecules are not going to show the IR radiation compared to that if you have a carbon-monoxide which is actually the C double bond O that is actually going to be a diatomic molecules but it is actually going to show you the molecular vibrations because the C and O are actually going to vibrate at a different frequencies and C and O are actually going to generate a dipole moment within the molecule and because of that this particular molecule is going to absorb the IR radiations.

So in the carbon monoxide that cause a change in dipole moment and that is why it is IR activity. The vibration of a bond in involving the 2 atoms that have the large electro negative difference is usually the IR activity, IR active. So looking at the molecule you can be able to say whether that particular molecule is going to be IR active or not. What is the condition? The condition is that it should be a diatomic molecule which means it should contains the 2 atoms and these 2 atoms should be connected through a bond and those 2 atoms should have very long, very large electronegative differences.

Which means, because of that they when they will vibrate they are they are going to generate a change in the dipole moment. So an IR active vibration of a particular frequency absorbed IR radiation of the same frequency. Let us calculate the position of absorption band for a carbonyl stretching frequency. So the carbonyl stretching frequency is 5 into 5.1 into 10 to power 13 vibration per second in acetone.

So if you were if you want to calculate where this molecule is going to absorb in the IR radiation, the formula is very simple, the frequency (divided) is equivalent to 1 by lambda which means the you can just put this number and what you are going to get so nu by c okay and what you are going to get is 1700 centimeter minus 1 which means in IR absorption spectra it is going the carbonyl bond is going to absorb at 1700 centimeter minus 1.

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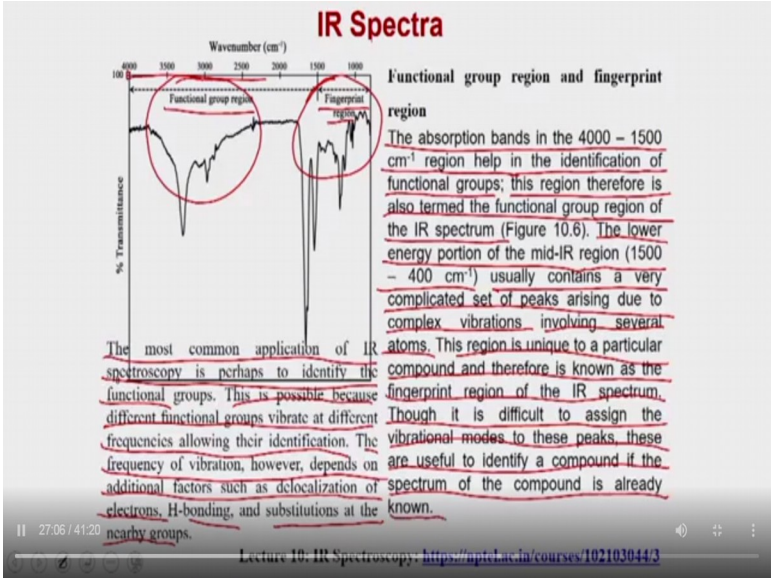

So this is a typical IR spectra where you have t functional group region and the fingerprint region. So these are the 2 region which are important for the identification of the molecule as well as for identification of the different functional group present in the molecule. So the most common application of IR spectroscopy is identifying the functional group. This is possible because the different functional groups vibrate at different frequencies allowing their identifications.

So as I think we, as we discussed in our previous lecture as well that the different functional groups are actually having the different types of atom attach associated with it and because of that it is actually going to generate the different amount of dipole moments and because of that it is actually going to absorb differentially at different places in the IR absorption spectra and because of that it can be possible to characterize the presence of those functional group within the molecule.

The frequency of vibration however depends on the additional factors such as delocalization of electrons, hydrogen bonding and substitution at the nearby group. So as I said, you know when you have the 2 atoms associated with a single bond, what is the most important thing is the change in dipole moment and the change in dipole moment is going to be affected by the environmental conditions. If you have more electronegative atoms or suppose electronegative

atom (associate) is bound to another atom then you are actually going to have the different types of absorption spectra or different types of IR spectra.

So nearby functional groups are also could be able to influence the IR spectra. The absorption spectra is in the range of 4000 to 1500 centimeters region helps in the identification of the functional group. This region therefore is also termed as the functional group region of the IR spectra which means from 4000 to 1500 that is the region which is actually been called as the functional group region.

The lower energy portion of the mid IR region which is 1500 to 400 usually contains a very complicated set of peak arising due to the complex vibration involving several atoms. This region is unique to a particular compound and therefore it is known as the fingerprint region of the IR spectra, which means this is the region which is actually where you have the multiple peaks and these multiple peaks are corresponding to a particular compound.

And as a result, these peaks are actually going to tell you the compound whereas these peaks are going because these peaks are going to give you the presence of a functional group. Therefore, it is difficult though it is difficult to assign the vibrational modes to these peaks, these are useful to identify a compound if the spectrum of a compound is already known. So as I said not a single technique is complete on its own and that is why it is important that you use the multiple techniques.

So if you have already pre-known IR spectra of this particular molecule then you can be able to assign these peaks and you can be able to characterize the compound.

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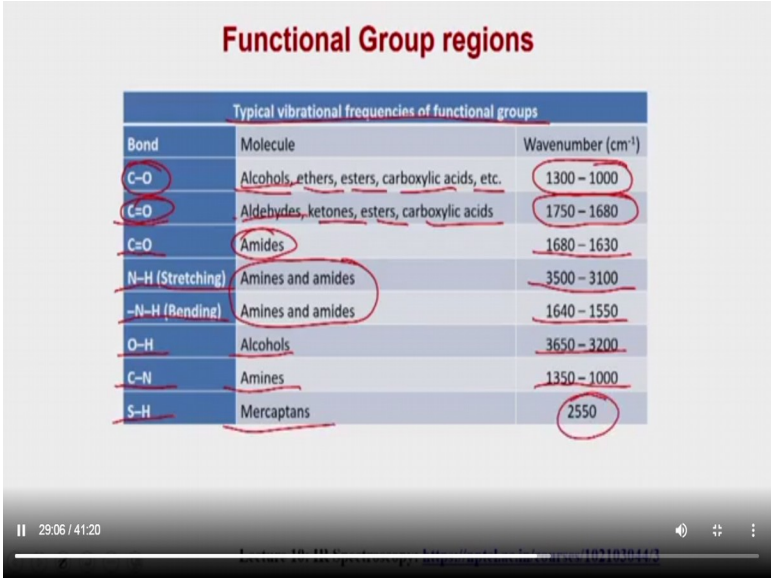

Now what you see is the vibrational frequencies of the functional groups, what you see is alcohol. So if you have the bond what you have is C-O bond which is going to be present in alcohol, ether, ester, carboxylic acid, etc and that is actually going to absorb in the range of 1300 to 1000 depending on the different types of molecules. Then you have the C double bond O which is the carbonyl groups, carbonyl bonds and that is present in aldehydes, the ketones, esters and carboxylic acid and that is going to absorb in the range of 1750 to 1680.

Then you have the carbonyl bond which is present in the amides and that is going to absorb between the range of 1680 to 1630. Then you have the NH stretching or NH bending. The NH stretching and bending both are present in the amines or the amides and that is actually going to absorb in the range of 3500 to 3100 or 1640 to 1550.

Then you have the O-H which is present in alcohols and that is going to absorb in the range of 3650 to 32. Then you have the CN which is the present in amines and that is going to absorb in range of 1350 to 1000 and then you have the sulphutryl or SH group which is going to present in the mercaptans and that is present in the 2550. So with the help of these kind of (vibrate) these kind of information where you have the information about if this particular bond is present it is going to absorb in this particular range or it is going to give me the peak in this particular range.

You can be able to predict whether this particular functional group is present in your compound or not or whether this particular type of functional group is present in your molecule or not.

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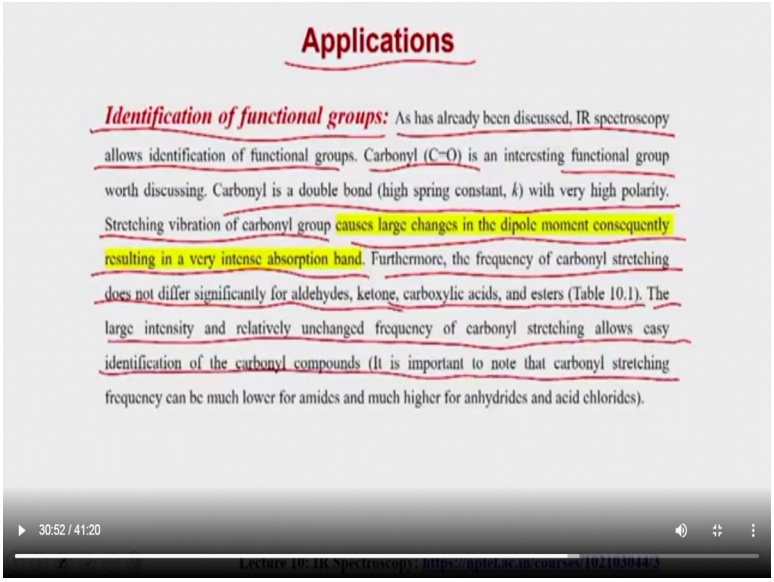

So what is the application of IR spectroscopy? So as I said the first and the foremost of application of the IR spectroscopy is that it will allow you to identify the functional group as it has been already we have discussed that IR spectroscopy allows the identification of functional group. For example, the carbonyl, carbonyl is a functional group, carbonyl is a double bond with high energy polarity. So, stretching vibration of carbonyl bond causes large change in the dipole moment consequently resulting in a very intense absorption band.

Furthermore the frequency of carbonyl stretching does not differ significantly for aldehyde, ketone or carboxylic acid or ester. As we discussed in the past that if you have the some of the functional groups and if you have the nearby some other groups that may actually change the electronic vibrations or that may change the vibrations. But the carbonyl bond is very-very strong and that is why it actually does not get affected by the nearby groups.

The large intensity and the relatively unchanged frequency of carbonyl stretching allows the easy identification of the carbonyl compound.

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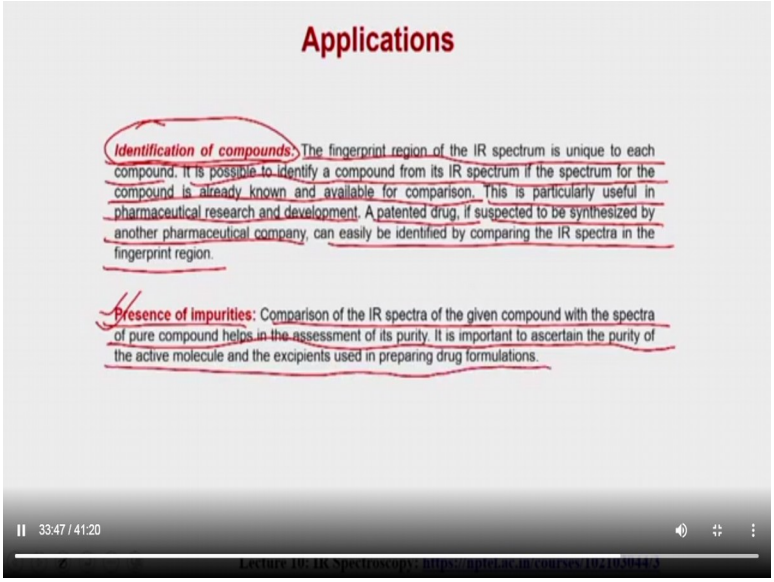

Then you have the second application that is the identification of the compounds. The fingerprint region of IR spectroscopy is actually unique to each compound. It is possible to identify a compound from IR spectra if the spectra from the compound is already known and available for comparison. So you cannot be able to identify the compound by a (())(31:19) IR spectroscopy because then it is very difficult to define the peaks which are present in the fingerprint region because you are going to have multiple peaks and it is very difficult to define.

But if you have a pure compound for which you have collected the IR spectra and you are known that these are the fingerprint peaks are going to appear in this particular type of compound then if you do the similar kind of analysis with the compound isolated from the host cells and it actually gives those peaks then actually it is going to confirm that this is the actually the same compound present in the, this is the same compound which is been isolated from the host cells.

So that is very important that you should have a standard IR spectrum for comparison purpose if you would like to use the IR spectroscopy to identify the compound. This is particularly useful in pharmaceutical research and development. A patented drug if suspected to be synthesized by another pharmaceutical company can easily be identified by comparing the spectra of the fingerprint region, which means if suppose I have discovered a drug and which is actually gone into the market and if the same drug is being synthesized by another company with a different name or different generic name or different brands.

Then in those cases you can easily isolate that particular you can easily procure that particular tablet and do a fingerprint analysis in the IR region and you can compare that with your compound which you have used and that actually will very easily be able to tell you that whether the two compounds are different or the same. Because if the other pharmaceutical company is synthesizing the same compound without taking a permission from the previous company then you can be able to find out that particular information.

Then you can, since you can easily calculate or you can easily know the presence of impurities. So comparison of IR spectra of a given compound with the spectra of pure compound will help you in terms of the assessment of it impurities. It is important to ascertain the purity of active compound before for the drug formulations.

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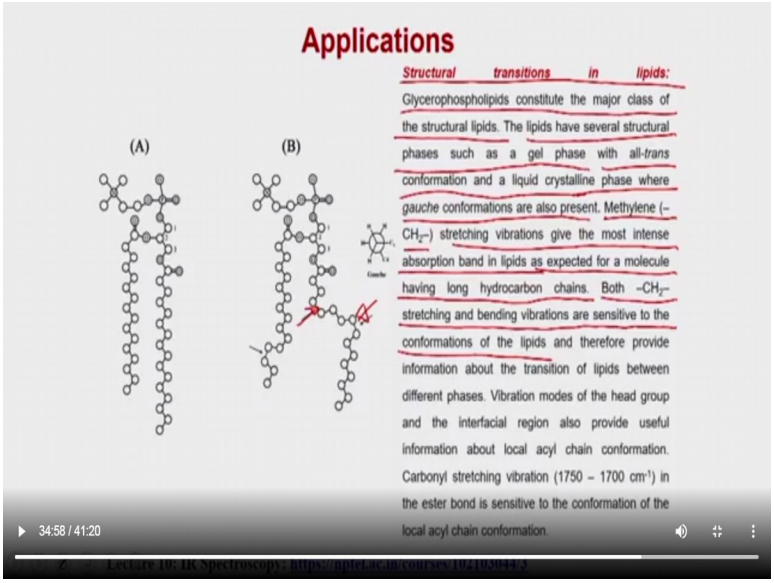

Then you have the structural transitions in lipids. So glycophospholipids are considered are the major class of the structural lipids. The lipids have the several structural phases such as gel phase with the all trans confirmations and unique liquid crystalline phase where the gauche confirmation are also present. So the methylene the CH 2 stretching vibration gives the most intense absorption spectra in lipid as expected for a molecule having long (hydrophobe) hydrocarbon chain.

Both the CH 2 stretching and bending vibrations are sensitive to the conformation of the lipids. So example, if you have any kind of the structural changes in the lipid that actually is going to affect the final IR spectroscopy because it is actually going to change the local environment and because of that you can be able to and study the structural changes in the lipids.

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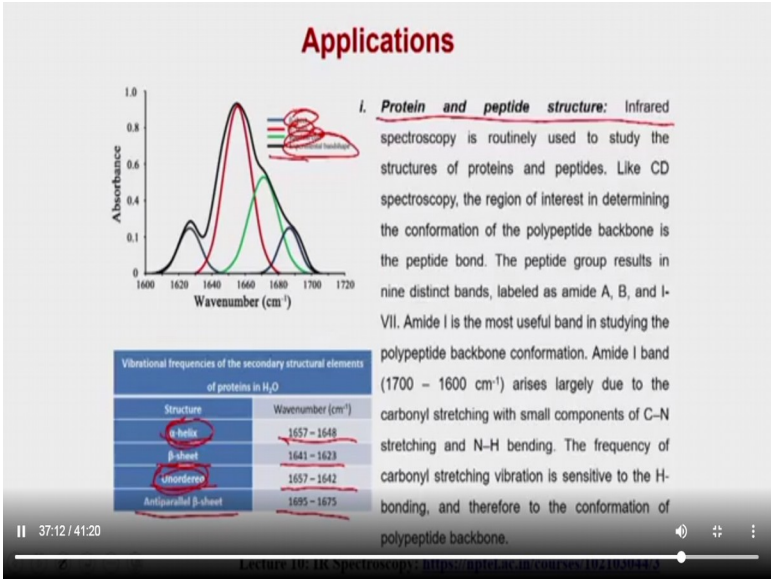

Then you can be able to characterize different types of the secondary structures present in the proteins because all the different stuff secondary structures actually provides they are containing the carbonyl, as well as the amine groups and these carbonyl and amine groups, are been placed in a different positions and as a result they actually are going to give you the different types of IR spectra and you can be able to categorize.

So, for example if you have the alpha helix, it is actually going to absorb in the range of 1657 to 1648. Whereas if you have the beta sheets, it is actually going to absorb in the range of 1641 to 1623 but if you have the loops or the un-ordered proteins the un-ordered regions then it is going to absorb in the range of 1657 to 1642. So you can see that the unordered region or the alpha helix are actually absorbing in the overlapping regions.

And if you have the anti-parallel beta sheets, it is going to absorb in the range of 1695 to 1675. So all this is possible to characterize because simply by looking at the spectra or absorption spectra and you can be able to see that for example the beta sheets or alpha helix or random coils

or the experimental band. So what you are going to do is you can calculate, you can actually collect the IR spectra of your experimental band and then depending on the presence of the different types of the secondary structures.

If you collect the IR spectra of your experimental sample, you can be able to calculate the proportion of alpha helix, proportion of beta sheets and proportion of unordered structures by comparing the different types of spectral bands for IR spectroscopy. The only issue is that the unordered region as well as the alpha helix are very difficult to distinguish in the water.

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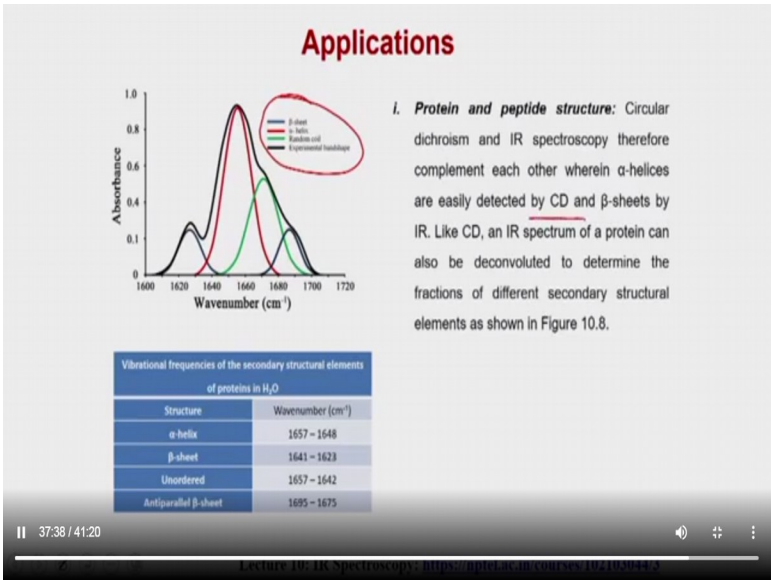

So that is why it is important that you should not simply rely on the IR spectroscopy to calculate the relative proportion or relative abundance of the secondary structure present in your proteins. You can actually verify or you can easily verify simply by doing the CD spectroscopy of the same protein and that actually is going to confirm the IR finding as well.

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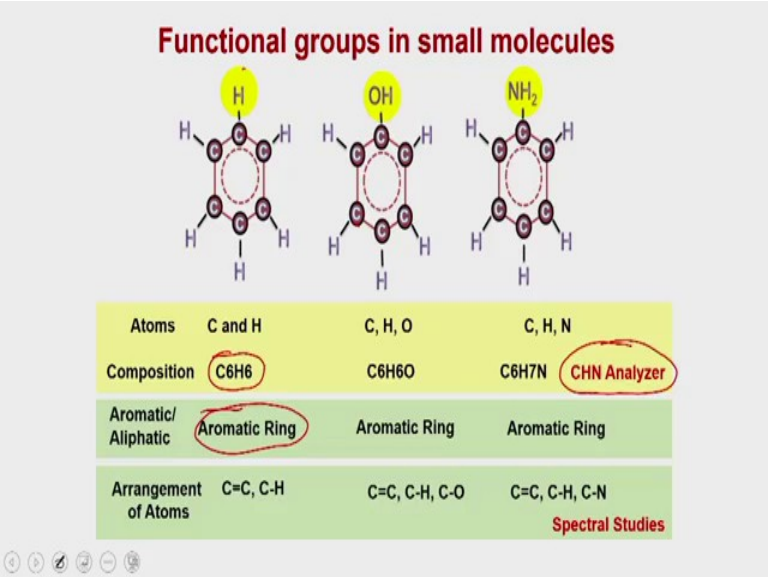

So with this we have concluded our lecture here and what we have discussed so far, we have discussed about how you could be able to characterize the molecule using the different types of techniques. So we have taken an example of benzene phenol and aniline and what we have said is that you have to go through with the systemic explorations and when you do this kind of analysis, you always have to keep in mind that you are actually knowing that what compound you are looking for.

For example, if I am if I know the structure of my final compound like benzene okay, so I know that all the information associated with this particular compound which means I am going to have the standard IR spectra, I am also going to have the standard absorption spectra, I am also going to have CHN analysis, I am also going to have the NMR spectra and so on.

So that is why when you are going to get this product from your host cells, what you are supposed to do is you are simply going to do first the CHN analysis and that actually will say okay the CHN analysis will say that, the compound is C6 X6 so that will say, okay, this is actually could be possibly a benzene then what you are going to do? Then you are going to do the sum of the groups (function) some of the reactions to know whether the ring which is why I am getting is aliphatic or the aromatic and once that will say okay this is a aromatic compound.

So that it will be going to say and then subsequently what you can do? You can do the simple the IR spectroscopy or you can do a UV visible spectroscopy of this compound and you can put the pure benzene as well as a standard compound and then you overlay and if the overlay also matches then it will say okay, it could be a benzene.

Then what you can do is, you can collect the IR spectroscopy and if that also matches with this particular compound then that will actually going to say that okay the compound what you have isolated from the host cells is definitely be a benzene. If you would like to do further confirmations, you can do the NMR spectroscopy, you can collect the 1H NMR or you can collect the carbon NMRs and that actually will definitely will tell you that this is benzene where how the atoms are attached to each other and how what are the functional groups are attached to which carbon and so on.

So by submission of the different techniques whether it is the CHN analyzers or the UV visible spectroscopy or the IR spectroscopy or the other kind of spectroscopy technique such as the NMR or the X-ray crystallography you can be able to characterize the compounds and so with this we would like to conclude our lecture here. Thank you.