Enzyme Science and Technology

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Module - VIII
Enzyme Assay System and Kinetics
Lecture - 36
Enzyme Assay System (Part-II)

Hello everyone, this is Dr Vishal Trivedi from Department of Biosciences and Bioengineering IIT, Guwahati. And what we were discussing? We were discussing about the different properties of the enzyme in the course, Enzyme Science and Technology. And in this context, in the current module, we are discussing about the different ways in which you can be able to measure the enzyme activity.

So, if you recall, in the previous lecture, we have discussed about the different aspects and how and different types of assays, what you can actually be able to set up the to measure the enzyme activity. Now, when you talk about the enzyme activity, you are actually going to talk about the several types of enzyme catalyzed reactions.

This enzyme catalyzed reaction could belong to the catabolic reactions or the anabolic reactions. So, anabolic reactions are the reactions which are going to be used for synthesizing the new molecule, whereas, the catabolic reactions are the reaction which are where a you know the biomolecule is going to be broken down or oxidized to generate the energy.

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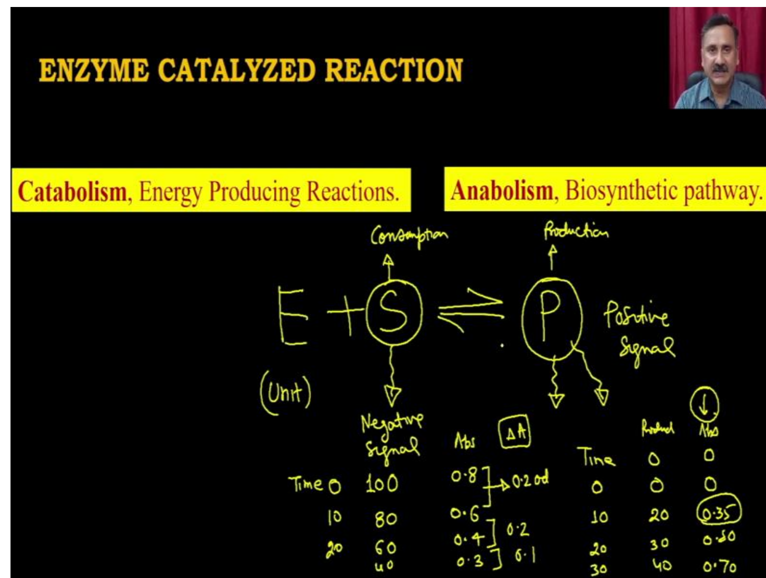

Now, when we talk about the enzyme activity, this means, we are actually going to talk about that the enzyme is actually processing the substrate and in that process, the enzyme is actually going to generate the product. Ok and if you want to measure the enzyme activity and if you recall, in our previous lecture, we have discussed what are different ways in which you can be able to express the enzyme activity.

So, one of the classical way of expressing the enzyme activity is that you are going to express the enzyme activity in the units, right? And every unit is actually going to be defined as the amount of enzyme required to convert a one-micromolar substrate into the product in a given time or one minute. And so, this we can calculate. So, enzyme activity can be calculated if we want to, if we can be able to measure the consumption of substrate or the production of the product, right?

Now, if I want to, if I want to measure the substrate or the product, I could actually be able to use the exclusive property associated with this particular substrate or exclusive properties associated with the product, right? So, in that case so, in many case, we can actually be able to see the disappearance of the substrate.

In some cases, you can be able to see the appearance of the product, right? When you see the appearance of the product, it is actually going to give you the positive signal, ok. So, this is actually going to give you the positive signal, which means you are actually going to see a increase in some value, right?

So, it is actually going to give you a positive signal. Whereas, when you are going to see a disappearance of the substrate, then it is actually going to give you the negative signal, right? What is mean by the negative signal is that your starting molecule. For example, if you started with 100 molecule of substrate, then after so, at time 0, for example, right? At time 0, you have started with 100 molecules of substrate, right? After 10 minutes, it is actually going to be 80, right?

So, suppose 100 molecules of, and suppose we are, you know, measuring the absorbance of this particular substrate, right? And which is actually exclusive, right? So, and does not have any kind of cross reactivity from the product or the enzyme. Then for example, if you have the absorbance of this 100 molecule as 0.8, for example, then 80 molecule will actually going to show you an absorbance of 0.6. After 20 minutes, you are going to have an absorbance of 60, 40, like that, and ultimately it is going to be 0, right?

So, similarly, you can actually be going to have a decrease in absorbance, right? This means, you are actually going to know what is the amount of substrate going to be consumed if you are actually so, you started with 100 molecules. Now, you have 80 molecules, right? So, you can actually be able to take this value and you will say, ok, 0.2 od substrate is being consumed in 10 minutes. And that is the way you can be able to use these values to calculate the enzyme activity.

Similarly, when you are going to talk about the positive signal, you are actually going to say that same way, right? You can have the time, you can actually be able to say at the starting, you are going to have 0 molecules and you are going to have 0 absorbance. So, it is actually going to say absorbance, number of product molecules, right? And like that, ok? So, time 0, you are going to start, there will be no product form, right? Because the enzyme has just started the reactions and then absorbance is also going to be 0, right?

Then after 10 minutes, you are actually going to see 20 molecules, right? This is not exactly what is happening, right? 20 molecules are going to be consumed, right? So, 20 molecules are going to be produced, right? So, 20 molecule are going to show you an od of suppose 0.35, ok. Similarly, you can have 20, 30 like that and it is actually going to show you 30 molecules, 40 molecules like that. So, it is going to show you an absorbance of 0.5 and its going to show you an absorbance of 0.7 or something.

So, this is actually the direct measurement, right? Here you are actually going to plot this. So, 0.35 is the concentration of the substrate product, right? And you can actually be able to say that this is the this is amount of the product is being formed and that is why this is the same amount of substrate is being consumed so, that you can actually be able to use for calculating the enzyme. So, that is why you see when you are measuring the sub-product, it is easy and straightforward.

Whereas, when you are measuring the substrate, you are actually going to go through with the another, you know, function, right? You are have to always calculate the delta absorbance, right? You have to calculate how much absorbance is gone down, right? So, in this case, 0.8 to 0.6, then for example, here to here, again 0.2, from here to here it is going to be 0.1 like that.

So, here you have additional step and when you have the additional step, more steps means, you are actually going to increase the number of chances of incorporating the errors. And that is why it is advisable to go with the positive signal rather than the negative signal or I will say you should not measure the disappearance of the substrate rather than you can actually be able to measure the appearance of the product.

So, depending on the different types of properties of the substrate or the product, the enzyme assays can be done by the utilizing the different types of techniques. What are these techniques?

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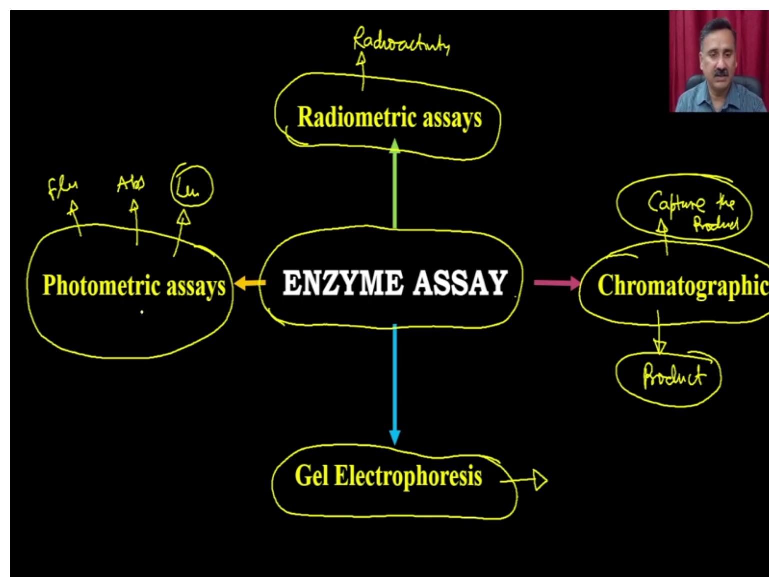

You can actually be able to use the photometric assays. So, you can actually be able to use the photometric assays where you can actually be able to change in absorbance or you can actually be able to change in the fluorescence and you can actually be able to say luminescence or all that. Then you can also have the radiometric assays so, these radiometric assays where you are going to see the change in the radioactivity of the substrate or the product.

And then you can also have the chromatographic techniques which you can also use for measuring the enzyme assays. And here you can actually be able to capture the substrate or the product right and you can actually be able to measure that with the help of either the radiometric assays or the photometric assays. And then you can also have the gel electrophoresis.

So, you can actually be able to you know visualize the product onto the electrophorestic gel and that you can also be able to use for measuring the enzyme activity or enzyme assays. So, enzyme assay is a very, very complicated you know recipe or I will say enzyme assay is a complicated protocol where you are going to use, not only one type of assay you can may have the possibility of using the multiple combinations or different types of techniques.

For example, you can actually be able to use the chromatography techniques to isolate the product right and you can actually be able to use this product and you can actually be able to quantitate this product with the help of either the photometric assays or radiometric assays. Same way you can actually be able to use the electrophoretic assays or electrophoretic system to visualize the substrate and then you can actually be able to use either the photometric assays or radiometric assays to quantitate the product right.

So, let us start first with the photometric assays and we will discuss different aspects of photometric assays, how you can be able to what are the different precautions you should take when you are setting up the photometric assays and when we will discuss about the you know UV visible spectroscopy.

And as well as the fluorescence spectroscopy, how you can be able to exploit these techniques for setting up a photometric assays and photometric assays and how you can be able to utilize that for measuring the enzyme activity.

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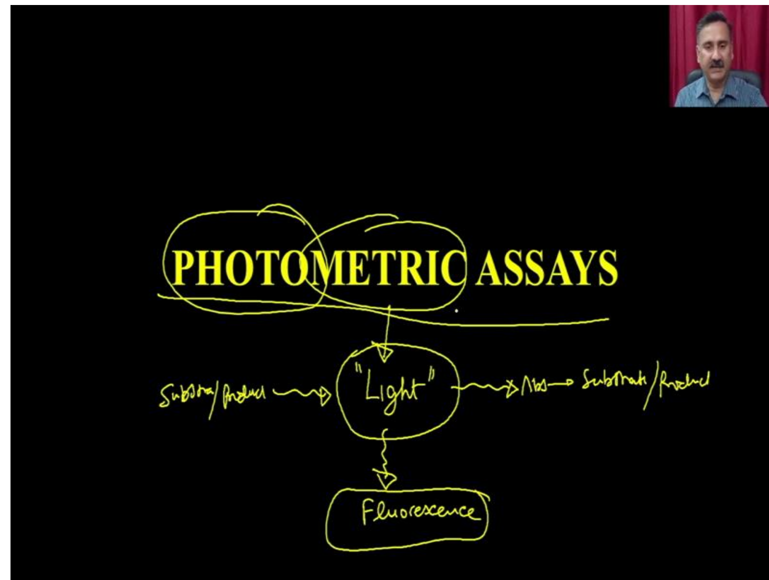

So, photometric assays as the name suggest photometric assays means, you are actually going to absorb or you are actually going to see something with related to light right. Photometric means photo means light, metric means measuring right. So, you are actually going to do the light measurements whether the light is going to be absorbed by the substrate right or product or it is actually going to be that the substrate or the product is actually going to produce the light right.

Sometimes you can actually have to you know we will be able to see that if there is a product which is formed, it is actually you know giving the light right or sometime you can actually have the alternative right where you are going to excite the molecules and then it is actually going to give you the fluorescence right. So, fluorescence is also of phenomena where you are actually going to measure the light intensity only. Ok. So, let us discuss and start discussing about the photometric assays.

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Introduction

- Photometric methods are the most commonly used type of enzyme assay. They are convenient and capable of producing accurate and reproducible results on large numbers of samples in a short period of time.
- The chemical transformations that accompany the enzyme-catalyzed conversion of substrate to product frequently result in useful changes in the optical properties of the system under investigation. 'S' 'P'
- When the reaction catalysed by the enzyme under assay does not produce a useful change in optical properties, the addition of appropriate additional reagents frequently allows the reaction to be photometrically monitored.
- Other photometric methods are less commonly used than enzyme assays based on changes in the light absorbed by the solution as the reaction progresses.
- Changes in fluorescence and turbidity of the solution, on the other hand, provide the foundations for useful optical methods for assaying enzymes.

So, as the name suggests the photometric assays are the most commonly used type of enzyme assays. They are convenient and capable of reducing the accurate and reproducible result on large number of sample in a short period of time. So, photometric assays are the most popular and most important and it is easy to perform and in terms of infrastructure, in terms of instruments, in terms of the reagents, they all are very much you know cheap actually. So, it is economically very viable.

The chemical transformations that accompany the enzyme catalyzed conversion of substrate to product frequently results in a useful change in the optical property of the system under the investigation. So, either there will be a change in the substrate absorbance or there will be a change in the product absorbance. Ok.

So, either you can have a very exclusive product which actually can give you the exclusive you know the light absorption phenomena or you can actually have a substrate which is going to (Refer Time: 12:47) your disappearance. When the reaction is catalysed by the enzyme under assay do not produce a useful change in optical property addition of the appropriate additional reagent frequently allow the reaction to be photometrically a monitor.

So, these are actually going to be couple of examples where neither the substrate nor the product are exclusive, then you can actually be able to add some coloring agent and that coloring agent is are going to either react with substrate or the product and then

ultimately it is actually going to give you the colored change actually and that is you can actually be able to monitor with the help of the photometric systems.

The other photometric methods are less commonly used than enzyme assay based on change in light absorbed by the solution as the reaction processes. Change in the fluorescence and the turbidity of the solution on the other hand provide the fundamentals foundations for useful optical method for assaying the enzyme. So, before we discuss and you know understand the phenomena of the photometric assays and how you can be able to design the assays let us discuss some basics of the photometry and how it actually works.

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ABSORPTION

Radio
Microwave
Infrared
Ultraviolet
X-ray
Gamma
Visible light
"UV-Visible"
UV-region

- Visible light is of wavelength between 400 and 750 nm, compounds that absorb light in this range are colored, many colorless compounds also absorb light in the ultraviolet(UV) range, 200-400 nm.
- Light absorption occurs when electrons in irradiated absorbing molecules are promoted to a higher energy level because the frequency of the electronic oscillation in the molecule coincides with the frequency of the irradiating light.
- The wavelength at this frequency is that at which compound absorbs maximum amount of light and is dependent on the structure of that particular molecule.
- The amount of light absorbed depends upon the probability that the electronic transition occurs.

So, first thing what we have to discuss is we have to discuss about the absorption phenomena. So, what you know is, this is the this is actually the UV will spectra right and what you see is this is the radiation of the different wavelengths right. So, you have started from the radio waves you can actually be able to talk about microwaves then you have infrareds.

And on this side, you have the ultraviolet X-ray and gamma right. So, as you go from this side to this side there will be decrease in wavelength and that is how there would be an increase in intensity. And this is the region which is called as the visible light or this is going to start from the somewhere around 400 nanometer to 700 nanometer right.

So, that is will be the called as the UV visible spectroscopy and next to the 400 nanometer is go up to the 250 nanometer or sometime it is going to be 190 nanometer and that is going to be called as UV region ok. So, UV region also can be used for photometric assays. So, you can actually have the UV visible range of the spectrum and that can be used for the all the photometric assays. So, visible light is of wavelength between the 400 to 750 nanometer compounds that absorb light in this range are colored.

Many colorless compounds also absorb light in the UV range that is from the 200 to 400 nanometers and light absorption occur when the electron in a irradiated absorbing molecules are promoted to a high energy level because the frequency of the electronic oscillation in the molecule coincide with the frequency of the irradiating lights.

The wavelength of this frequency is that it which compound absorb maximum amount of light it depend on the structure of that particular molecule. The amount of light absorb depend on the probability that the electronic transition occurs. And if you recall in our in a couple of module back when we were discussing about the you know the different types of UV visible spectroscopy.

We discussed about that when we were saying that how you can be able to use the spectroscopy for measuring the enzyme-substrate interaction right. That time we discuss about how the absorption is changed even if when you have a slight modification of one group or there is a you know substitution of the few more groups right. So, remember that when we were talking about how the absorption phenomena is changing when the benzene is getting you know benzene is getting substituted at different unit values and so on.

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Absorbance

$A = -\log_{10}(I/I_0)$

I_0 = light transmitted by solution not containing chromophore.

I = light transmitted by solution that does contain chromophore.

A = absorbance.

Since absorbance is derived from ratio, it does not have any units.

Conversion of absorbance to concentration.

Absorbance is directly proportional to concentration(c).

Absorbance is also directly proportional to path length(l).

Combining these two relationships we get,

$A = \varepsilon cl$

(Beer-Lambert law)

ε = molar absorbance coefficient (units = l $mol^{-1}cm^{-1}$)

$c = A/\varepsilon$

(when path length of cuvette is 1cm, which is usually the case)

So, second thing what we have to discuss is about the absorbance. So, absorbance is A is equal to minus log I by I 0 and where I 0 is the light transmitted by the solution not containing the chromophore right. So, I 0 is the light transmitted by the solution not containing the chromophore which means right this is and the I is the light transmitted by the solution that does contain the chromophore ok and A is the absorbance.

So, A is actually a ratio of I by I 0 and because this is a ratio it does not have any unit. Because so, that is very important right many times in even in the different types of exams they will actually going to ask what will be the unit of absorbance. So, it is a unit less property. How you can convert the absorbance to the concentrations right.

So, absorbance is directly proportional to the concentration right. So, if you recall from the Beers Lambert law what Beers Lambert law is says that absorbance is directly proportional to the concentration and absorbance is also directly proportional to the path length.

This means, if you combine these two absorbance is going to be equal to the epsilon cl right and epsilon is called as the molar absorption coefficient and unit is mole per centimeter right. So, if you keep or if you keep the path length constant right if you keep the path length constant then the absorbance then the concentration is going to be the absorbance divided by the epsilon ok.

So, when the path length of the cuvette or path length of the light path is 1 centimeter, it is going to be constant right. So, if it is a constant event, we can actually be able to ignore this right and that is how the amount of as you will increase the concentration, there will be an increase in absorbance. And you can actually be able to calculate the concentration of the molecule with the help of the absorbance divided by epsilon ok.

This means, if you can have the molar absorption coefficient of the molecule you can take the you know defined you can take the molecules put it into the cuvette and then if you measure the values right. If you measure the absorbance and then you can actually be able to just divide that value by the molar absorption coefficient it will actually going to tell you what will be the concentration in terms of moles right and that is the very very you know its powerful tool right to measure right.

There are deviations from the beer Lambert law and that we are not going to discuss here because there are saturations and then there will be a product dimerizations and you know this defragmentations and all that. So, that is actually going to change the this particular relationship.

But that is are called as the deviation from the Beers Lambert law and that you can actually be able to study from any of the classical biochemistry book that or biophysics books like I think the if you go through with the book I Wilson and Goulding right.

So, if you think about this you can actually be able to know this right. So, you can actually be able to study about the deviation from the Beers Lambert law and that is going to be helpful to understand under what conditions the law will not be applicable and then we should be little more careful ok.

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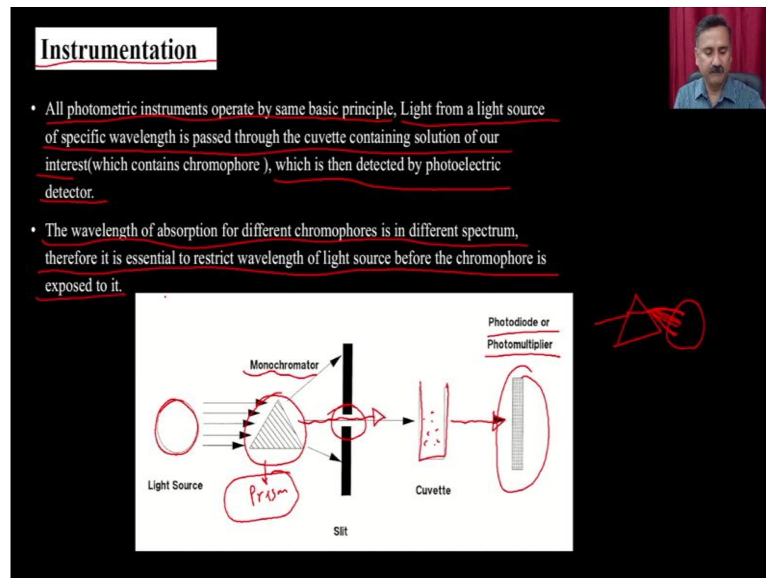

Now, let us talk about the instrumentation part so, instrumentation of the spectrophotometers. So, all photometric instruments operated by the same basic principles like from a light source of a specific wavelength is passed through the cuvette containing solution of our interest right molecule which you for which you want to measure the absorbance which is then detected by the photometric detector right.

So, what you see here is, you are going to have a light source then in front of this light source you are going to have the monochromators. So, monochromator is nothing but a prism right. So, it is going to have a prism and you know that when the light goes through the prism, it actually diffract and that is how it is actually going to show you the different wavelength right.

And remember that when the white light goes into the prism it actually going to split into different seven color right and since it got fragmented into seven color, you can actually be able to rotate this prism and that is how you can be able to select the wavelength of your choice. So, same is here you can actually be able to rotate this and that is how the your wavelength of your choice will enter into this particular slit right.

The slit will be a small gap between and or the hole and from there you can actually be able to allow the entry of this particular array right. And then there will be a place where you can actually be able to keep the cuvette and that is how the cuvette is actually going to have the molecule right for which you want to measure the absorbance and that is how

the unabsorbed light will actually going to pass through the cuvette and it is actually going to be detected by the detector.

And detector could be of many types, it could be photo diodes or photomultipliers. So, wavelength of the absorption for different chromophore is different right and therefore, it is essential to restrict the wavelength of light source before the chromophore is exposed to it.

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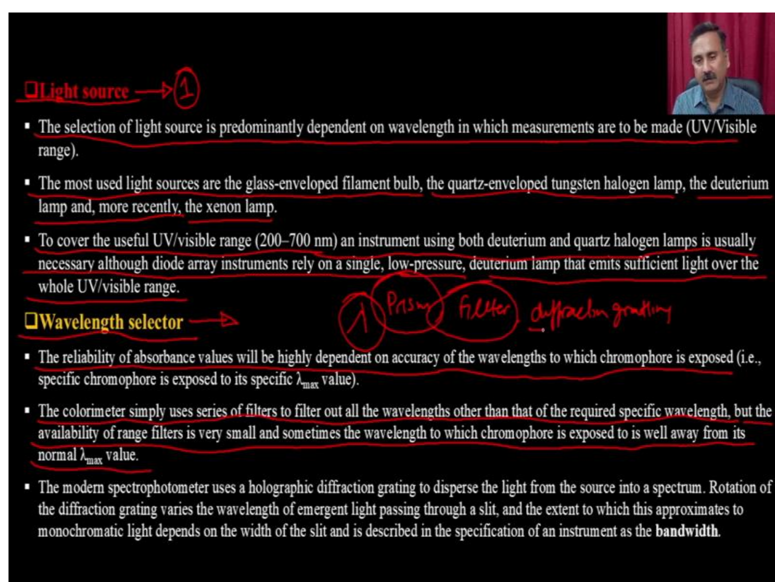

Now, we will discuss about the different components. So, first component is the light source right the selection of the light source is predominantly depend on the wavelength in which the measurement are to be made so, either the UV visible or UV range or the visible range. The most used light source for the glass enveloped filament tube bulb the quartz enveloped tungsten halogen bulb or the deuterium lamp and more recently the xenon lamp.

So, many of these lamps are available you can actually use the tungsten lamp or you can use the halogen lamp, you can use the deuterium lamp and you can also use the xenon lamp. And all they are actually capable of providing the light of the different wavelengths so, that you can actually be able to use according to your choice. And sometime what happen is that in some of these spectrophotometer you may have the two lamps rather than single lamp right.

So, you can have the lamp for the visible range you can have the lamp for the UV range. To cover the useful UV visible range that is from 200 to 700 nanometer an instrument using both deuterium and the quartz halogen lamp is usually necessary although diode array instrument rely on a single use. So, low pressure deuterium lamp that emits sufficient light over the whole UV visible range ok.

So, you have the choice you can actually be able to use the two lamps you can actually be able to use the single lamp. Then we have the wavelength selector. So, this is called as a diffraction ratings right or the prism right. So, the reliability of the absorbance value will highly be depend on the accuracy of the wavelength to which the chromophore is exposed that is the specific wavelength right.

So, selection of the lambda is very important right and that is why the wavelength selector is a very very important component of this spectrophotometer. The calorimetric simply use series of filter to filter out all the wavelength other than that of a required specific wavelength. But the availability of range filter is very small and sometimes the wavelength to which the chromophore is exposed is all is a well away from its normal lambda max values ok.

So, there are many ways in which you can be able to use the wavelength selector, you can have the filters, you can have the diffraction gratings you can have you know the prisms right. So, depending on the price of the instrument you can actually have the different types of things right and that is how you can actually be able to have the precision in terms of selecting the particular wavelength.

And if you are going to have multiple wavelengths, it is actually going to you know it is actually going to affect the final absorbance phenomena. The modern spectrophotometer uses a holographic diffraction grating to disperse the light from the source into a spectrum.

Rotation of the diffraction grating varies the light or wavelength of the element emergent light passing through a slit and to extend to which this approximate to a monochromatic light depend on the wavelength of the slit and it is describes in the specification of the instrument as the bandwidth. So, bandwidth is the deviation from the selected wavelength ok.

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❑ Determination of absorption spectra

- Many photometric enzyme assay problems can be solved more quickly if the absorbance spectrum of the solutions involved can be determined rapidly.
- This can only be accomplished in an instrument that uses physical movement of the diffraction grating to irradiate the sample with monochromatic light.
- Modern instrumentation allows a full spectrum to be determined in less than 3 seconds.
- A diode array instrument captures all of the points on the spectrum at the same time, and spectra can be measured at 1 second intervals.
- One major draw back of this is, since sample is exposed to all possible spectra problems may arise.

❑ Cuvette holder

- Maintaining constant temperature is essential for continuous assays, electrical heating is preferred over circulating water (because of danger of flooding the instrument).
- Many instruments have multiple cuvette holders, allowing multiple continuous assays to be performed at the same time via automatic mechanical movement of the carriage between measurements.
- It is also ideal in having a cuvette holder which accept cells of path length other than 1 cm.

Then you have the determination of the absorption spectra. So, many photometric enzyme assay problems can be solved more quickly if the absorption spectra of a solution involved can be determined rapidly. This can only be accomplished in instrument that uses physical movement of the diffraction grating to irradiate the sample with the monochromatic lights.

This means, you can actually be able to absorb you can actually be able to have the absorption spectra of the molecule right. So, you can actually have like lambda on this side and absorbance on this side and that is how you can actually be able to know where this particular molecule is absorbing and that is how you can actually be able to choose this and this wavelength which where there have the maximum absorbance can is called as the lambda max.

So, if you have a that kind of instrument where the diffraction grating can very precisely be able to move around from one from the different positions and that is how it can actually be able to illuminate the sample with the monochromatic light of different wavelengths, then it can actually be able to give you very precise value for the lambda max and that is how it actually can help you to decide what lambda max I should use for the detection purpose.

Modern instruments allow a full spectrum to be determined in less than 3 seconds. A diode array instruments capture all of the point on the spectra at the same time and

spectra can be measured at 1 second intervals. One major drawback of this is that since sample is exposed to all possible spectrum problems may arise ok. Then we have the cuvette holders. So, maintaining the constant temperature is essential for the continuous assay.

Electrical heating is preferred over circulatory water bath. This means, you can actually have the you know the electrical heating rather than water circulation because water circulation is the fluctuation is going to be more.

Many instruments have multiple cuvette holders allowing the multiple continuous assay to be performed at the same time while the automatic mechanical movement of the carriage between the measurements. It is also ideal in having a cuvette holder which accepts cells of the path length other than the 1 centimeter.

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And the main important thing is the limits and the source of errors. So, when you are doing the enzyme assays you always have to be very very careful about this particular phenomena that you are going to have limits until you are. So, you are actually going to have a range in which you are actually going to be utilized or you can be able to use the assay and then what are the different sources through which you are actually going to get the error.

Because if you know the sources you can actually be able to minimize or you can be able to taken into account so, that you can be able to correct for those errors; one is the nonlinearity arising from the stray light ok. So, the light that originates from the instrument and passes directly to the photo detector without even passing through the cuvette is the source of this error.

Remember that when you are actually having a bulb right and from the bulb you have a filter you have a prism right then you have a slit and then you have a cuvette right. So, if you have the solution, right. So, there are actually going to be. So, it is actually going to enter right and there will be some rays which are actually going to go directly and they will actually going to hit the detector ok.

And these direct light, which goes through the detector are actually going to provide a source of this error. The deviation of the linearity between the concentration and absorbance is predicted by the Beers Lambert law is often due to the instrument rather than the complex behavior of the chromophore.

Despite the best effort this error will always occur and it is accounted by the passing the light to the empty cuvette with the solids, opaque object in the and nothing the absorbance. Ok; that means, how you can be able to correct this what you can do is just add just put this cuvette will just not contain any sample. So, if that happens there will be some amount of light which is actually going to be passed through without this sample is going to absorb and that you can actually be able to measure.

So, imagine that if that light is I s. So, if I s is the amount of light which is actually directly be getting into the detector, then you should subtract that amount into that like you should actually be able to add onto the intensity. So, what you are going to do is, your apparent absorbance would be that minus log I plus I s divided by I 0 plus I s ok and that is actually going to somehow or some way actually going to correct the error what is going to be incorporated from the instrument.

Then we have the instrumental noise. So, this is random fluctuation in the photo detector output that originates within the instrument and it is and this caused this is and it is not caused by the solution ok. Then we have a zero drift. So, in a single beam instrument this is slow steady change in the apparent absorbance. It is caused by the time dependent change in the photo detector and a lamp which causes the true value of I 0 to change.

Whereas, I 0 value used to calculate absorbance is at that measured when the absorbance 0 was set ok. So, this is the problem of single beam instrument right when the single beam instrument what happen is that when you have two different types of spectrophotometer you have the single beam instruments you can have the double beam instruments.

So, if you have a double beam instrument you can continuously be able to monitor what is the intensity coming from the bulb and what is the intensity the detector is detecting. But you have if you have a single beam instrument what will happen is that, initially you put the cuvette and you measure the absorbance having the no chromophore right.

And that value you are always subtracting from the your measured value, but that value which you have measured 10 minutes back may actually change over the course of time because the bulb is actually going to get warm up or there are some other artifacts which are actually going to happen.

So, those things cannot be mapped in a single instrument right single beam instruments and because of that the double beam instruments are more preferred because they are actually going to give be you know more helpful. So, when using a diode arrays spectrophotometer this problem can be avoided by the internal referencing or subtracting the absorbance reading taken at a wavelength when there is a no absorbance change due to the reaction. This problem is also avoided by using a split beam instrument that determine the absorbance from the I and I 0 value measured simultaneously.

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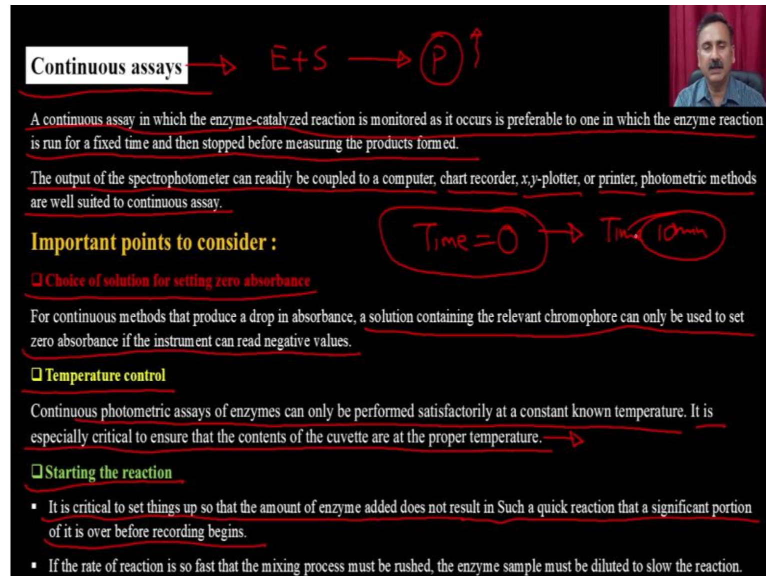

Then in the photometric assays you can have the continuous assay or you can have the discontinuous assay. So, when you are setting up the continuous assay which means you are actually going to have the enzyme you will add the substrate and then you are actually going to have the generation of product and that you can actually be able to measure.

So, either you measure the substrate or the product. The continuous assay in which the enzyme catalyzed reaction is monitored as it occurred is preferred to one in which the enzyme reaction is run for a fixed time and then stopped before measuring the product formed. The output of the spectrophotometer can readily be coupled to a computer chart recorder or x, y plotter or printer and photometric methods are well suited to the continuous assay.

But before you set up the continuous assay you have to consider the following points. The choice of solution for setting up the zero absorbance: So, for continuous that system that reduce a drop in absorbance a solution containing relevant chromophore can also be used to set the zero absorbance in the instrument can read the negative values. Then we have the temperature control.

So, because the spectrophotometer will have the bulbs and other kinds of electronic instruments, electronic components they will actually going to have the increasing temperature. So, continuous photometric essay of enzyme can only be performed

satisfactorily at a constant temperature. It is especially critical to ensure that the content of the cuvette are at a proper temperature.

You know that the temperature is actually going to change the enzyme activity number 1 and the temperature can also actually be able to change the dissociation of that substrate and temperature can actually be able to even make lots of changes right. So, it that is why the a constant temperature in the measuring cuvette is very important and then starting the reactions because time zero is very important.

When you are actually setting up or when you are starting the reaction that is very important. So, it is critical to set the things so, that the amount of enzyme added not result in such a quick reaction that a significant portion of it is over before even recording reading begins. If the rate of reaction is so fast that the mixing process must be rushed, the enzyme sample must be diluted to slow the reaction.

So, time zero is very important because you will going to say after time T after time you know 10 minutes what will be the errors, but if you add like a large quantity of enzyme, if you add large quantity of, if you set up the reaction in such a way that it is going to you know complete before you go to that point or before you even start measuring then it is actually going to give you more errors.

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Now, let us see the examples of enzyme assays by the absorbance change. So, direct observation of reaction using the natural substrate right. So, a limited number of enzyme catalyzed reaction results in a usual change in absorbance.

For example, the oxidation of NADH or the NADPH as well as the opposite reaction caused a significant change in absorbance at 340 nanometers because it has the epsilon 340 as 6200. And this simplifies the direct continuous absorbance assay of a large and important group of enzyme known as dehydrogenases.

So, for dehydrogenases what you can do is, you can just add the this NADH substrate and you can actually be able to monitor the disappearance of the NADH or reappearance of the molecules. So, for example, you can actually be able to measure the lactate dehydrogenase.

So, lactate dehydrogenase is one of the most commonly measured enzyme activity enzyme because its present in the serum after tissue damage aids in the clinical diagnosis. Because the reaction catalyzed is freely reversible the assay can be performed either in the direction of lactate oxidation by NAD plus or in the reverse direction by the pyruvate reduction by the NADH.

Reduction of pyruvate the preferred direction for the assay. For example, this, right. So, pyruvate plus NAD plus NH plus, give you the lactate and NAD plus. So, it is actually consuming the NADH right and you know that the NADH is actually going to absorb at 340 nanometer. So, if you plot this what will happen is that you are actually going to start like for example, you start from one right and over the course of time you will see that it is actually going down.

So, this is actually going to be like time versus product right. So, this is time versus substrate, right this is the NADH what you are measuring. So, if you are actually going to see 10 molecules of NADH which is going to be consumed this means, the 10 molecules of pyruvate are also going to be consumed and that is how there will be a 10 molecule of lactate this is going to be produced.

And that is how you can use these information to calculate the activity of lactate dehydrogenase. The enzyme may be satisfactorily assayed at 30 degree Celsius pH 7.2 in

50 millimolar Tris with substrate concentration of 0.15 millimolar NADH and 1.2 millimolar sodium pyruvate.

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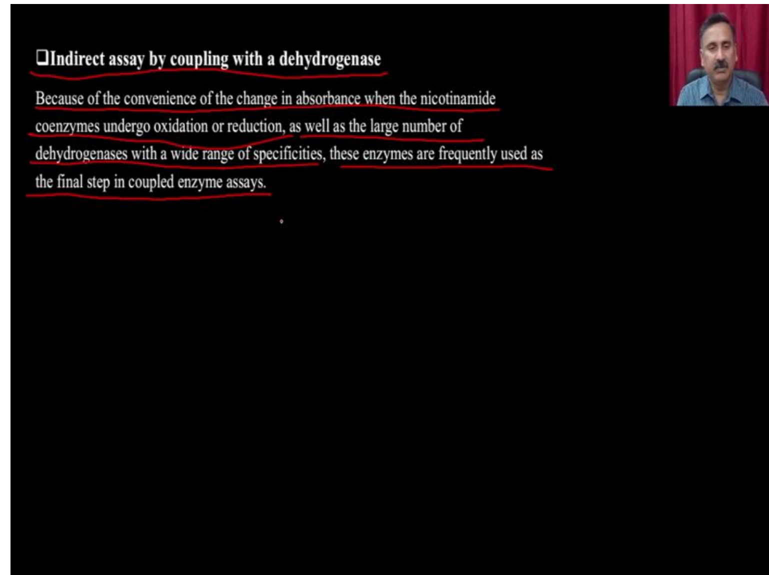

Then we have the indirect assay by coupling with the dehydrogenase. So, because of the convenience of this change in absorbance when the nicotinamide coenzyme undergo oxidation on reduction as well as the large number of dehydrogenase with a wide variety of specificity these enzymes are frequently used as a final step in a coupled enzyme assays. So, you can actually be able to couple these assays with the other enzyme and that is how you can be able to measure the activity.

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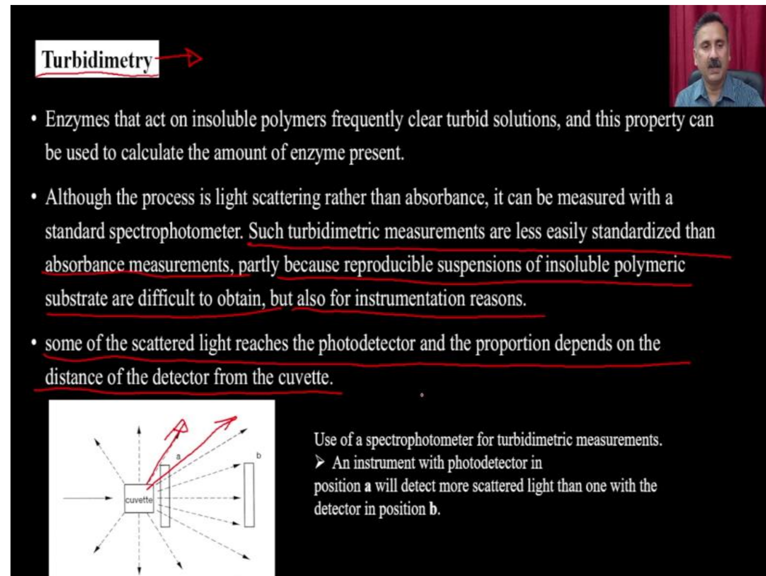

Now, let us talk about the another way of doing the enzyme assay. So, the another enzyme assay way of doing the enzyme assay is turbidimetry. So, enzyme that act on insoluble polymers frequently clear the turbid solution and this property can be used to calculate the amount of enzyme present.

So, turbidimetry means, the scattering of the light ok. So, although the process is light scattering rather than absorbance it can be measured with a standard spectrophotometer. Such turbidimetric measurements are less easily standardized than the absorbance measurements partly because the reproducible suspension of the insoluble polymers substance are difficult to obtain, but are also for instrument reasons ok.

So, these are the places where you are not measuring the absorbance, but you are measuring the scattering ok yes. This means, scattering of the light and although the final result would be that you are going to see a disappearance of the intensity from the detector and that is how you can actually be able to use this, but it has a limitation that it is not going to be very reproducible.

Because it depends on the how good your instrument is and how good your that scattering material is actually be remain in the solution. Some of the scattered light reaches the photo detector and the proportion depends on the distance of the detector from the cuvette.

So, this is what is going to happen when you put the molecule in cuvette and it is having the scattering properties; it is actually going to scatter the light in all the directions right. And some of the light is actually going to enter into the detector which is actually going to affect the final results.

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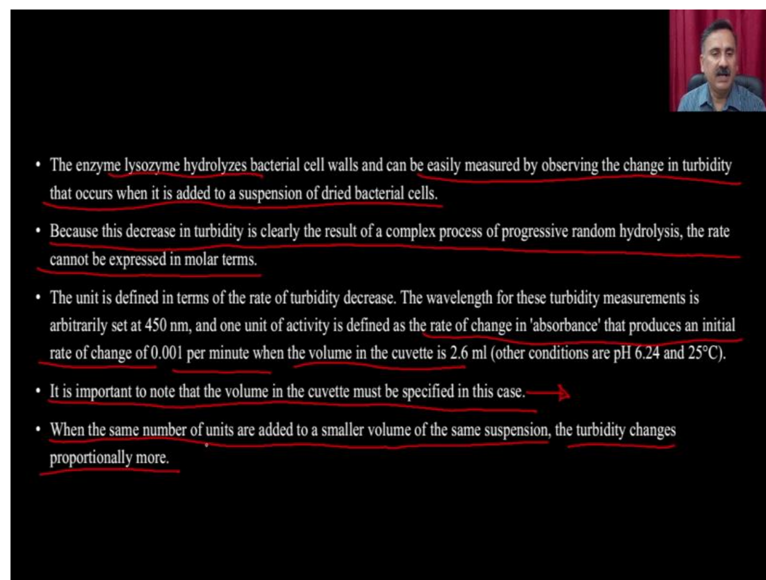

So, the enzyme lysozyme for example, you can be able to you hydrolyzes the bacterial cell wall and can be easily measured by observing the change in turbidimetry that occur when it added to a suspension of dried bacterial cell. Because this decrease in turbidity is clear the result of a complex process of progressively progressive random hydrolysis the rate cannot be expressed in molar terms.

So, the unit is defined in terms of the rate of turbidity decrease. The wavelength for these turbidity measurement is arbitrary set at 450 nanometer and the one unit of activity is defined as the rate of change in absorbance that produced an initial rate change of 0.001 per minute and when the volume in the cuvette is 2.6 ml ok.

It is important to note that the volume in the cuvette must be specified in this particular case because that is actually going to affect the settling process. When the same number of units are added to a smaller volume of this suspension that turbidity change proportionally more.

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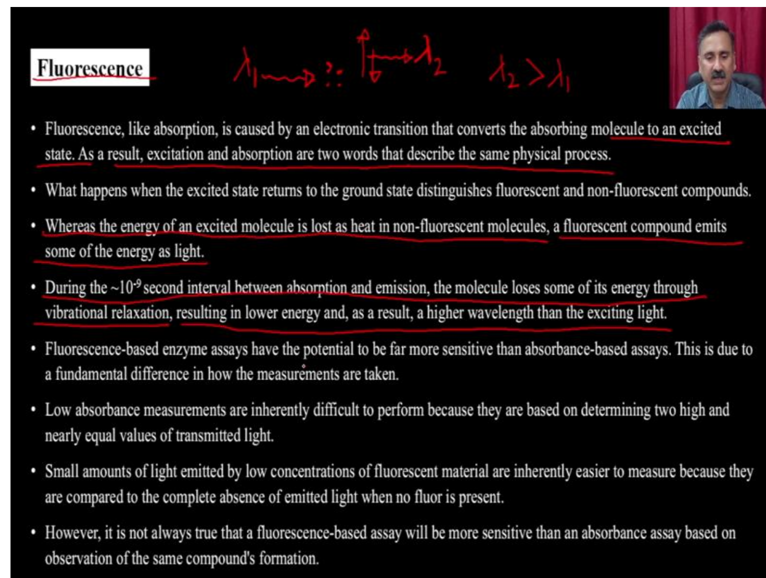

Then we have the third phenomena that is also can be used to measure the substrate or the product concentration is the fluorescence. So, fluorescence like absorption is caused by a electronic condition that converts the absorbing molecule to an exciting state. As a result, the excitation and absorption are two word that describe the same physical process.

What happen when the excitation state return to the ground state distinguishes the fluorescent and non florescent compound right? So, when you have a compound for example, if you have a you know the some compound right and if you illuminate this with high beam of light what will happen is that the electron in this is actually goes into a higher energy state and then they come back to the energy.

And in this process and after that they are actually going to give you a light emitting of light from these. So, this lambda is going to be lambda 1, this is going to be lambda 2 and then lambda 2 is actually going to be bigger than lambda 1 because in this process there will be a some loss of energy and that is how the lambda 2 is going to be bigger than lambda 1.

So, when whereas, the energy of an exciting molecule is lost as heat in a non-fluorescent molecule of fluorescencent compound emits some of the energy as light right. So, during that 10 to the power minus 9 second interval between the absorption and emission the molecule loses some of its energy through vibrational relaxation resulting in a lower

energy and as a result a higher wavelength than the exciting wavelength. Ok. So, fluorescence can be used and if you want to measure the fluorescence you also you are going to have the different set of instrumentations.

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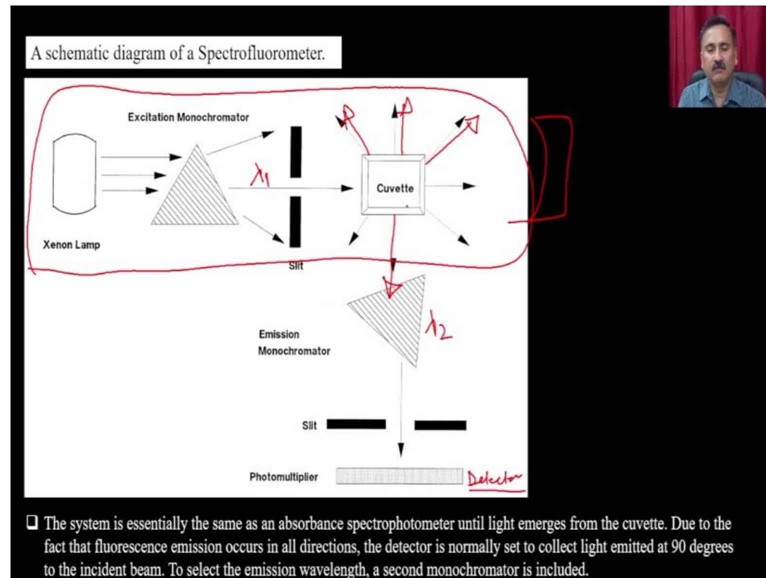

So, this is a schematic diagram of a fluorometer. So, till this it is actually be same as the spectrophotometer. So, you are going to have a light source you are going to have the monochromators and then you are going to have a slit and then you are going to have a cuvette. And this cuvette is actually going to show you the light right because it is going to show you the light in all the direction.

And then since you in the spectrophotometer you have a detector on this side. So, you can actually be able to measure the light what is actually being absorbed with by this molecule and, but in this case what you are going to do is you are going to put another monochromator so, that you can be able to select the wavelength what you are actually going to see and then you are going to have a slit and then you are going to have a detector. Ok.

So, what you are actually basically doing is you are actually detecting the wavelength of the light for which. So, this is actually going to be a lambda 1, this is going to be the lambda 2 and on a specific lambda 2 you are going to see whether that light is coming out from the sample or not.

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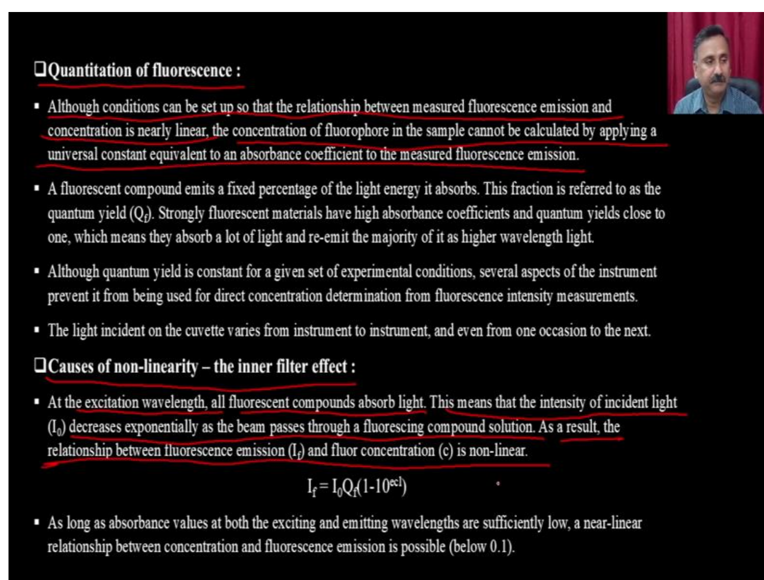

So, the quantitation of the fluorescence: so, although the condition can be set up so, that the relationship between the measured fluorescent emission and the concentration is linear, concentration is linear. The concentration of flurophore in the sample cannot be calculated by applying a universal constant equivalent to absorbance coefficient to the measured fluorescence emission. Ok.

So, fluorescence is not like absorbance very straightforward that you can be able to just put the you know the molar absorption coefficient and then you can actually be able to calculate the concentration of the compound. So, a fluorescent compound emit a fixed percentage of the light it absorbs, the that fraction is referred to as the quantum yield.

And strong fluorescent material have the high absorption coefficient and in a quantum yield close to 1 which means they absorb a lot of light and re-emit majority of it as a higher wavelength. Whereas, in other cases the quantum yield is going to be low and that is how it is actually going to emit only a fraction of that absorb light.

Although quantum yield is constant for a given set of experimental conditions several aspect of the instrument prevent it from being used for direct concentration measurement from fluorescent intensity measurements. The causes of nonlinearity or the inner filter effect is that at the exciting wavelength all the fluorescent compound absorb light. This means the light density of incident light decreases exponentially as the beam passes through a fluorescent compound solution.

As a result, the relationship between the fluorescence emission and the fluorophore concentration is non-linear this means the I is equal to I o Q f 1 minus 10 to power ecl. As long as the absorbance value at both the exciting and emitting wavelength are sufficiently low a non-linear relationship between concentration and fluorescence emission is possible. This means, if you want to use the fluorescence as a assay to measure the substrate or the product you always have to use the diluted samples.

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Examples of fluorimetric enzyme assays

- Direct observation of the natural reaction
  - NAD(P)H-dependent systems: glucose-6-phosphate dehydrogenase :
    - Glucose-6-phosphate dehydrogenase is assayed by adding 50 μl of enzyme sample to 3 ml of 0.1 M Tris–HCl, pH 7.8, containing 3 mM MgCl2, 10 mM glucose-6-phosphate and 7 mM NAD^+ (λ_{ex} = 340 nm, λ_{em} = 465 nm).
  - Porphobilinogen deaminase :
    - Uroporphyrin I is a highly fluorescent compound formed by the oxidation of uroporphyrinogen I, an intermediate in the pathway that leads to haem synthesis. Its fluorometric determination serves as the foundation for an assay for porphobilinogen deaminase and other enzymes in the same pathway.
  - Anthranilate synthase :
    - Conditions for assay are 20 mM L-glutamine, 10 mM $MgCl_2$, 0.1 mM chorismate, 25 mM mercaptoethanol in 50 mM potassium phosphate, pH 7.4. The reaction is followed at excitation wavelength 325 nm and emission wavelength 400 nm. Anthranilate is used as standard.

Chorismate + glutamine ⇌ anthranilate + pyruvate

(anthranilate synthase)

Examples of the fluorimetric enzyme assay. So, you can have the direct observation of the natural reactions. For example, the NADPH dependent glucose 6 phosphate dehydrogenase. So, remember that we have used the photometric assays also to measure the dehydrogenase assays same way you can actually be able to use the fluorimetric assays.

So, you can actually take the glucose 6 phosphate dehydrogenase by adding the 50 microliter enzyme sample to a 3 ml of 0.1 molar Tris pH 7.8 containing 3 millimolar in magnesium chloride glucose 6 phosphate and 7 millimolar of NAD plus. And for NAD plus the lambda excitation would be the 340 nanometer and lambda emission would be 465 nanometers.

So, this means you are going to excite it plus you will keep the sample into the cuvette then you will excite it at 340 nanometer, right. So, in the monochromator 1 you are going to set as 340 nanometer and in the monochromator 2 you are going to put as the 465.

Then we have the porpho bilinogen deaminase. So, this is also another enzyme through which you can also be able to use to measure the enzyme activity. Then we have the anthranilate synthase.

So, condition for the assay is given here and anthranilate synthase is catalyzing this reaction where the chorismate plus glutamine is getting converted into anthranilate (Refer Time: 48:40) and the pyruvate and you can be able to measure this activity with the help of the fluorescence.

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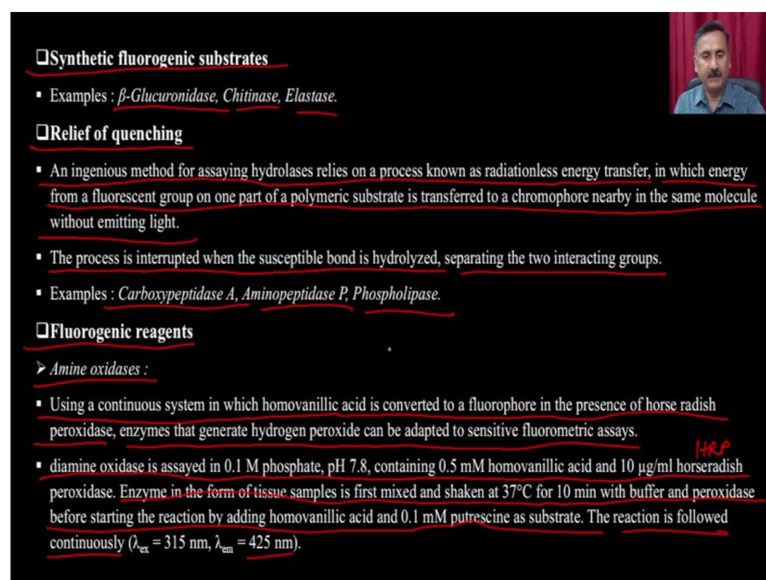

Then sometime we also use the synthetic fluorogenic substrate like the beta glucuronidase chitinase and elastase. So, in many of the protease also are going to be used where you are using the fluorogenic protease substrate and this when you are actually having the reactions if this fluorescence is actually going to be released this fluorescent you know probe is actually going to be released and that is how it is actually going to give you the increase in fluorescence.

Relief of the quenching: So, an indigenous method for assaying the hydrolases rely on a process known as the ratio less energy transfer in which from a fluorescent group on one part of polymeric substrate is transferred to a chromophore nearby in the same molecule without emitting the light. The process is interrupted when the susceptible bond is hydrolyzed separating the two interacting groups examples are carboxy peptidase, amino peptidase and phospholipase.

Then we can also have the fluorogenic reagents like the amino oxidases. So, using a continuous system in which the homovanillic acid is converted into a fluorophore in the presence of HRP in the enzyme that generate the hydrogen peroxide can be adapted to a sensitive fluorometric assays.

So, diamine oxidase is assayed in a phosphate buffer containing homovanillic acid and 10 microgram per ml HRP. So, this is HRP right a horseradish peroxidase. Enzyme in the form of tissue sample is first mixed and shaken at 300 degrees, 37 degrees Celsius for 10 minutes with buffer and the peroxidase before starting the reaction by adding the homovanillic acid and 0.1 molar putrescine as substrate.

The reaction is followed continuously at the lambda excitation of 315 nanometer and lambda emission as 425 nanometer. So, there are many ways in which you can be able to adopt the photometric assays and that is why you can be able to measure the enzyme assays or enzyme activity. But as I said you know you should be very very careful when you are doing these kind of measurements because it is a kind of a black box.

What you are getting the absorbance it has to be validated very carefully by running the control reactions and other kinds of things. So, this is all about what we have discussed about the photometric assays and in the photometric assays we have discussed about the UV visible spectroscopy, we discussed about the fluorescence spectroscopy and we also discussed about the turbo (Refer Time: 51:25) method.

And all of these approaches are you know are can be a potential way in which you can be able to use to calculate the concentration of the substrate or the product and that is why you can be able to determine the activity of the enzymes. So, with this I would like to conclude my lecture here.

In our subsequent lecture we are going to discuss more about the radiometric assays and then we will discuss more about how you can be able to measure the activity in the gel itself and so on. So, with this I would like to conclude my lecture here.

Thank you.