Enzyme Science and Technology
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Module - VIII
Enzyme Assay System and Kinetics
Lecture - 37
Enzyme Assay System (Part-III)

Hello everyone, this is Dr. Vishal Trivedi from Department of Biosciences and Bioengineering IIT, Guwahati. And in the course Enzymes Science and Technology, we are discussing about the different aspects of the enzyme. And in the current module, we are discussing about the different types of Assays to measure the Enzyme activity.

So, in this context, if you remember that we have said that the enzyme is playing a crucial role in running the anabolic reaction and as well as the catabolic reaction. And both of these reactions are important for the survival of the host. And if you want to monitor the anabolic reaction or the catabolic reaction, you have to develop a suitable assay system so that you can be able to measure the activity of an enzyme.

In this context, we have discussed so far what we have discussed? We have discussed about the photometric assays. And in the photometric assays, we have discussed about how you can be able to measure the absorbance of the system and how you can be able to use that information to calculate the concentration of the substrate or the concentration of the product utilizing the Bears Lambert law.

And then we can also be able to use the photometric assays where you can actually be able to use the fluorescent substrate or the fluorescent product. And that is how you can be able to use the photometric assays. In today's lecture, we are going to discuss about the chromatographic, we are going to discuss about the radiometric assays and as well as the assays how you can be able to perform the assay of an enzyme in the using the gel electrophoresis. So, let us start discussing about the radiometric assays.

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So, as the name suggest, the radiometric assays mean the assays where you are actually going to use the radioactive or radioactivity as the monitoring substance, right. Just like and we will discuss about the photometric assays, we were discussing about the absorption and excitation and all those kind of phenomena. In the radiometric assays, you are actually going to measure the radioactive counts.

And remember that the radioactive assays are going to be done under the strict guidance or the strict rules and regulations. So, that you should not to have a radioactive wastage and as well as you should not have the radioactive spillage. And I have already discussed all these aspects, what are the different types of precaution you should take when you are doing to do the radiometric assays in my another MOOC course called Experimental Biotechnology.

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So, in that case, we have discussed about the different types of precautions and other kinds of you know the things what you should actually able to (Refer Time: 03:43) So, for example, when you are doing the radiometric assays, you always have to have the radiometric you know radioactive batches right.

And you should also use the lead walls and other kinds of sheets and so that you should actually be able to protect yourself while you are doing radioactive assays. So, as its name suggest, radiometric assays means you are actually going to use the radioactivity.

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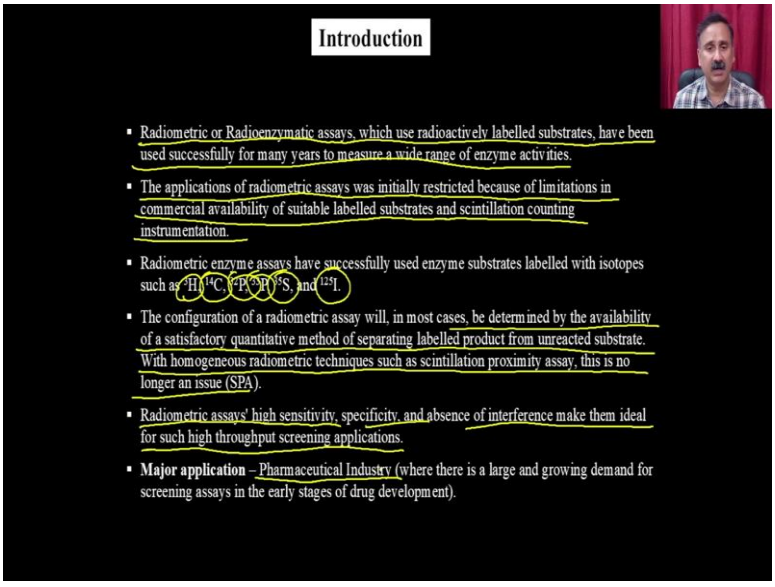

So, the radiometric or the radioactive enzyme assays which use the radioactively labelled substrates have been used successfully for many year to measure a wide variety of enzyme activities. The application of radiometric assays were initially restricted because of the limitation in the commercial availability of suitable labelled substrate and the scintillation counting instruments.

Radiometric enzyme assay have successfully been used for enzyme substrate labelling with its isotope like Tritium 14 C, 32 P, 33 P 35 S and I 125. So, these are the radio labelled nuclei because they are actually going to give you the radioactivity and you can be able to make the substrate along with the either the Tritium labelled hydrogen.

Tritium labelled 14 C carbon, phosphorus and so on, ok. So, depending on what kind of atoms are present in your substrate, you can be able to use either of these. The configuration of radioactive assay will in most cases be determined by a satisfactory quantity method of separating the labelled product from the unreacted substrate with homogeneous radiometric techniques such as scintillation proximity, this is not a longer issue actually.

So, in one of the major chunk or major task of performing radiometric assay is that you are actually going to have the separation of unreacted substrate from the labelled substrate or the product. But with the help of the scintillation proximity assays system which we are actually going to discuss in this particular lecture, there is no need to verify actually. Radiometric assay has highly sensitive, specific and absence of interference make them ideal for such high throughput screening applications.

Pharma in the pharmaceutical industry where there is a large and growing demand for the screening assay in the early stage of development. So, radiometric assays are very popular in the pharmaceutical industry where you would like to use these kind of assays in the high throughput screening to screen the different types of inhibitors or drug like molecules.

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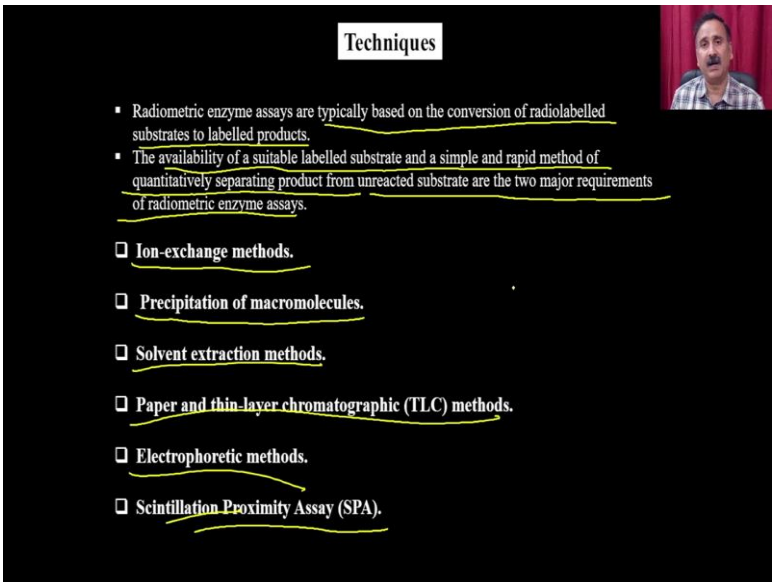

When you are going to do the radiometric assays, you are actually going to use the different types of associated techniques. So, radiometric assays are typically based on the conversion of radiolabelled substrate to the labelled product. The availability of suitable labelling substrate and a simple and rapid method of quantitatively separating the product from the unreacted substrate are the two major requirement of radiometric enzyme assays.

So, when you want to perform the radiometric assays you are actually going to use all these assays or all these techniques. You are going to use the ion exchange technique, you are going to use the precipitation, solvent extractions, paper and thin layer chromatography, electrophoretic method and as well as the scintillation proximity assays to purify the labelled product from the unreacted substrate.

Because unreacted substrate is also radioactive your labelled product is also reactive, right. So, that is why you are supposed to purify the labelled product.

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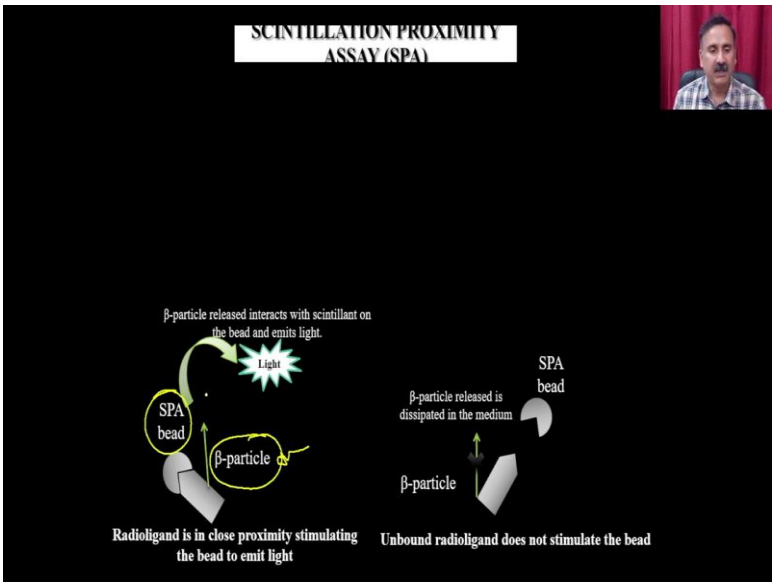

Scintillation proximity assay, the scintillation proximity assay is a assay where you are actually going to where you have the beads and these beads or the scintillation proximity beads. And these beads when they are you know receiving the beta particles from the radioactive material, they are actually going to give you the light, ok.

In a ideal setting when you want to do the radioactive measurements what you are going to do is you are actually going to take the radioactive active nuclei and put it into the scintillation cocktail and then you are actually going to put the vial into the beta counters or the gamma counters depending on the type of radioactive nuclei what you have used. But in the case of scintillation proximity assay you are actually going to have the spa beads or scintillation proximity beads and these scintillation proximity beads.

When they are actually going to receive the beta particles from the radioactive nuclei, they are actually going to give you the light. And they this excitation of scintillation proximity assay scintillation proximity beads depends on the distance of these beta particles. So, it is actually going to give you a very specific signal.

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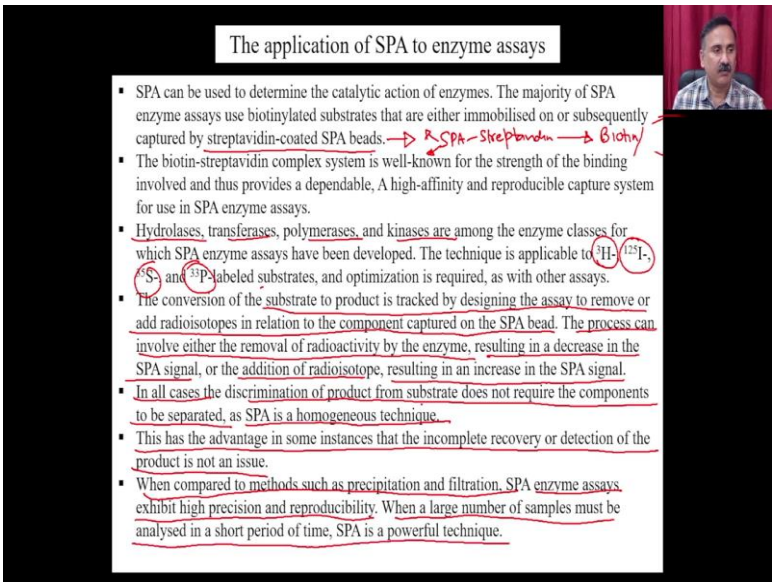

What is the application of the scintillation proximity assays to enzyme assays? So, SPA can be used to determine the catalytic action of the enzyme. The majority of the SPA enzyme assay uses the biotinylated substrate that can either be immobilized or subsequently captured by the streptavidin coated SPA beads, right. So, you can have the SPA beads which is actually going to be coupled with streptavidin and you know that the streptavidin has a very strong affinity for biotin.

So, if you have the biotin on your substrate as soon as the substrate is going to be captured by the SPA beads the SPA beads are actually going to start emitting the lights and that is why you can be able to quantitate that light and you can be able to calculate the concentration of the enzyme.

You can actually be able to use the SPA based enzyme assays to measure the activity of hydrolysis, transferases, polymerases, kinases and the technique is applicable to the tritium labelled, iodine 125, sulphur 35 and the phosphor 33 labelled substrate and optimization is required as with the other enzyme assays.

The conversion of the substrate to the product is tackled by designing the assays to remove or add the radioactivity in relation to the component captured on the SPA beads. The process can either involve the removal of radioactivity by the enzyme resulting in a decrease in the SPA signal or the addition of radio isotope resulting in the increase in the SPA signal.

So, you can actually be just like as we actually discussed right, either you can be able to measure the substrate concentration and you can actually be able to monitor the decrease in substrate concentration or you can be able to see the increase in product and then you can actually be able to see the. So, that is why the SPA beads can be is a very convenient way of measuring the either the initial concentration of the substrate.

And then you will going to see the decrease in in initial concentration of the substrate or you are going to see the increase in the concentration of the product. In all cases the discrimination of the products on the substrate does not require the component to be separated as SPA is a homogeneous technique.

This has the advantage in some instance that incomplete recovery or detection of the product is not an issue. When compared to the methods such as precipitation and filtration, SPA enzyme assay exhibit high precision and reproducibility. When a large number of sample must be analyzed in a short period of time SPA is a very very powerful technique.

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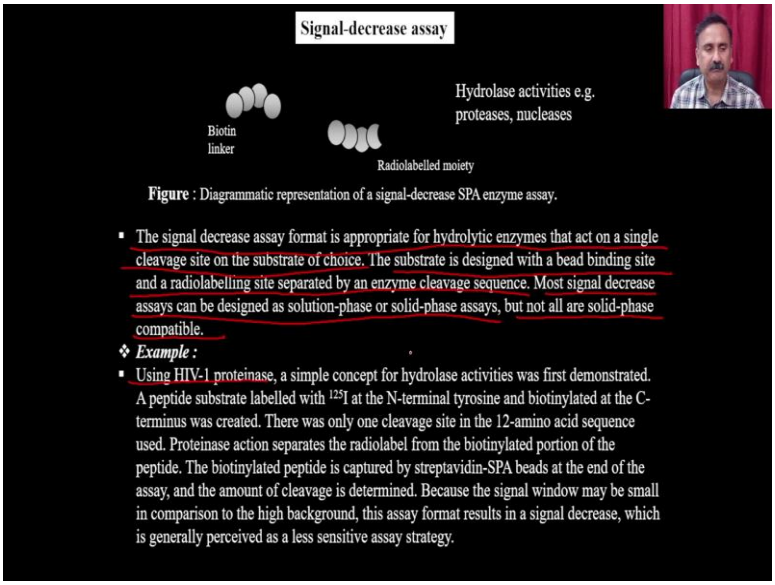

Then we so, this is what it is showing is that how you can be able to use. So, either you are going to have the increase in the signal or you are going to have the decrease in signal. So, the signal decrease assay format is appropriate for the hydrolytic enzyme that acts on a signal cleavage site on the substrate of the choice. The substrate is designed

with a bead binding site and a radio labelling site separated by a enzyme cleavage sequence.

Most signal decrease assay can be designated as a solution phase or solid phase assay, but not all are solid phase compatible. For example, you can be able to use the SPA based proteinase assay to measure the HIV-1 proteinase, ok.

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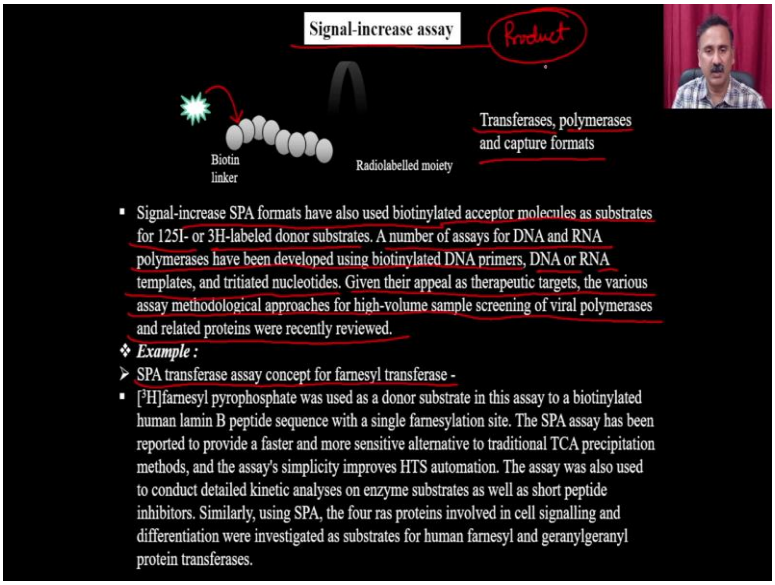

Then we can also have the signal increase assay. So, in a signal increase assay you are going to first, you know, this this is good for the transferases, polymerases and the capture format, ok. So, signal increase SPA format has been used biotinylated acceptor molecule as a substrate for iodine related or tritium labelled donor substrate.

The number of assays for the DNA and RNA polymerases have been developed using the biotinylated DNA primers, DNA or RNA templates and the tritiated nucleotide. Given their appeal as a therapeutic targets the various assay methodology have been approached approaches for high volume screening of virus polymerases and related protein were recovered.

For example, you can actually be able to use the SPA based transferase assays for the farnesyl transferase. So, this is the assay what I am going to show. And what you are going to do is you are going to take the substrate and then you are going to have the increases which means you are actually going to measure the product.

In the signal decrease assay you are going to measure the substrate. So, substrate you are as the substrate is getting cleaved there would be a decrease in signal. Whereas in this case there will be a transfer of the radioactivity onto the biotinylated substrate and that is how you are actually going to have the increase in product.

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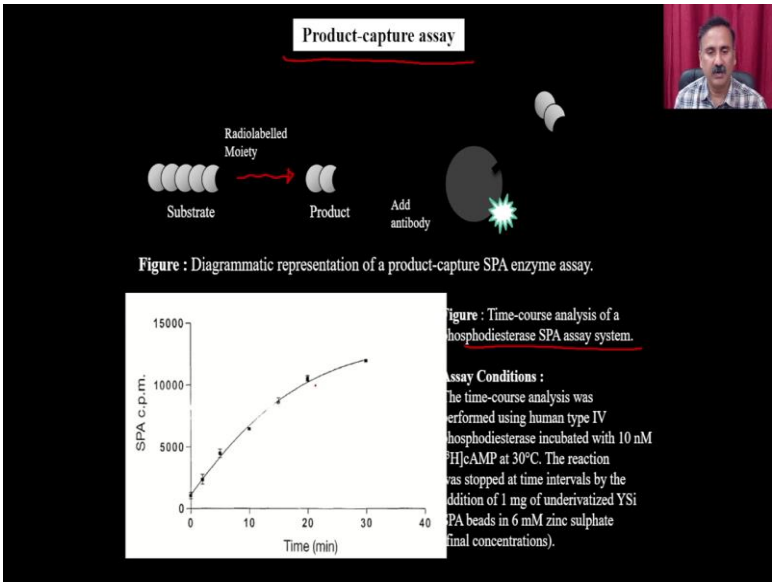

Then we have the product capture assays. So, in the product capture assays what you are going to do is you are going to do a time course analysis of the phosphodiester SPA based assay. So, the time course analysis was performed using the human type 4 phosphodiesterase incubated with 10 nanomolar cyclic AMP at 30 degree Celsius.

The reaction was stopped at time intervals by the addition of a 1 milligram of underivatized YSi spa beads in 6 millimolar zinc sulphate. So, you can actually be able to use the capture product capture assays where you are actually going to have the substrate which is actually getting converted into the product and then you can actually be able to add the antibodies and you can actually be able to add the SPA beads and this is how you are actually going to see a signal.

And when you add the SPA beads it is actually going to terminate the signal and that is how you are actually going to see a signal, right. And using this signal you can be able to calculate the kinetics of the enzyme assays you can be able to calculate the concentration of the products and you can be able to calculate the other kinetic parameters as well.

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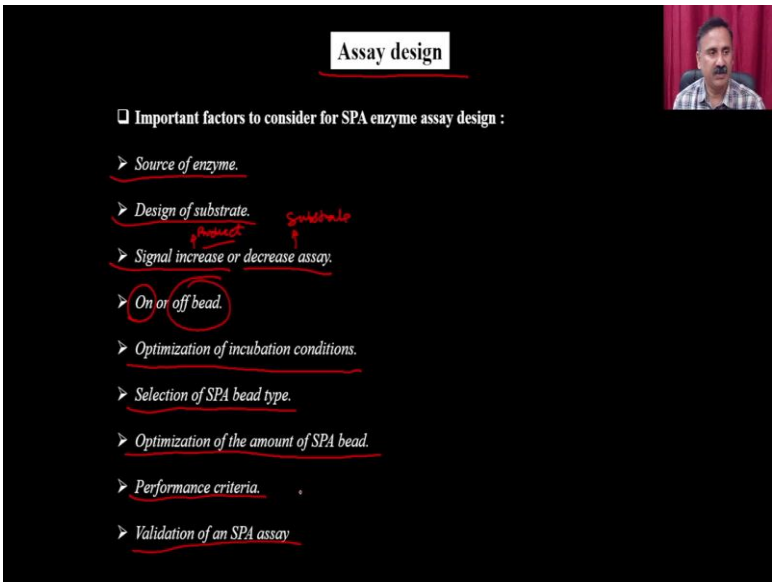

Then we have the assay design. So, important there are important factors what you are going to consider when you are going to design the SPA based radiometric assays. First is source of the enzyme, the second is design of the substrate, then you are going to decide whether you want to use the signal increase or the decrease assay; which means whether you are going to signal increase means you are actually going to say whether the it is easy to measure the product or signal decrease means it is whether it is easy to measure the substrate.

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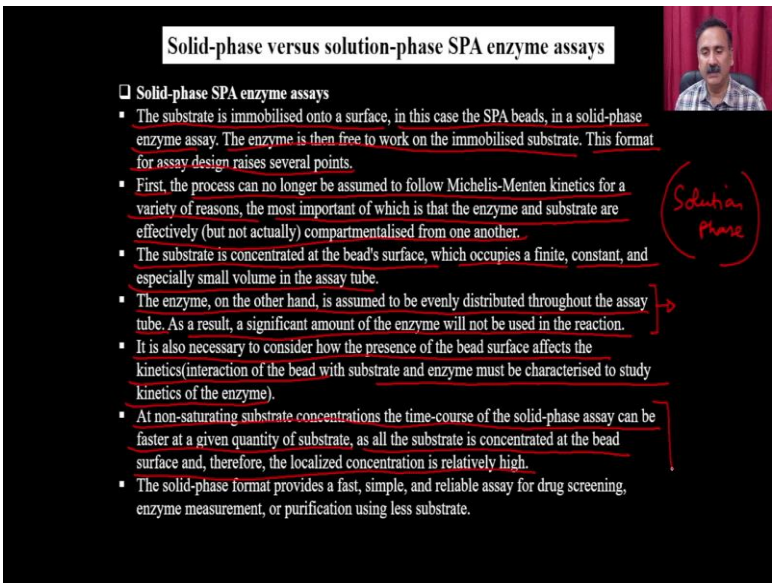

Then you whether you want to make the on bead or the off bead which means whether you want to capture the on signal or the off signal. Then you also going to have the optimization of the incubation concentrations, then you are actually going to have the selection of the different types of SPA beads and then you can also have the optimization of the amount of SPA beads what you are going to use for every reactions.

And then you also going to have the performance criteria and the validation of the SPA assays. Then we have the solid phase versus solution phase SPA enzyme assays. So, far what we were discussing we were discussing about the solution phase SPA assays where you are actually adding the SPA beads and that is how you are actually going to collect the cap signal from the SPA beads and you can convert that into the signal.

And that is how you are going to convert that into the concentration of the substrate or the product. But we can also use the solid-state SPA enzyme assays. So, the substrate is immobilised onto a surface in this case the SPA beads is a in a solid phase enzyme assays.

The enzyme is then free to work on the immobilized substrate this format for assay design raises several points. First the process can no longer be assumed to follow the Michelis-Menten kinetics for a variety of reason. The most important of which that the enzyme and substrates are effectively compartmentalised from one another, ok. So, there are many issues when you are going to do the solid phase SPA assays because it is not giving the free movement of the substrate and that is how they will not be able to follow the Michelis-Menten kinetics.

The substrate is concentrated at the bead surface which is occupied a finite constant and especially a small volume in a assay tube. The enzyme on the other hand is assumed to be evenly distributed throughout the assay tube. As a result, a significant amount of enzyme will not be able to used in the reactions, right.

This means it is actually not going to give you the real picture of how you are going to use the you know the enzyme assays or damaging the enzyme activity. It is also necessary to consider how the presence of the bead surface affects the kinetics interaction of the beads with the substrate and the enzyme must be characterised to study the kinetics of the enzyme.

At non-saturating enzyme substrate concentration, the time-course of the solid-phase assay. Can be faster at a given quantity of substrate, as all the substrate is concentrated at the bead surface and, therefore the localized concentration is relatively high. So, in this case one of the major drawback of the solid phase is that the substrate is not evenly distributed. It is very concentrated.

So, the initial rate of reactions are going to be very fast because the substrates exchange is very high. But the later on it is actually going to be very low because the substrate is the concentration is going to be very low because it is already being consumed, right. So, you are actually not going to get the equilibrium state actually.

The solid-state format provides a fast, simple and reliable assays for the drug screening, enzyme measurements or the purification using the less substrate. So, one of the major advantage of the solid phase is that you are not going to lose the assay, you are not going to lose the substrate.

So, you can be able to reuse those beads again and again because they are immobilized onto the surface. So, you can be able to even measure the activity, we can be able to reuse actually the same thing again and again.

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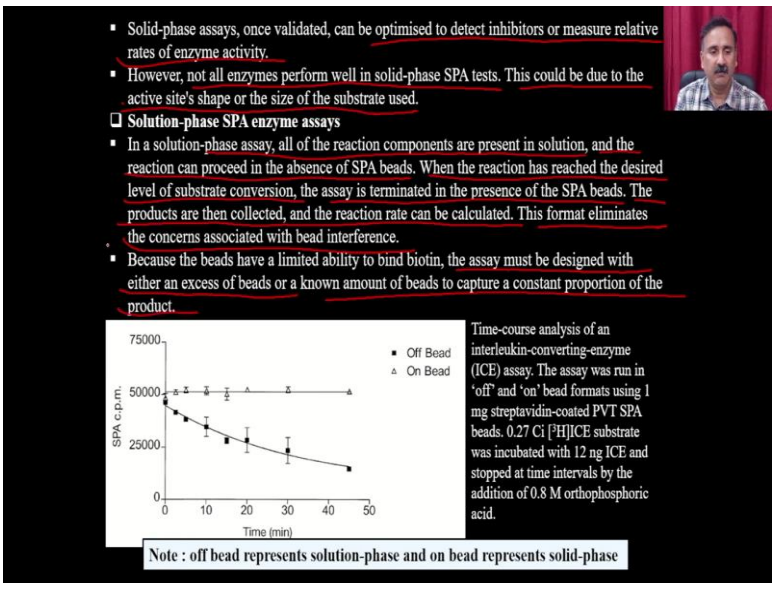

Then the solid phase assay once validated can be optimized to detect inhibitor or more relative rates of the enzyme assays. However, not all enzyme perform well in solid phase

spa test. This could be done due to this this could be due to the active sites shape or the size of the substrate used.

So, this is very important because not all the enzymes are going to accept the substrate which is captured onto a particular solid support. Because it is possible that the region what you have used for you know linking to the solid support may be very crucial for the enzyme to recognize the substrate.

May not be for catalysis, but for reorganisation of the substrate or binding of the substrate. And that is why the some of the enzymes are relaxed, some of the enzymes are flexible. So, they will be able to use, but not for all. Solid-phase SPA assays: in a solid phase assay all of the reaction component are present in solution, right.

And the reaction can proceed in the absence of SPA bead. When the reaction has reached the desired level of substrate conversion the assay is terminated in the presence of SPA bead. The product are then collected and the reaction rate can be calculated. This format eliminates the concern associated with the bead interference.

Because the beads have a limited ability to bind the biotin, the assay must be designed with either an excess of bead or the known amount of bead to capture constant proportion of the product. So, this is a very very important point that you should have the enough amount of bead. So, that you should be able to capture all the substrate or the product so that you should be able to give the real representation of the concentration of the substrate at that particular moment. If there will be a shortage of the beads.

Then the substrate actual substrate concentration is going to be very high. But the relative, but the observation what you are going to get from the reactions are going to be on the lower side.

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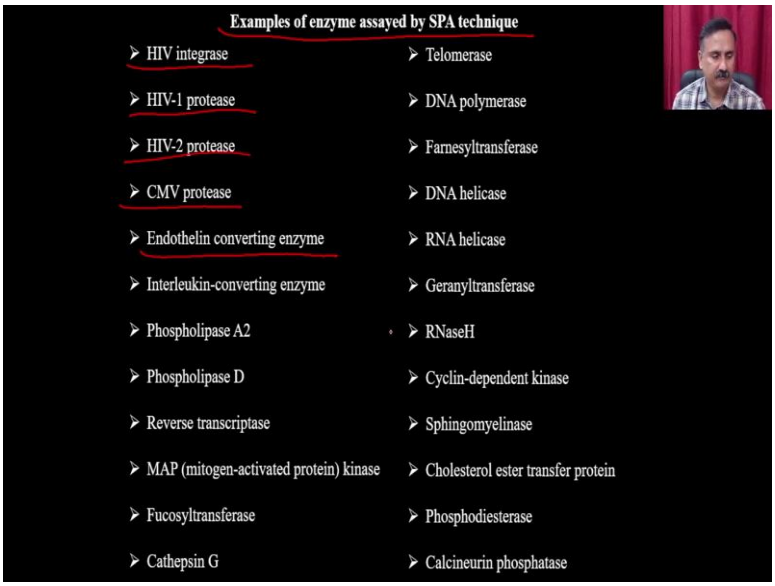

So, examples there are so many enzymes what you can actually be able to use or what you can actually be able to use for enzyme assays. You can use the HIV integrase, HIV protease, HIV-2 protease, CMV protease, endothelin converting enzyme and so on. So, these are the some of the enzymes for which people have already developed the spa beads enzyme assays.

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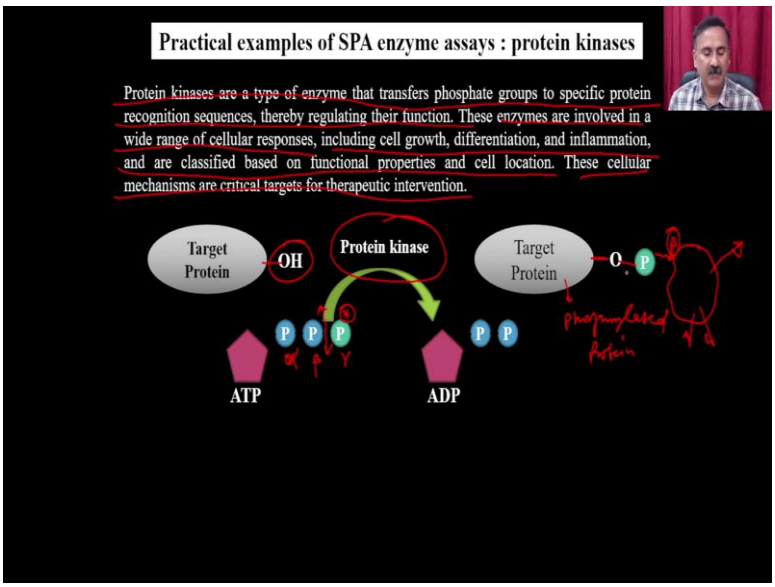

For example, you can have the kinase assay. So, kinase are protein kinase are a type of enzyme that transfer the phosphate group to specific protein, recognizes the sequence

thereby regulating their function. These enzymes are involved in a wide range of cellular processes including cell growth, differentiation, inflammations. And are classified based on the functional property and cell location. These cellular mechanisms are crucial target for the therapeutic interventions.

So, for example, this is the target protein on which is going to be recognized by the kinase and it actually has a free OH group. So, what will happen is when you have the protein kinase it is going to take it is this as a target and it is actually going to have the radio labelled phosphate group, right.

So, this is the gamma phosphate, this is the beta phosphate and this is the alpha phosphate sorry alpha phosphate, right. And this gamma phosphate is actually going to be you know broken down from here and it is actually going to be transferred onto this OH and that is how you are actually going to have the O phosphate, right.

And that is how this is actually going to be the phosphorylated product right, the phosphorylated protein. Now, if you have the scintillation beads right, the scintillation beads are actually going to receive the beta particles and that is how they were actually going to give you the lights. They are going to give you the lights and that is how you can be able to use that.

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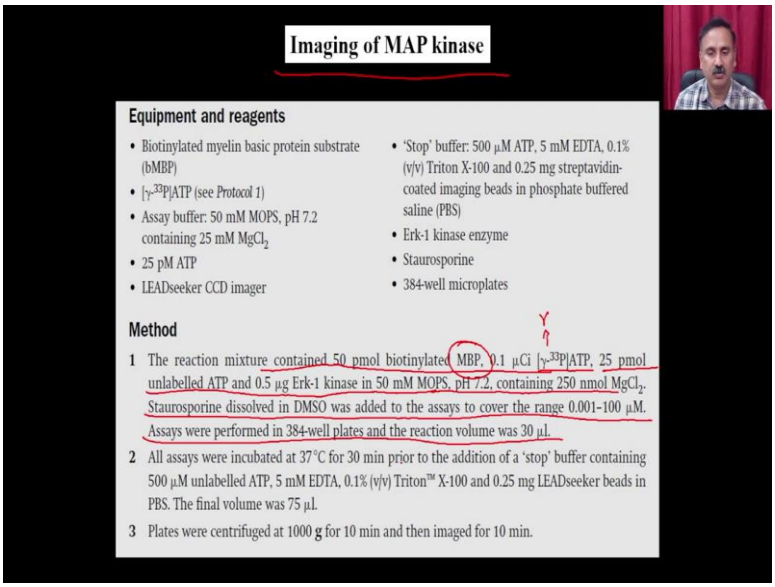

One example is, you can actually do the imaging of the MAP kinases, right. You can actually be able to use this and you can be able to do the imaging of the MAP kinases. So, the reaction mixture contain the 50 pico mole biotinylated MBP. So, MBP is a protein, the maltose binding protein.

You can actually have the radio labelled ATP. So, you see the this is the radio labelled ATP where you have the phosphate onto the gamma phosphate. Then you can have 25 pico mole unlabelled ATP and 25 microgram Erk kinase. So, this is the kinase, what you are going to use in a MOPS buffer, right.

And you can have this staurosporine dissolved in DMSO was added to assay to cover the range and assay were performed in 384 plates and reaction volume was 30 microlitre. All assay were incubated at 37 degree Celsius for 30 minutes and all that. And then you are actually going to measure the activity.

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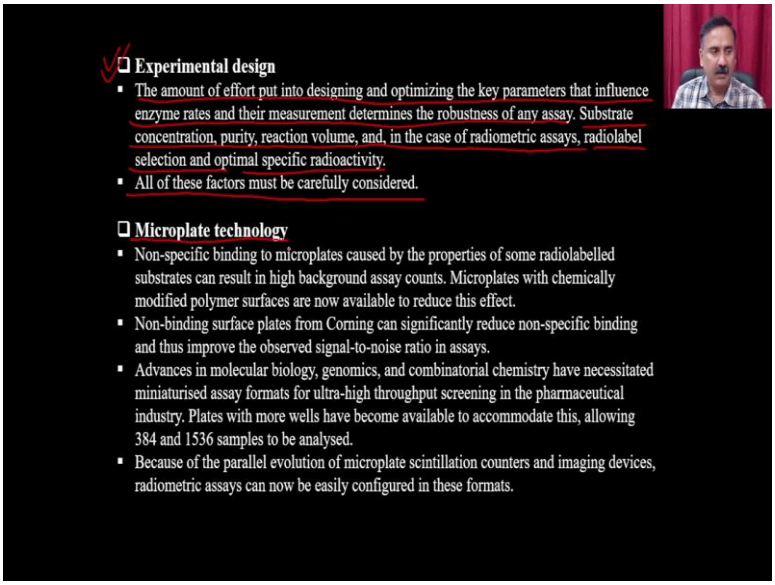

So, in the experimental design, what you are going to do is you are actually going to do the amount of effort put into the designing and optimizing the key parameter that influence the enzyme rates and their measurement determine the robustness of any assays, right. Substrate, concentrations and the POT reaction volume and in case of radiometric assays, the radio labels selection and optimal specific activity. All of these factor must be careful considered carefully.

So, when you are want to perform the radiometric assays, you have to design the experimental very nicely so that you can be able to reduce the background of the reactions and you can also be able to you know choose the different types of radiometric assays, radio labels whether you want to use the tritium labelled or carbon 14 or any other molecules right sulphur, right. And then you are going to use the technology, what technology you are going to use.

Whether you are going to use the microplate technology or and ideally people are going to use the (Refer Time: 25:53) assays. So, we can actually be able to use the radio labelled techniques for the microplate technologies.

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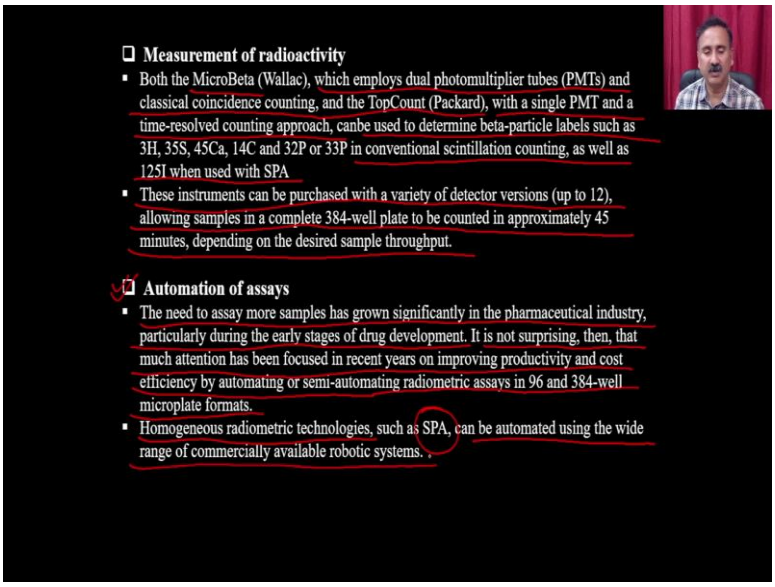

Then we can also use see whether what kind of measurement techniques are available in your laboratory. So, that also will going to decide which what kind of radio radiometric assays you want to use.

So, you can actually be able to use the MicroBeta which employs the dual photomultiplier PMTs and the classical coincidence counter and the top count. With a single PMT and a time resolved counting approach can be used to determine the beta particle label such as tritium label, sulphur, calcium, carbon, phosphorus or the in a conventional scintillation counting and as well as the 125I when used with the SPA.

These instruments can be purchased with a variety of detector versions allowing the sample in a complete 384 well plate to be counted in approximately 45 minutes depending on the desired sample throughout. Then you can also be able to because the radiometric assays just require the incubate setting of the reactions and then you can actually be able to have some kind of separation technique.

So, that you can be able to separate the radio labelled substrate from the normal substrate or radio labelled substrate from the product and then you can be able to just have the technique to measure, right. So, that is why you can actually be able to think about the automation of the assays. So, the need to assay both sample will grow significantly in the pharmaceutical industries particularly during the early stage of development.

It is not surprising then that the much attention has been focused in the recent year on the improving the productivity of cost efficiency by the automatic or semi-automatic radiometric assays in the 96 and the 384 microplate formats. Then you can have the homogeneous radiometric assays such as the scintillation proximity assay can be automated using the wide range of commercially available robotic systems.

So, this is all about the radiometric assays. What we have discussed? We have discussed about what are the different way in which you can be able to set up the radiometric assays and how you can be able to utilize or exploit the scintillation proximity assay based system to measure the activity of the enzyme without even going through the TDS process of separating the substrate or the radio labelled product.

So, this is all about the radiometric assays. Let us now move on to the another approach where you are actually going to use the gel electrophoresis.

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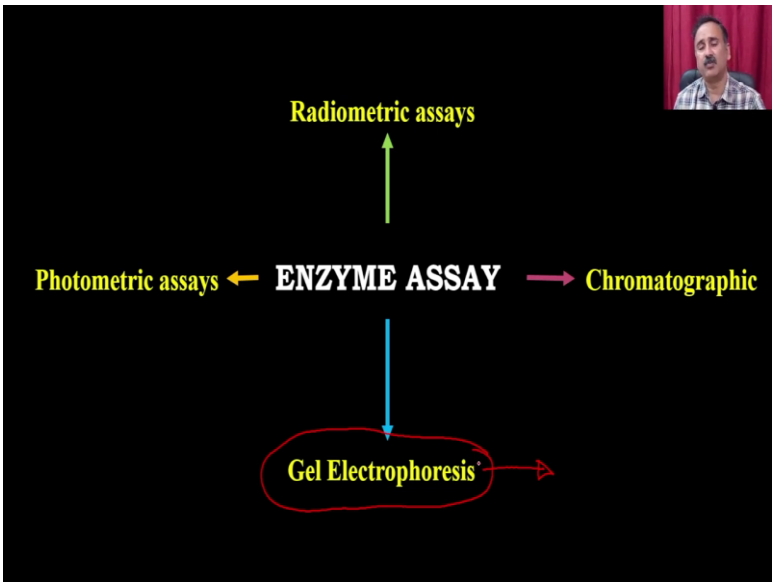

So, the gel electrophoresis is a another approach where you are actually going to run the enzyme on to the electrophoresis or you are actually going to use the electrophoresis to separate the labelled substrate from the non-labelled substrate or labelled product from the unlabelled product.

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So, enzyme assays after the electrophoresis.

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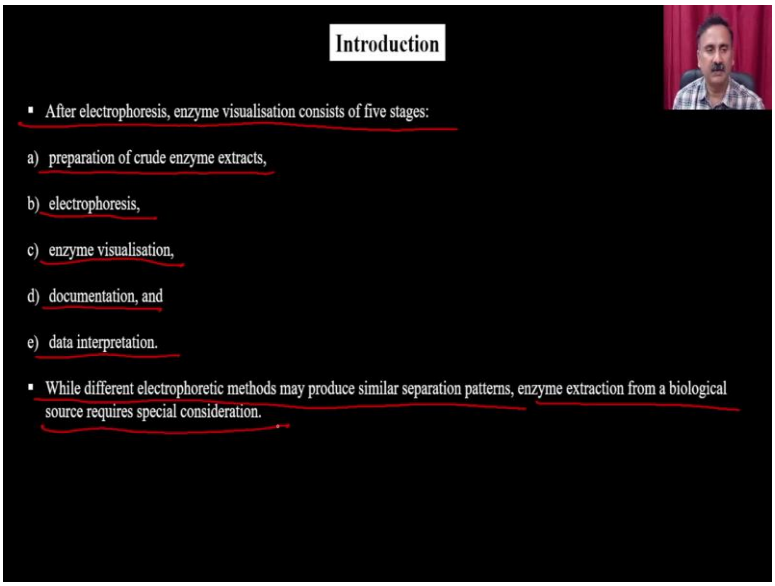

So, after electrophoresis enzyme visualization consist of five stages. Stage one preparation of crude enzyme extracts. Then you require the electrophoresis you can have the enzyme visualizstions, documentation and data interpretations. While the different electrophoretic method may produce similar separation pattern, the enzyme extraction from the biological source require the special considerations. So, let us discuss the first step.

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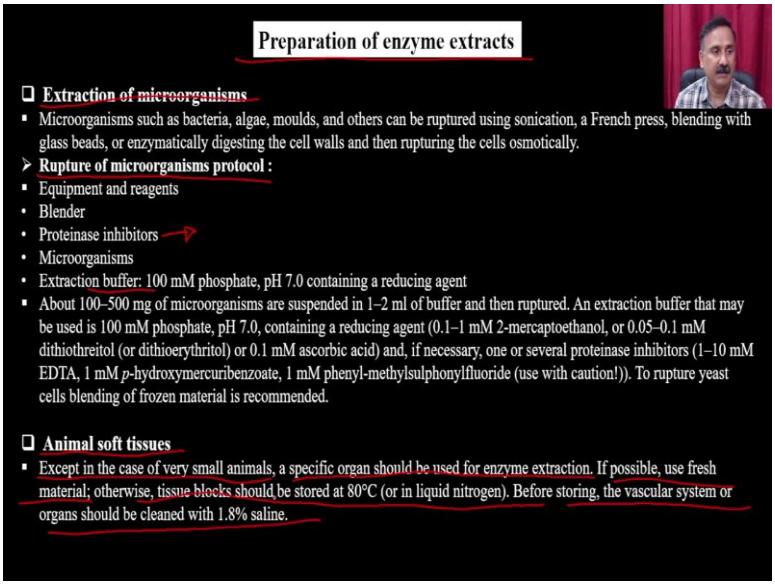

First step is the preparation of the enzyme extract. So, the extract of the micro-organism, all of these techniques or all of these approaches we have already discussed when we were discussing about the extraction of the enzyme from the different host. So, you can actually be have the rupture of the microbial micro-organism protocol.

So, you can use the different types of proteinase inhibitor so that there should be no degradation of the enzyme. And then you can use the extraction buffers and then you can also use the procedures and that is how you can actually be able to prepare the extract of the micro-organisms.

If it is a animal or the soft tissue to accept in case of very small animals, specific organ should be used to enzyme extractions. If possible, use the fresh material otherwise tissue block should be stored at 80 degrees before storing the vascular system or organ should be cleaned with the 1.8 percent saline.

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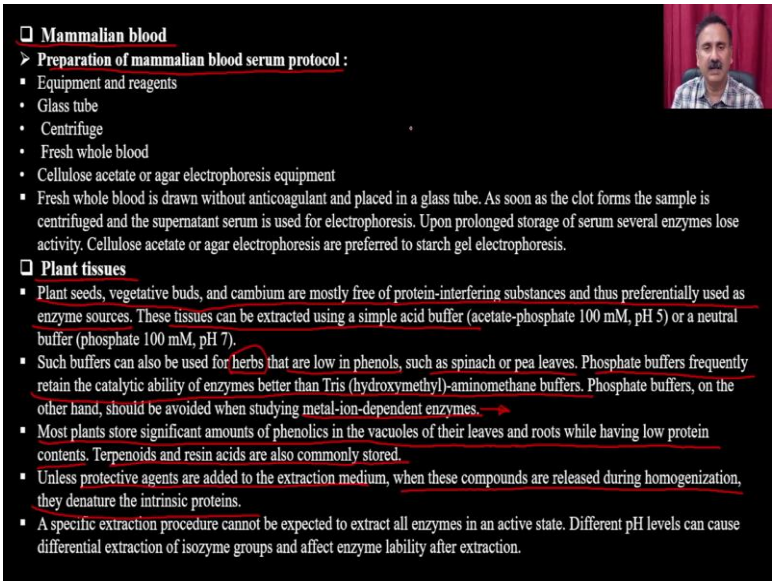

Then you can also be able to have the processing of the mammalian bloods. So, preparation of the mammalian blood serum protocol. So, you can actually be able to follow the same protocol. This protocol we have already discussed when we were discussing about how you can be able to prepare the serum, right.

When we were discussing about how to produce the antibodies. And the lastly, we have plant tissues, right. So, plant seeds, vegetables, buds and cambium, are mostly free of

protein interfering substance and thus preferentially used for enzyme source. These tissues can be extracted using a simple acid buffer or a neutral buffer, right. So, 100 mili molar pH 7. Such buffer can be used to used for herbs right, small plants right, that are low in phenol such as spinach or the pea leaves.

Then you can use the phosphate buffer, frequently to retain the catalytic ability of the enzyme better than the tris, right. So, as far as the buffer is concerned you should always try to use the physiological buffer such as phosphate buffer rather than the chemical buffer like tris. And the phosphate buffer on the other hand should be avoided when you studying the metal ion dependent enzymes. And phosphate buffers are also should be avoided when you are trying to measure the activity of the kinases.

Most plants store significant amount of phenolics in the vacuoles of their leaves and roots while having low protein content. So, terpenoids and resins, resin acids are also commonly stored. Unless protective agents are added to the extraction medium when these compounds are released during homogenization, they denature the intrinsic protein. So, that is why we should be very very careful from the phenolics and tannins and all other kinds of the secondary metabolites from the plants.

So, we should have a sufficient amount of the antioxidant molecules and the reducing agents so that we should not they should not be able to affect the proteins. Now, once you prepare the extract you can actually be able to run them onto the electrophoresis and then you can actually be able to do the different types of visualization techniques. So, principle of enzyme visualizations.

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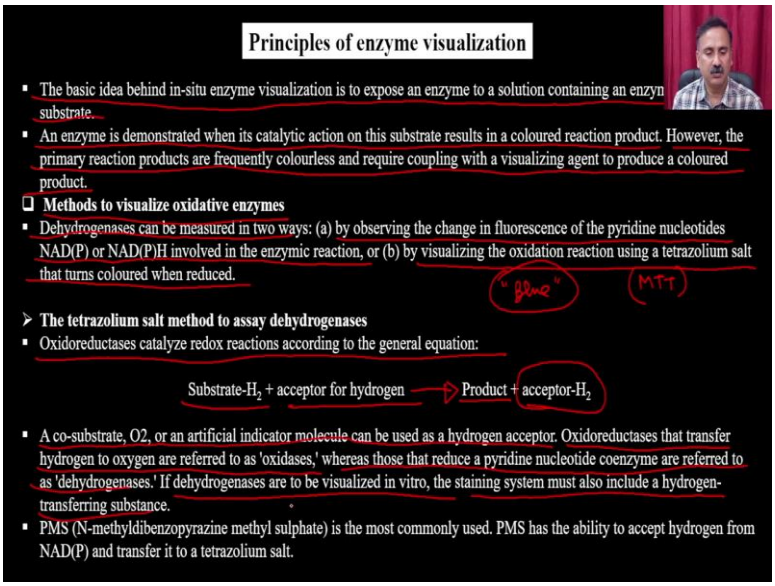

So, the basic idea behind the in-situ protein visualization is to expose an enzyme to a solution contains the enzyme specific substrate. The enzyme is demonstrated when its catalytic action on the substrate result in a coloured reaction product. However, the primary action products are frequently colourless and require coupling with a visualization agents to produce a coloured product. There are methods to visualize the oxidative enzymes. For example, dehydrogenases can be measured in two ways.

By measuring the change in the fluorescence of pyridine nucleotide NAD P H or NAD H involved in the reaction or by visualization of the oxidation reaction using a tetrazolium salt that turns coloured when reduced. So, you can actually be able to use either of these method to measure or to monitor the dehydrogenases what are present in the gel. Either you can use the fluorescent method to measure to see how the NAD H or NAD P H is getting reduced or you can be able to see whether you know you can actually run the enzymes.

You can perform the reactions and then you can be able to use the tetrazolium salt. For example, you can use the MTT and that actually is going to give you the blue colour wherever it is actually going to find the enzyme, which is of the dehydrogenases. So, oxidative reductase catalytic reactions according to the general equation is that it is going to take the substrate, the acceptor for the hydrogen and then it is actually going to give you the product plus acceptor which contains the hydrogen.

And in this process, it is actually going to process the MTT and that is how it is actually going to give you the colour. A co-substrate O2 or an artificial indicator molecule can be used as an hydrogen acceptor. Oxidoreductase that transfer the hydrogen to oxygen are referred as ‘oxidases’, whereas, those who reduce a pyridine, nucleotide, coenzymes are referred to as dehydrogenases. If the dehydrogenases to be visualized in vitro, the staining system must include a hydrogen transferring substance.

Then we have the PMS that is the most commonly used sub substrate and PMS has the ability to accept the hydrogen from the NAD plus and transfer it to a tetrazolium salt.

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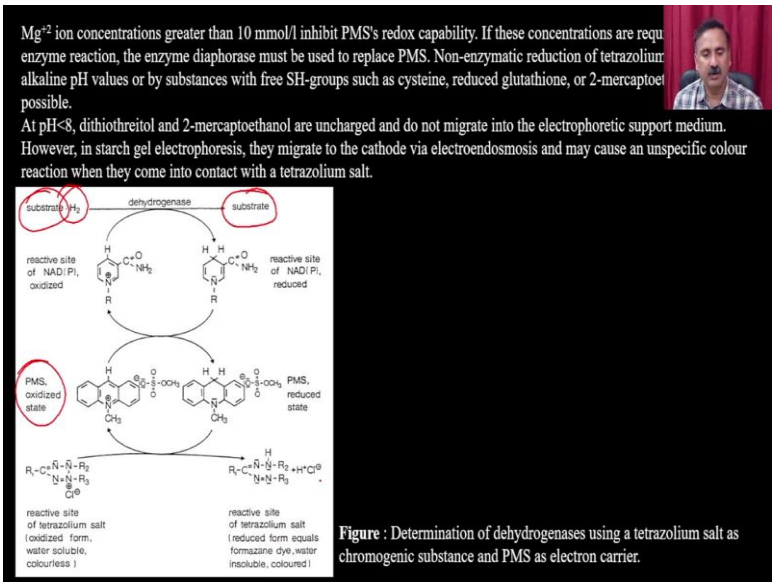

Figure : Determination of dehydrogenases using a tetrazolium salt as chromogenic substance and PMS as electron carrier.

Then we have the this kind of system where you have the substrate and hydrogen and it is actually going to product get converted into the substrate and this hydrogen is going to be received or accept by the reactive site reactions site NAD plus and that in terms is going to oxidize the PMS.

And while the PMS is going to receive this hydrogen, it is actually going to give that hydrogen back to the tetrazolium salt. And that is how the tetrazolium salts are going to be get converted into the reduced form. And the reduced tetrazolium is actually going to give you the colour.

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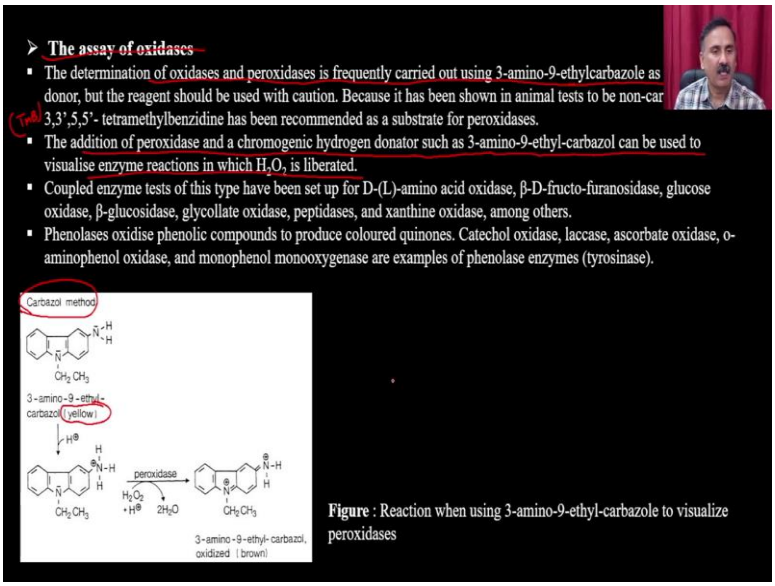

Then we can have the assay of oxidases. So, the determination of oxidases and peroxidases is frequently been carried out using the 3-amino-9-ethylcarbozole as a electron donor. But the reagents should be used with cautions because it has been shown in animal tested to be non-catagenic. Then you can use the 3, 3', 5, 5' tetra methyl benzidine has been recommended as a substrate for.

So, this is also short form it is called as TMB, right. And the addition of the peroxidases and the chromogenic hydrogen donor such as 3-amino-9-methyl c arbozol can be used to visualize the enzyme reaction in which the H 2 O 2 is liberated. So, this is the Carbozol method where you can actually have the carbozol which is a yellow-coloured product and it is getting converted by accepting the hydrogen after the reactions.

And then when the peroxidases are converting the hydrogen peroxide into the water, they are actually taking up this molecule and that is how it is converting a yellow coloured carbozol into a brown coloured carbozol and these brown coloured carbozol can be visualized.

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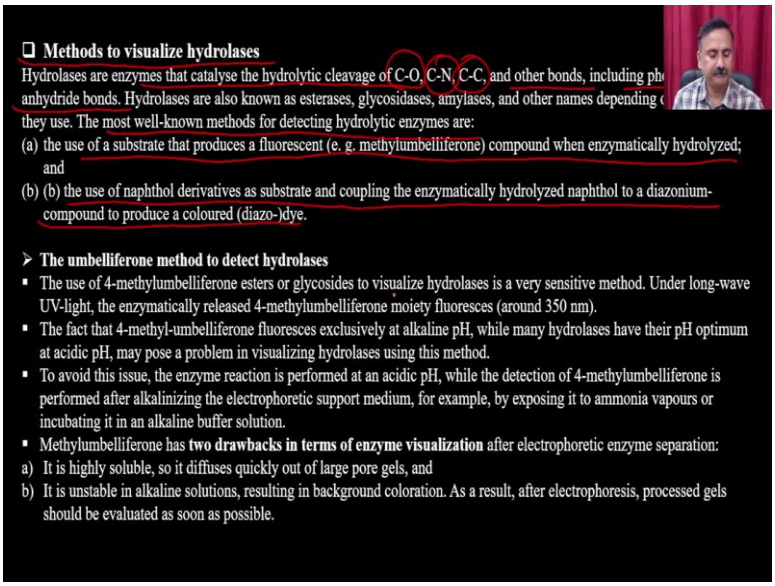

Then we have the methods to visualize the hydrolysis, right. So, hydrolysis are the enzyme that catalyze the hydrolytic cleavage of C-O, C-N, C-C and all other kinds of bonds including the phosphoric anhydride bonds.

So, the well-known method for detecting the hydrolytic enzymes are the use of substrate that produces a fluorescent compound when enzymatically hydrolyzed and the use of naphthol derivative and substrate and coupling the enzyme hydrolyzed, the naphthol to a diazonium compound to produce a coloured diazo dyes.

The umbelliferone methods to detect the hydrolysis. So, this is the method what I have given to measure or to detect the activity of the hydrolyses. And here what you are going to do is you are going to have the substrate when it is being cleaved, it is actually going to be you know be fluorescent and that is how you can be able to measure the fluorescence.

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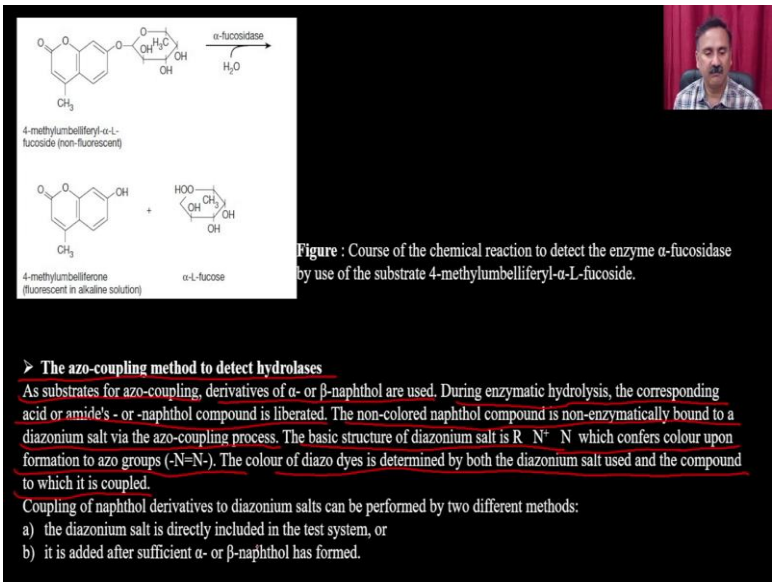

So, then you can have the azo coupling method to detect the hydrolysis. So, as the substrate for azo coupling derivative of alpha, beta and naphthols are used. During enzymatic hydrolysis the corresponding acid or amide or naphthol compound is liberated. The non-coloured naphthol compound is non-enzymatically bound to a diazonium salt via the azo coupling process.

The basic structure of the diazonium salt is R N R right, which confers colour upon the formation to a azo groups. The colour of azo dye is determined by both the diazonium salt used and the compound to which it is coupled. Coupling of naphthol derivative to the diazonium salt can be performed by the two method. The diazonium salt is directly included in the test reaction or it is added after the sufficient alpha or beta, naphthol has formed.

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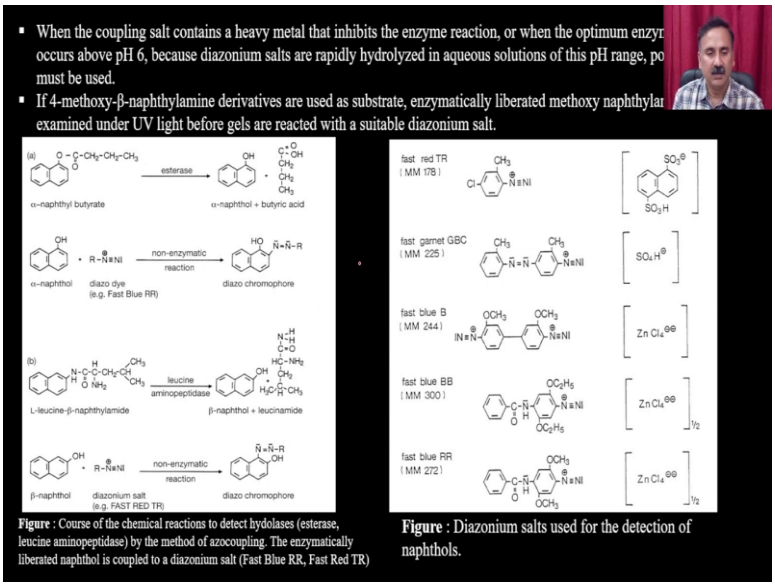

Figure : Course of the chemical reactions to detect hydolases (esterase, leucine aminopeptidase) by the method of azocoupling. The enzymatically liberated naphthol is coupled to a diazonium salt (Fast Blue RR, Fast Red TR)

Figure : Diazonium salts used for the detection of naphthols.

So, these are the different types of assays were which can be used by the you know the electrophoresis.

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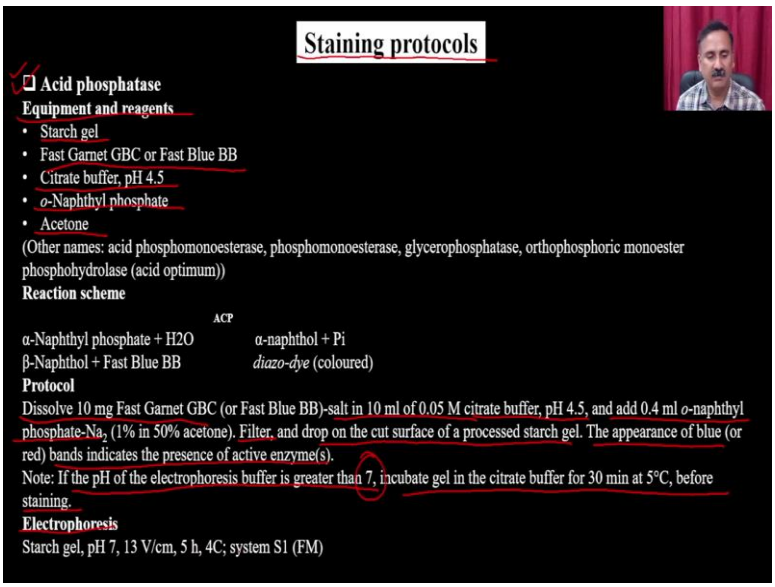

And then we have the staining protocol. So, once you have the staining protocol for the acid phosphatases. So, you can have the equipments and the reactions, right. So, you can have the starch gel, you can have the dye, which is called fast blue, (Refer Time: 39:51) blue. You can have the citrate buffers, alpha naphthyl phosphate and acetones.

So, in this assay what you are going to do is you are going to run the reactions and then you are going to do the staining protocol for the acid phosphatases. Then you what you are going to do is you are going to dissolve the 10 milligram of fast garnet GBC salt in the 10 ml of citrate buffer pH 4.5 and add the 4 ml of azo naphthyl phosphate and you filter and drop on the cut surface of the process the starch gel.

The appearance of blue band indicate the presence of an active enzyme. Note if the pH of the electrophoresis buffer is greater than the 7. The incubate gel in the citrate buffer for 30 minutes at 5 degree Celsius before staining. Electrophoresis, you are going to run the starch gel with the pH 7 and 13 volt per centimeter 5 hours at 4 degree Celsius.

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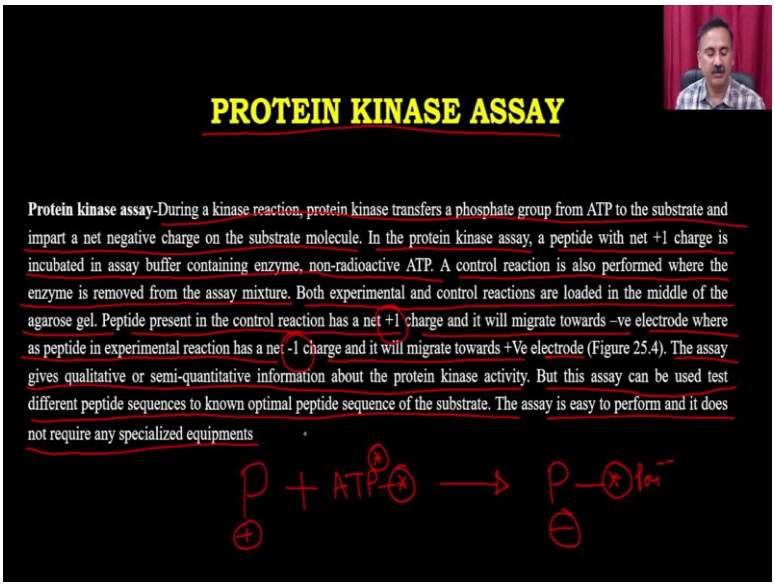

Then you can also use the measuring protein kinase assays. So, remember that the protein kinase assay is actually going to do this, right. It is actually going to take the proteins. Then you are actually going to add the ATP. So, ATP is actually going to have the radio level, right. Or it is actually going to have the gamma phosphate, right.

And then what will happen is that the protein is getting converted into a phosphate which is actually going to impart. So, this is actually going to be either positive recharged or neutral. Whereas this is actually going to be negatively charged because it is actually going to have the phosphate group, right.

So, this negatively charged group is going to run towards the different side where the positively charged group is going to run on the other side. So, during a kinase assay. The protein kinase transfer phosphate group from ATP to the substrate and impart a negative charge onto the substrate molecule. In the protein kinase assay, a peptide with positive with a net plus 1 charge is incubated in the assay buffer containing enzyme non-radioactive ATP.

A controlled reaction is also performed where the enzyme is removed from the assay mixture. Both experimental and controlled reactions are loaded in the middle of the agarose gel. So, the peptide present in the controlled reaction has the plus positive charge and it will migrates towards the negative electrode.

Whereas, the peptide in the experimental reaction has the net minus charge and it will migrate towards the positive electrode. The assay gives the qualitative or semi-quantitative information about a protein kinase assay. But this assay can be used for test different compound, different to know where the optimal sequence of the substrate. The assay is easy to perform and it does not require any specialized equipment.

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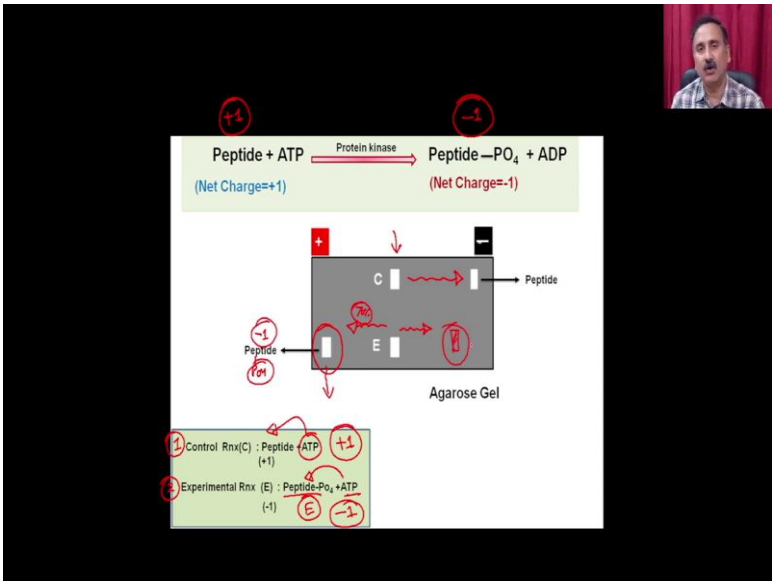

So, this is what you are going to do is you are going to take the peptide which is actually going to have the net positive charge and because it has a positive charge, it will runs towards the negative electrodes. Whereas when it is getting phosphorylated with the help of the phosphate group from the ATP, the peptide is going to have the net negative

charge. So, it is actually going to have the minus 1 charge and because it has a minus 1 charge, it will run in the different directions, right.

So, we are going to run the two reactions. One is you are going to run the control reaction. The second is you are going to run the experimental reaction. So, in the control reaction, you are going to have the peptide plus ATP, right. So, there will be no transfer of phosphate group from the ATP to the peptide and that is why it is the peptide is going to remain as plus 1. Whereas in the experimental reaction, you are going to have the peptide plus ATP and with the enzyme, right.

So, you are going to have the enzyme. So, because you have the enzyme, there will be a transfer of the phosphate group from the ATP. And that is why here the you are going to have the minus 1 charge. And then what you are going to do is you are going to load these two in the middle of the agarose gel, right.

So, what will happen is that the control reaction which is actually going to have the positively charged peptide molecules will run towards the negative electrodes. Whereas the peptide which is from the experimental conditions is going to have the phosphate group, right.

So, it is actually going to have the minus 1 charge and because of that, it actually going to run in this direction. And amount of peptide which is going to be migrated towards this side versus the amount of my amount peptide which will migrates towards this side. Because from this reaction, it will not be 100 percent. So, imagine that you have the 70 percent phosphorylations, 30 percent non-phosphorylation. So, non-phosphoryled peptide will run in this direction whereas, the phosphorylated peptide will run in this direction.

So, if you measure the activity or if you measure the intensity of these bands which are you going to get on both the side, you can be able to calculate the activity of the particular protein kinase assays. So, this is all about the enzyme assays, how you can be able to set up the different types of enzyme assays utilizing the different properties of the substrate or the product so you can actually be able to use the photometric based assays.

Where you are going to see how the substrates or the products are absorbing the lights. And then you can also be able to use the radiometric assays where you are going to use

the radioactive molecules or the nuclei's. And that is how you are going to measure the activity of the kinases, transferases and hydrolyses. And lastly, we have also discussed about the electrophoretic method where you can actually be able to run the enzyme onto the electrophoretic gels.

And then you can actually be have the different types of staining methods to stain the substrate or the product or sometime you can also be able to have the flexibility of staining the co-factors and that is why you can be able to identify the different types of substrates. So, with this I would like to conclude my lecture here. In our subsequent lecture we are going to discuss some more aspects related to enzymes.

Thank you.