Enzyme Science and Technology
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Module - VIII
Enzyme assay system and Kinetics
Lecture - 35
Enzyme Assay System (Part-I)

Hello everyone, this is Dr. Vishal Trivedi from Department of Biosciences and Bioengineering IIT, Guwahati. And what we were discussing, we were discussing about different properties of the enzyme in the course, Enzyme Science and Technology.

And what we have discussed in the previous module is that we can actually be able to study the interaction between the enzyme and the substrate or enzyme and the different type of ligand what are interacting with the enzyme with the help of different types of techniques.

So, in this context, what we have discussed, we have discussed about the chromatography techniques, whether the chromatography techniques are going to be used for measuring the change in shape or size of the molecule or whether it can be used for measuring the or detecting the different types of charges onto the enzyme structures.

And in addition to that, we have discussed about the couple of spectroscopy techniques, we have discussed about the isothermal titration calorimetry, and then in the previous lecture, if you recall, we have also discussed about the surface plasmon resonance technique.

So, all of these techniques are very easy to perform and they are actually been, and majority of these techniques are label free techniques. So, they can be very easily or economical in terms of usage. So, with this, we have understood that the enzymes are interacting with the substrate and enzymes are catalyzing the different types of reactions.

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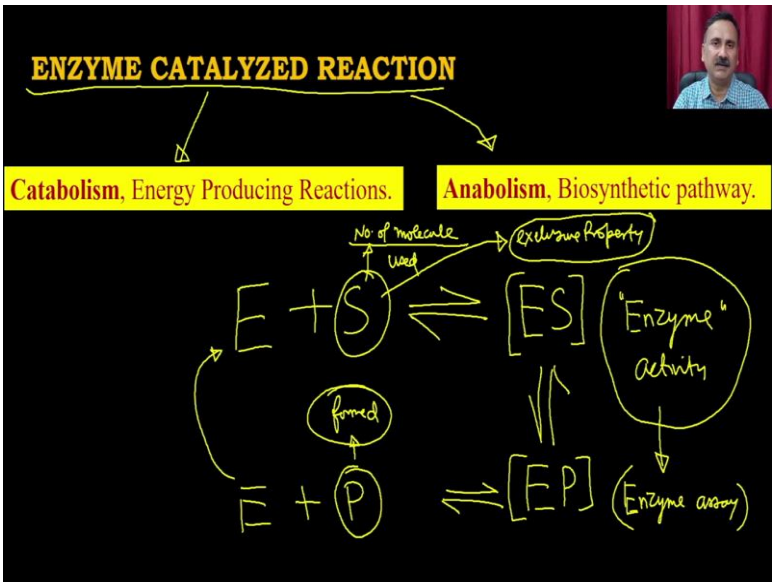

So, if you recall, we have discussed about the different types of enzyme-catalyzed reactions, we have discussed about the catabolic reactions and we have also discussed about the anabolic reactions. So, when an enzyme is processing a substrate, what it is doing is enzyme is reacting with the substrate, ok, and then it is actually forming the transition state which is called as enzyme-substrate complex.

And then this transition state is getting converted into another transition state which is called as the enzyme product transition state, right. And from the enzyme product, you are actually going to have the release of enzyme and you are actually going to have the generation of product, right.

And this enzyme will again go into the cyclic manner and it will actually go and receive the another molecule of substrate. Now, what how we can be able to measure the enzyme activity, right. So, the ability of an enzyme to process the number of substrate molecule is actually going to be a direct measurement of the activity, right.

So, number of molecules. So, if you want to measure the enzyme activity, you have two options. Either you can be able to count how many number of molecules of substrate are being used or you can be able to say how many number of molecules of products are being formed, right.

So, either you can be able to measure the formation of the product or you can be able to measure the depletion of the substrate and then only you can be able to measure the enzyme activity, right. Now, if you want to do this, you are actually going to have a very well documented or well managed infrastructure so that you can be able to measure the substrate or the product. And if you want to do that, you are actually should have the exclusive properties related to either the substrate or the product, right.

So, you have the exclusive properties, what is mean by the exclusive property is that these exclusive properties will not be present or will not be present in any of the species throughout this particular cycle, which means if you are measuring the substrate, it should not have that you are measuring the substrate, but at the same time, the product is also very much similar and it is also giving you the similar reactions.

And that is why if you want to do the enzyme activity, you require a or you actually have to standardize a system which is called as enzyme assay. And in the enzyme assay, you are actually going to use the different types of criteria or different types of conditions in which you are actually going to either measure the depletion of the substrate or you are actually going to form measure the formation of substrates.

So, in today's lecture, we are actually going to discuss about some of these aspects and how you can be able to set up the enzyme assay to measure the enzyme activity.

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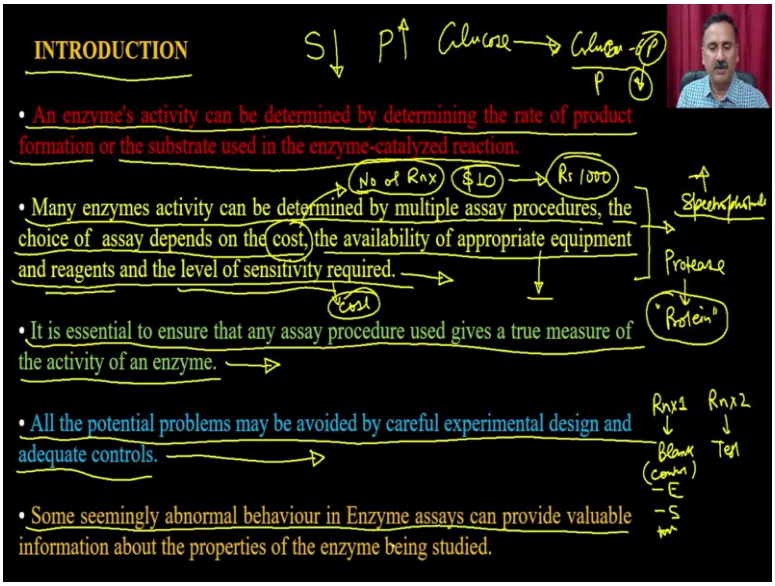

So, as the name suggests, the enzyme assay is a assay where you are either going to measure the depletion of the substrate or the generation of the product. So, the enzyme's activity can be determined by determining the rate of product formation or the substrate used in the enzyme-catalyzed reactions.

Many enzymes activity can be determined by the multiple assay procedures, the choice of assay depends on the cost, the availability of appropriate equipment and the reagent and the level of sensitivity required. So, this is very, very important parameter what you are supposed to consider when you are trying to develop an enzyme assay for measuring the enzyme activity.

So, it depends on the multiple procedures. It depends on the availability of the equipment's such as the if you have the spectrophotometer or not, right. So, if you have the spectrophotometer, then only you can be able to or you should actually set up a assay where you are actually going to have the change in the UV region, right?

If you have the fluorimeter, you can actually be able to set up the or design the enzyme assay accordingly. So, depending on the equipment, you are actually going to set up the enzyme assays. Then you also require the different types of reagents. Suppose for example, I am trying to develop a protease assay.

So, actually I require the different types of proteins, right. Because that is going to be the substrate, right. So, whether I should have that protein or not, then only I can be able to use or develop a protease assay, right.

And then what is the level of sensitivity required? If you require an assay which actually can work in the micromolar range or millimolar range, then or versus whether you are looking for a sensitivity where you are actually going to measure the differences between the substrate and the product in the range of nanomolar range and so on. So, depending on the sensitivity, the cost is also will actually going to be you know go up.

So, all these are and the most important thing is cost actually because when you are trying to develop the enzyme assays, the cost is one of the very, very important parameters because that is actually going to decide how many number of reactions you can be able to perform, right.

So, if you are actually going to run an assay where the cost is 10 dollar for example. So, if you are actually going to have an assay which has 10 dollar cost for every assay you are going to do like your, so this means you are actually going to use approximately rupees 1000 for performing single reactions, right? So, this is going to be very, very costly.

So, that also is actually going to be a limiting factor to go and adopt that particular type of enzyme assay because if you can measure the enzyme activity, even with the cheaper options, maybe you may have to compromise with the sensitivity and other things, but you will actually at the end you will get the answer, right.

Then we also, it is essential that you ensure that any assay procedure which gives a true measure of the activity of an enzyme. So, that is very, very important that when you develop the assay, it should actually give you the right readouts and it should give you the real assay or real procedure.

So, in this procedure, you should actually first figure out what are you are going to measure and whether there will be a cross reactivity because as I said in the past also, right in the previous slide also that the substrate and products are not very different, but they are different in terms of some of the properties.

For example, if you are measuring an assay where glucose is getting converted into glucose 6 phosphate, these two are very, very different except that you are actually going to have a phosphate group which is attached to this substrate, this product actually, right. So, you are actually can only use this as a probe to measure, right? So, that is how you should have a very good procedure in which you should not get misguided and you are measuring something which may not be reflecting the activity of an enzyme.

All the potential problems may be avoided by careful experimental design and adequate control. So, when you are trying to develop the enzyme assays, you are going to have two reactions, one reaction one which is actually going to be blank or I will say control, right. Where you are going to have everything except you can actually be able to remove the enzyme, then you are going to have the reaction two where you are actually or I will say this is actually the test reactions.

Sometimes you may also have the multiple types of blanks. For example, you can have the control for enzyme, you can also have the control for substrate you can also have the control for other things, right? So, if that is the case, you should be little more you know careful and you should actually be able to set all these reactions, then only you can be able to measure the enzyme activity.

Some seemingly abnormal behaviour in enzyme assay can provide valuable information about the properties of enzyme being studied. So, when you perform the enzyme assay, it is actually going to say how the substrate is going to be processed and how it is actually going to convert that into the product.

And this is actually a very very important information. It actually going to tell you the behaviour of that particular enzyme because if you do a subtle change in pH or subtle change in temperature or pressure or other kind of environmental parameters, it is possible that the same enzyme probably may be start processing the different types of substrate, it may start you know it generating the different types of product and so on.

So, that would be a very, very abnormal behaviour of the enzyme assays and that is going to be also give you the very, very critical informations. Now, when you try to develop the enzyme assays you also going to see the different types of behaviour of these enzymes right, enzyme assays. So, these behaviour is actually going to give you a lot of information about the enzyme and its interaction with the substrate and how it is actually processing the substrate to actually giving you the product.

So, let us see what are the different types of behaviour of the assay.

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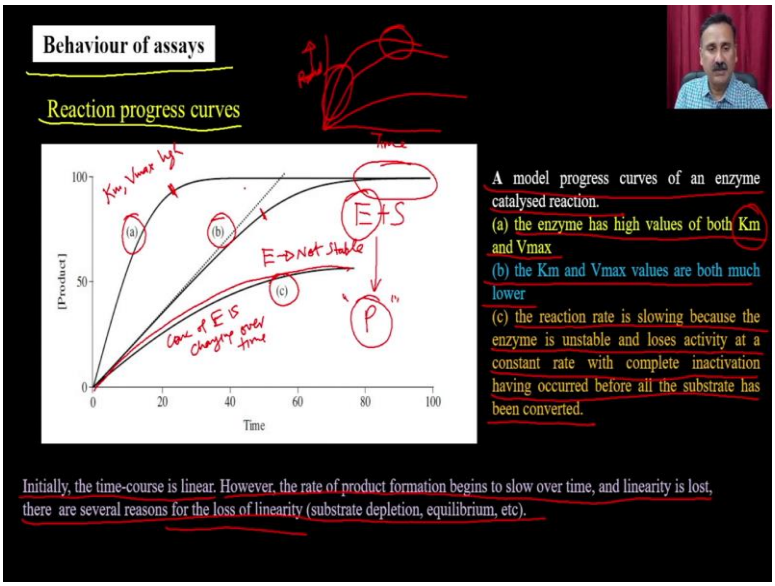

So, when you are going to study the behaviour of the assay, you can actually be able to expect that the reaction is actually going to process in these three way, right. It can either be curve a, it can follow a curve b or it actually can follow a curve c. In either of these cases, what will happen is that when you are actually going to run a enzyme assay for time versus product, right.

And suppose you are measuring the product, what will happen is that initially it is actually going to be a linear and then it is actually going to saturate, ok. So, this is actually the non-linear regions and this is the linear region, right. And it actually can follow different path. It may actually linear, it can be linear or it can be reaching to this point much faster and it can actually be able to not be able to reach this, ok.

So, that is how it is going to have the three different types of curves. So, when you do the reaction progress curve or you are going to monitor the enzyme assay and you are going to see how the enzyme is actually processing the substrate and it is actually going to give you the product. And if you are measuring the product, not the depletion of substrate, what you are going to see is you are going to see the multiple types of curves, right.

So, initially the time course is linear. So, what you see here is that in this area, the time course is linear. For example, in this one also up to this, it is linear actually and it started that plateau actually. So, this is the non-linear region. This is in fact, there is no change

in product, right? However, the rate of product formation begin to slow over time and linearity is lost. There are several reasons for the loss of linearity, ok.

So, these are the three types of model which can be possible. So, a model progress curve of an enzyme catalyzed reaction a, when you are going to get a, when the enzyme has the high value of both Km and the Vmax. So, if I think you should not be worry about the Km and Vmax, but these are the assays what you are going to see is when both the Km and as well as the Vmax is going to be very high.

Then the curve b you are going to get when the when the Km and the Vmax values are lower. So, these are the curve what you are going to get. And the curve c is the reaction where the reaction rate is slowing because the enzyme is unstable and it loses activity at a constant rate with a complete inactivation having occurred before all the substrate has been converted.

So, this is the reaction where you are expecting an enzyme which is not very stable. So, it is not stable and what happens is that the concentration of enzyme is also changing. So, here what happens is that the concentration of enzyme is changing over the time, ok. So, you can imagine if that happens then it is actually going to keep processing the lower and lower and lower substrates and that is how the enzyme, instead of increasing enzyme, instead of increase in product formation there will be a decrease in product formation and that is how this is not going to be the good.

So, you can actually have the three scenarios a, b and c and why there is a loss of linearity. So, there are multiple reasons why there is a loss of linearity in this particular system or I will say why the system actually gets reaches to the saturation.

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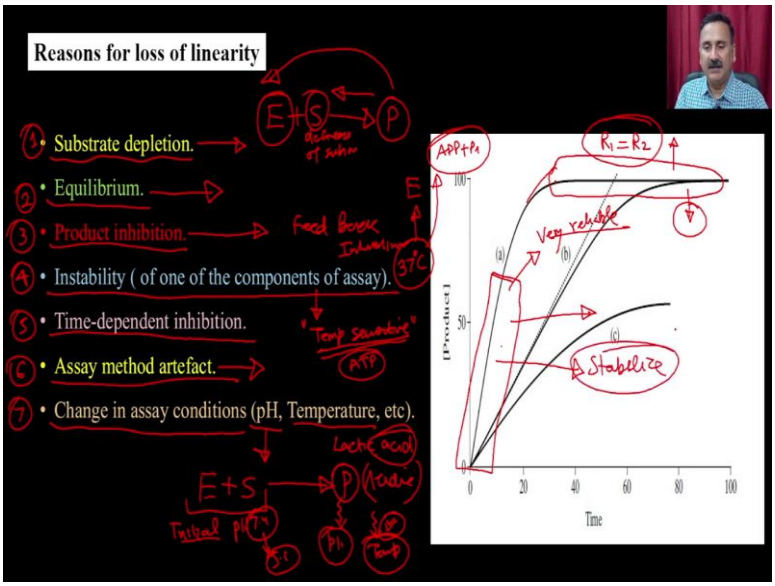

So, number one reason is that you are actually going to have the substrate depletion, right. Because enzyme what enzyme is doing is, enzyme is processing the substrate and it is generating the product, right.

So, if there will be a substrate depletion or if there will be a decrease in substrate concentration, right, because this is equilibrium, right. So, if the decrease in substrate concentration, enzyme will not have the adequate substrate so that the I mean if the binding should occur.

And that is how it is actually going to get converted into the product. Then it is also going to be because there is a change in equilibrium, right. So, because there is a, you know, because all these are under equilibrium, so product is getting converted or it is actually reversible actually.

So, at equilibrium, the concentration of, so initial concentrations are high, but when the system reaches to the equilibrium, the forward reaction and reverse reactions are equal and as a result, there you will see that it is actually going to adopt the plateau. For example, in this case, right.

Forward reaction and the reverse reaction is actually going to be equal and that is how it will not go down. So, it will go linearly to some extent and then it will actually going to

attain the equilibrium and that is how it is actually going to have the plateau or there will be loss of linearity.

The number 3 is the product inhibition. So, you can have the product inhibitions and this is actually also going to be called as feedback inhibition, right? So, when you have the, you know, some product is formed, right so this product could be a inhibitor for the enzyme and that is how initially it may not be having significant impact, but when the product, a lot of product is formed, it is going to inhibit the enzyme and that is how it is actually going to make the curve linear or there will be loss of linearity.

Then the fourth point is the instability of one of the component of the enzyme assays. So, when you are actually going to use the different types of components, some component could be temperature sensitive, for example, right. So, when you, and majority of these assays are being performed at 37 degree Celsius, right. Because that is the temperature at which most of the enzymes are, you know, showing the activity, optimal activity, right.

So, but the components could be temperature sensitive. For example, if you are using the ATP, right? So, ATP is good, ATP is stable molecule, but at 37 degree Celsius, the auto oxidation of the ATP to give you the ADP plus PI is very high, right? And as a result, there will be a degradation of ATP and that is how it may actually going to not available for, you know, for enzyme, for catalyzing the reactions and that is how it can actually be able to give you the nonlinearity or the product is stop, you know, going up right.

Then you can also have the time-dependent inhibition. So, there is a, in a time-dependent inhibition is also like product inhibition that after some time the system is reaching to the equilibrium or after some time the product is formed which is actually going to have the inhibitions. Then some time you can also have this kind of behaviour where you can actually losing the linearity because there is a artefacts in the system. So, it is actually, you know, artificially showing the, you know plateau, but it is actually not.

And the number 6 is change in the assay conditions so this is very important, right. You are actually going to have the change in pH or temperature. So, when you are actually going to have that, for example, in some enzymes, what happens is that when the enzymes are processing the substrate.

For example, at this site when you are actually doing it initially, right. So, in the initial reaction, you have set the pH everything right, but when the product is forming and suppose the product is acidic in nature, right. So, if the product is forming which is, you know, acidic, right so that is going to slowly going to start affecting the pH, ok.

In other cases, sometime you are actually going to have the assay where the assays are endothermic, right. So, when the product is forming, it is actually also affecting the temperature. Although majority of these assays are always being done in the incubators or they are actually being done in the water bath.

So, you always maintain the regular temperature, but the pH is always can be modulated, right. Because if you are forming some acid, like for example, if you are generating the lactic acid, right. So, lactic acid is a acid, right so, it is actually going to impact the pH of the media.

And as a result, initially the pH is 7.4, but when the lactic acid is started producing, the pH could actually come down to 5, right, for example so, if the enzyme is optimally active at 7.4, but it may not be active at 5 and that is how you might be able to see that there is a loss of linearity. So, these are the sum of the reason why you cannot be able to see a loss of linearity.

And because of this reason, majority of the people what they do is they are actually using this part of the curve only for measuring the enzyme activity. So, they do not allow or they do not use the whole curve for measuring the enzyme activity because this is the curve which is very, very reliable and compared to this.

Because at this point you are actually reaching to saturation. And at when you are reaching to a saturation, there are many things what can actually go wrong you can actually have the substrate depletions, you can actually have the product inhibition and so on.

And you can also have the, you know, sometime artefacts which are actually also possible. So, that is why this is the initial curve where you are actually going to do a lot of measurements. And majority of the enzyme kinetics people are using this particular curve. What are the different features of this initial measurements are?

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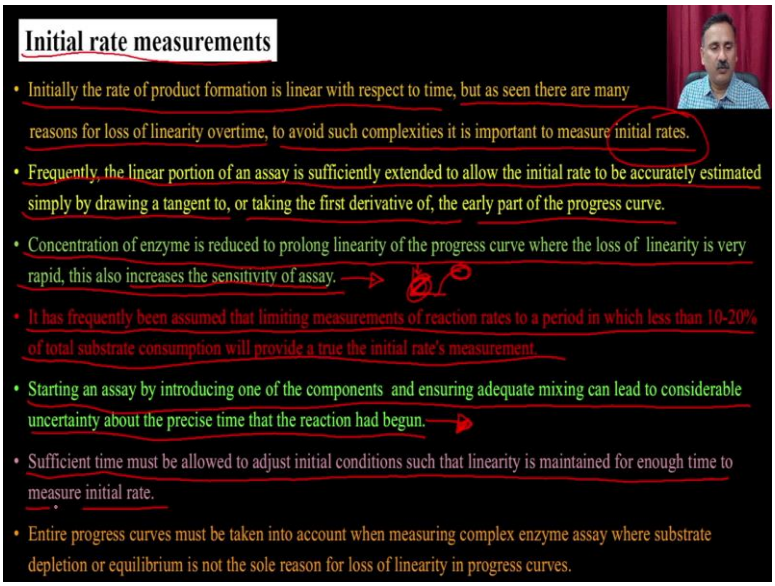

So, what you when you do the initial measurements, what you are going to do is initially the rate of product formation is linear with respect to time. But as there is there are many reason for the loss of linearity over time to avoid such complexity is it important to measure the initial rates ok. This means you are actually going to measure only in the initial part of the curve, right, because that is going to be linear and you know that a linear part is actually going to give you the accurate information.

Frequently, the linear portion of an assay is sufficiently extended to allow the initial rate to be accurately estimated simply by drawing a tangent or taking of first derivative or the early part of the progressive curve ok. So, this is very easy because when you are doing the initial curve.

It can actually be able to extended and then that is how or you can actually be able to derive the equations and that is how you can be able to use that part of the curve to calculate the different you know properties or different kinetic parameters of the enzyme.

The concentration of the enzyme is reducing to prolong the linearity of the progressive curve whereas the loss of linearity is very rapid. This also increased the sensitivity of the assay. So, when you are doing the initial rate measurements, you are also going to increase the sensitivity of the assay because in this particular range, in this particular curve, the you know the assay is very sensitive. Suppose you add any kind of inhibitor, this actually is going to get affected.

It is possible that the saturation point may not get affected, but this portion is going to get affected. It has frequently been assumed that the limiting measurement of reaction rate to a period in which less than 10 to 20 percent of the total subject volume will provide a true the initial rate measurements ok.

Starting an assay by introducing one of the component and ensuring adequate mixing can lead to the considerable uncertainty about the precise time and the reaction had begun. So, one of the major issue with the initial measurement is that you are not very sure about when you started the reaction because when you are going to add one component, the last component, then the reaction is going to start. But the precise time when the reaction had begun is very difficult to say right.

So, sufficient time must be allowed to adjust the initial conditions such as the linearity it is maintained for enough time to measure the initial rate. So, it is not only important that you are actually going to make the initial measurement. It is also important that you allow the enzyme to get stabilized because initially the enzyme is actually going to take time to recognize the substrate.

Then it is also going to you know start catalyzing it is not like it started jumping on all the substrate and then started eating them right or started converting them. So, it is actually going to be a molecular interactions and initially the molecular interactions are going to be slow and then it is actually going to catalyze. So, that is why it is not important that you do measurement in the as early as possible, you can actually let the enzyme to get stabilized, get you know started reactions and then you actually can measure ok.

And then you can have the entire progress curve must be taken into account when measuring the complex enzyme assays where substrate depletion and equilibrium is not the sole reason for the loss of linearity in the progress curve. Now, you can see that even if you are doing the measurement in the initial curve, there are many issues related to even the initial measurement as well.

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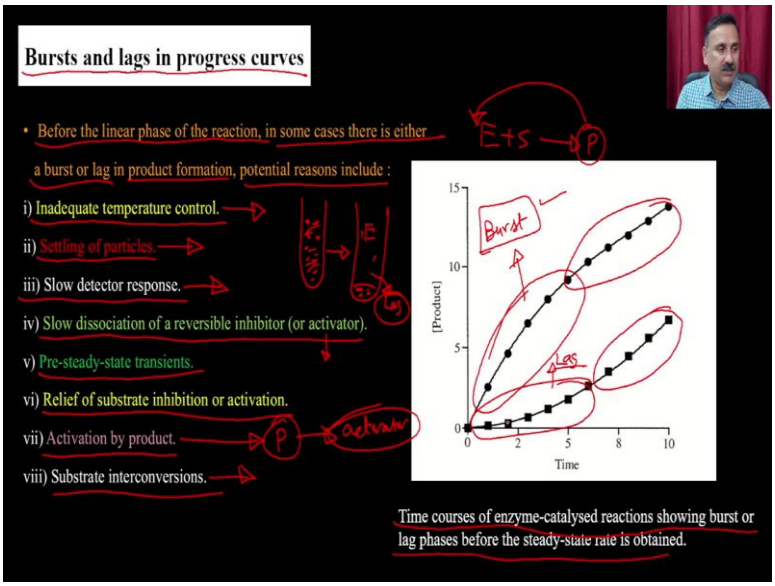

And the worst issue is the burst and the lag in the progress curve. So, what you can see here is that i am showing you the two curve where the later part of the curve, right this part of the curve is different from the this part of the curve right. This part of the curve is actually showing of a phenomena which is called as burst right this means the enzyme is starting very rapidly.

Whereas in this case what you see here is that this part of the curve is very different from the this part of the curve. So, this part is actually showing a lag phase which means the enzyme is taking very long time actually to recognize the substrate and actually going to catalyze the reactions.

So, if you are measuring the reactions in the initial time curve or initial very initial time curve, then you are actually going to make a mistake because if it is a burst conditions, you are actually going to do the overestimations ok. And if it is a lag kind of enzyme, you are actually going to make the underestimations. So, before the linear phase of the reaction, in some cases there is either a burst or lag in the product formation.

And the potential reason includes is inadequate temperature control. So, sometime what happen is that there is a you know temperature is not isothermic. So, it is actually going either be very high whereas in the case of burst or it could be very low in the case of lag in the case of lag. So, that actually is also going to control the activity of the enzyme.

Then sometime when you are set when you are doing the reactions, the reaction components may not be soluble into the buffers and they may be keep settling during the reaction. So, what happens is that suppose this is a test tube right and I have taken the enzyme and when I have taken the enzyme of the components. So, what happen is that some of the components are particulate right.

So, what happen is they will go so, after sometime what will happen is they will actually going to be settled down at the bottom right. And once the enzyme is present here right so enzyme is present in the solution. But there are these some of these particles which are also important for the reactions are settling down on the corners. So, that is how they were actually going to you know change the reaction rates and they may actually induce the lag or burst.

Then sometime we have the slow detector response. It means your measuring devices are also having the fault. So, they will be actually either measuring the very quickly or they were taking sign to measure and because of that enzyme is catalyzing its reaction, but you are measuring very slowly. So, that is why you can actually have this particular type of the curve.

Sometime you have the slow dissociation of a reversible inhibitor or the activator so, in that case if you have slow inhibitions, it may actually be able to you know have the lag phase a longer lag phase or if you have this slow dissociation of activator, you may have the burst and that is why these are the two phenomena which actually going to make the initial measurements wrong.

Then you can have the pre-steady state transients or relief of the substrate inhibition or activations. Then you can also sometime have the activation of the product. So, this is very very interesting because when you have the product which is actually been working as an activator. So, what happened is that in the lag phase what happened is the enzyme is you know converting a converting a substrate into the product and then the product is actually you know working as an activator.

So, Initially the curve is actually going to be slow, but as soon as some amount of product is formed it is actually going to activate the enzyme and that is how it is actually going to you know activate the activity of the enzyme. Then you can also have the sub state inter convergence which means the substrate are actually going to be converted to

each other and that is how they are also going to affect the burst or the lag phase in the progress curve. So, the time course of the enzyme catalyzed reactions showing the burst or the lag phase before the steady state rate is obtained.

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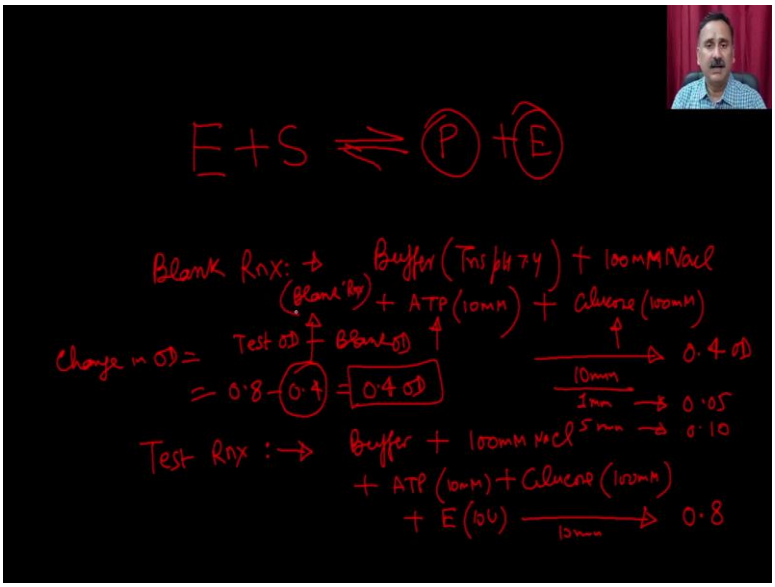

This is all about the enzyme. So, in an enzyme when you are actually going to do an enzyme catalyzed reaction what you are going to do is you are actually going to have these right. This means you are going to have the product and enzyme is being formed right and if I set up the reactions what I am going to do is I am going to set the blank reactions right.

So, in a blank reaction what I am going to do is I am going to take the buffer right where I am going to catalyze for example, this pH 7.4 right and I am going to add the any kind of salt or additives for example, in this case I am suppose keeping it 100 millimolar NaCl.

So, it is actually going to give me some provide some kind of biological environment and I am going to add the some amount of substrates. So, for example, if I if this is a ATPase for example so, I am going to add like ATP which is 10 millimolar right and that is it ok and then I am going to add the substrates. So, I am going to add the substrate like for example, glucose ok.

So, 100 millimolar for example, ok so, I am going to add the glucose. So, I am going to add the ATP and everything remember that I have not added the enzyme. So, this is actually going to be a blank reaction. So, imagine that I got an OD after 10 minutes for example, ok.

So, if I did the incubation after 10 minutes, I am going to get a OD of 0.4. So, this is the 0.4 absorbance ok. So, this is the OD values. Now, I am going to do a test reaction ok. So, test reactions now I am going to have everything I am going to have the buffer I am going to have the salt.

So, everything I am going to take from the blank reactions I am going to have ATP and I am also going to have the glucose right and at the end what I am going to do is I am going to add the enzyme. So, for example, I have added 10 units of enzyme. So, and then I will start the reactions and after 10 minutes I am going to have an OD of 0.8 for example, ok. This means if I want to know what is the enzyme catalyzed reactions OD. So, what is the change in OD right.

So, what I am going to do is I am going to do the test OD minus blank OD ok this means 0.8 minus 0.4 and that is the 0.4 OD what is been changed when the enzyme was added right and this is what you are actually going to calculate after every time points like 1 minute, 2 minutes, 5 minutes like that ok and what you will see is that this measure this value is also going to be changed for example, if I do it after 1 minute I may have a value of 0.05 if I do the after 5 minutes I will have the value of 0.1 and so on.

So, this value is very important because this is actually going to change your final values ok and that is why this value is actually going to be contributed by the blank rates or blank reactions right and the blank reactions are very very important in a enzyme assays.

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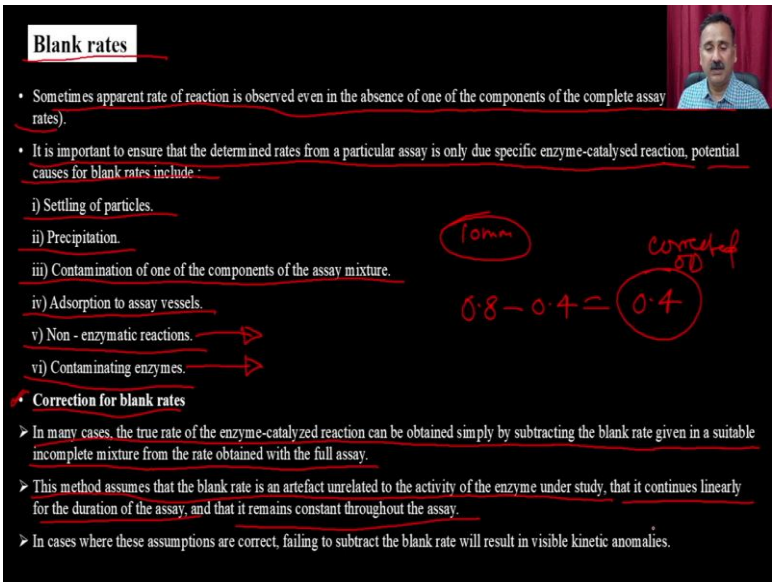

So, blank assays or blank rates. So, sometime the apparent rate of reaction is observed even in the absence of one of the component of complete assays which are called as and that are called as blank rates right. And blank rates are very important because they are actually going to be decide what would be your final rates of the enzyme because in the blank rates are very high for example, if I am getting a OD of 0.8 0.9 then your you know the sensitivity is actually will be very low because the blank rates are very high.

So, it is important to ensure that the determined rates for a particular assay is only due specific reaction is only due to the specific enzyme catalysed reactions. So, potential cause for the blank rates include settling all the particles, precipitation, contamination of one of the component of the assay react mixture, adsorption to the assay vessels and non-enzymatic reaction. So, this is the non-enzymatic reaction the blank what I am talking about or some of the contaminating enzymes, so that are also going to be responsible.

Now, how are you going to correct about the blank rates right in many cases the true rate of the enzyme catalyze reaction can be obtained simply by subtracting the blank rate given in the suitable incomplete mixture from the rate obtained with the full assay. So, that is why I said you know when you subtract the 0.4 from 0.8 that is what you are going to get.

So, this is actually the corrected OD values for this particular time point for example, 10 minutes right and that is how you can be able to overcome the blank rates, because

ideally when I am not adding the enzyme you know ideally there should be no formation of product, but in some of these cases you are actually going to see some of the enzyme which is going to be formed.

This method assumes that the blank rate is an artefact unrelated to the activity of enzyme under study that is continues linearity for the duration of an assay and that it remains constant throughout the assay. In some cases where these assumptions are correct failing to subtract the blank rate will result in the visible kinetic abnormalies.

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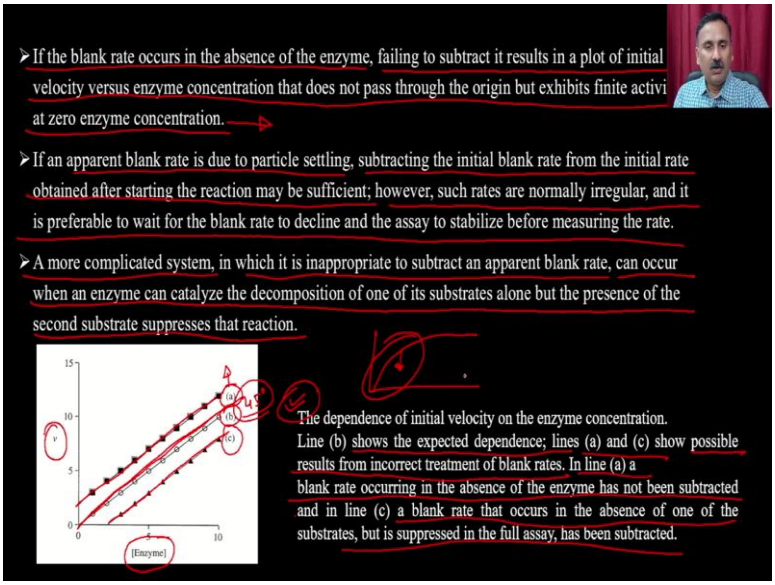

Then if the blank rates occurs failing to subtract it result in a plot of initial velocity versus enzyme concentration that does not pass through the origin, but exhibit finite activity as a 0 enzyme concentration.

If the apparent blank rate is due to the particle settling subtracting the initial blank rate from the initial subtracting the initial blank rate from the initial rate obtained after starting the reaction may be sufficient. However, such rates are normally irregular and it is preferable to wait for the blank rate to decline and the assay to stabilize before measuring the rates.

In a more complicated system, in which it is inappropriate to subtract an apparent blank rate, can occur when the enzyme can catalyze the decomposition of one of the substrate alone, but the presence of the second substrate suppresses these reactions.

So, the dependence of the initial rate on the enzyme concentration so, what you are going to see here is that the initial reactions are or initial measurements actually depends on the concentration of the enzyme and they are also going to be very very sensitive for the these blank rates because the blank rates ok because the blank rates actually can reduce the OD values. So, what you see here is that I have shown you how the enzyme concentration is actually going to change the rate of the product formations.

So, in the line b what you see. So, it is three conditions a, b and c ok. So, b it shows the expected dependent line which means it is actually going to show you a linearity at 45 degree angle. Ok so, this is actually going to be ideal, whereas a and c showed the possible result from the incorrect treatment of the blank rate.

In line a the blank rate occurred in the absence of enzyme has not been subtracted. So, this is actually the ideal rates what you are going to measure. In the absence or in the when there is no the you know blank rates, but if you have the blank rates and if you do not subtract those blank rates then you are actually going to expect a curve which is look like as the curve given in the a.

Whereas the c a blank rate occurs in the absence of one of the substrate, but it is suppressed in the full assay has been subtracted. So, this is actually the under representation this is the over representations of the same enzyme assays because the blank rates are affecting that.

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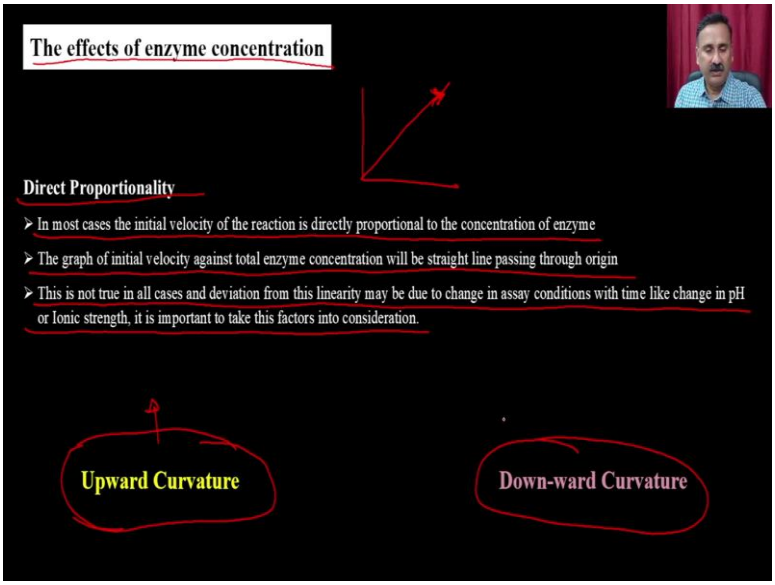

So, the effect of the enzyme concentration on the enzyme kinetics or the product formation can be of two types. It can be directly proportionality. So, in most cases the initial velocity of the reaction needs directly proportional to the concentration of the enzyme.

The graph of initial velocity against total enzyme concentration will be straight line passing through the origin. So, that is what I have shown you right. This is actually going to be like this. So, initial rates are very very sensitive for the concentration of the enzyme and this is not true in all cases of derivation from this linearity maybe due to change in assay condition and with the time like change in pH or the ionic strength, it is important to take this factor into considerations.

So, you can have the two different types of effects when you are going to see the effect of enzyme on the initial rate measurements. It can have the upward curvature or you can also have the downward curvatures. So, let us see what is the upward curvature.

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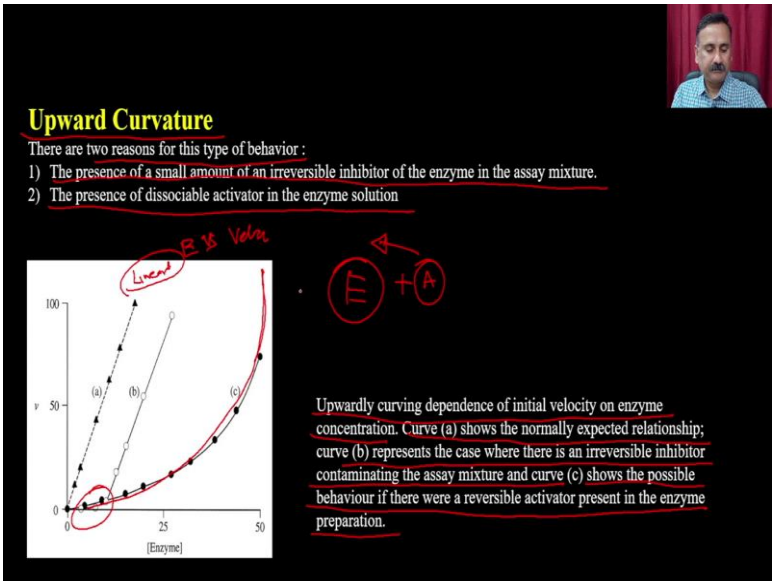

So, when you are actually going to see how the enzyme presentation is going to induce the upward curvature. So, there are two reasons for this type of behaviour. So, what you going to see is that when you are actually increasing the enzyme what you see is that there is an increase in upward curvature right which means the velocity is increasing and so these are the initial rates ok.

So, there are two reasons for this kind of behaviour, the presence of a small amount of an reversible inhibitor of the enzyme in the assay mixture and the presence of dissociable activator in the enzyme solution.

So, upward curving dependency of initial velocity on the enzyme concentration so, curve a shows the normal expected relationship right. So, this is the curve a which is going to show a linearity right. Linearity between the enzyme presentation versus verses you know velocity of the enzyme velocity of the reactions.

Whereas the b represent the case where there is a irreversible inhibitor contaminating the assay mixture. So, b is the time place where when you are actually going to have the irreversible inhibitors and because of that there would be a delay in initial kinetics and that is how it is actually going to show you the linearity afterwards. And then the c is the show the possible behaviour if there are reversible inhibitor activator present in the enzyme preparation.

So, when you are actually isolating the enzyme, it also contains one of the activator along with it. So, when this activator is present it is actually going to activate the enzyme activity over the course of time and that is why it is actually going to show you an upward curve. Then you can also have the downward curvatures.

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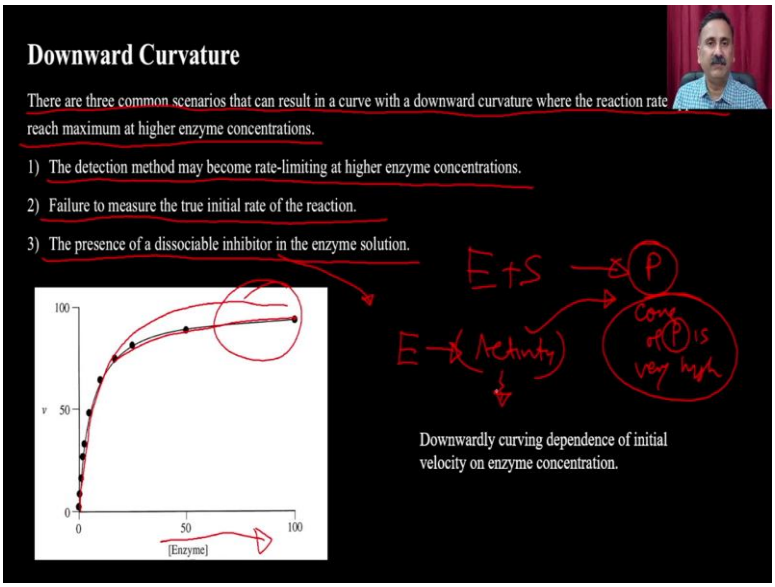

So, there are three common scenario that can result in a curvature curve with a downward curvature with a reaction rate appears to be reach maximum at a high concentration rate. The detection method may become rate limiting at the high rate concentration. So, these are the downward curvature depend on the initial velocity of the excursion. So, what you are going to have is when you are keep increasing the enzyme concentrations initially it will be linear, but later on it is actually going to be downwards ok.

And one of the major reason is that the detectors or the detection system what you are going to use for these kind of assays may not be you know vary the rate limiting which means they will not be able to measure the substrate or the product what is being formed which means when the enzyme is reacting with the substrate and it is forming the product the concentration of product is very high. So, concentration of product is very high and as a result it is actually reaching to a saturation level and because of that it is actually going to show you a downward curvature.

Then failure to measure the true initial rate of reaction because of the you know responses of these instruments and other kind of thing you will not be able to do the real initial measurements of the reactions and the presence of dissociable inhibitor in the enzyme solution. So, sometime you may have some kind of inhibitors and that may also getting dissociated and that is how it is actually going to show you the downward curvature.

Now, whether you are getting the upward curvature or whether you are getting the downward curvature the enzyme is actually going to show you activity right which means because of this activity the enzyme is going to convert the substrate into the product. Now, the question is how you are going to express this activity and how you can be able to use that information for determining the other types of parameters.

So, how you can be able to express this activity because you can have the you know enzyme from the multiple sources you can have an enzyme from animal source you can have the enzyme from plant source and so on. So, how you will compare which enzyme is more active and which enzyme is less active and so on. So, there is a universal activity what is or activity definition that is what required to compare the enzymes from the different sources even the same enzyme from different sources.

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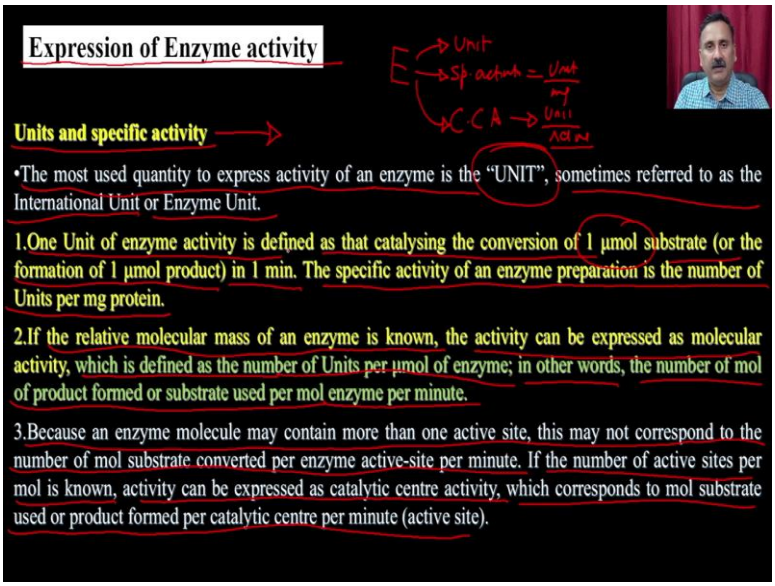

So the expression of the enzyme activity, so one of the units is unit and the specific activity that is the universally very very popular way of expressing the enzyme activity. So, what is the one unit of enzymes?

So, the most used quantity to express the activity of enzyme is the "UNIT" of enzyme sometime referred to as the international unit or the enzyme unit. What is the one unit of enzyme? It is defined as the catalyzing the conversion of one micromole substrate or the formation of one micromole product in one minute. The specific activity of an enzyme preparation is the number of units per milligram of proteins.

If the relative molecular mass of an enzyme is known the activity can be expressed as the molecular activity which is defined as the number of units per micromole of enzyme. In other words, the number of moles of product formed or substrate used per mole of enzyme per minute and that will actually going to give you the molecular activity.

Because an enzyme molecules may contain more than one active site this may actually this may not correspond to the number of mole substrate converted per enzyme active site per unit. If the number of active site per mole is known the activity can be expressed as the catalytic centre activity which corresponds to the moles of substrate used or product formed per catalytic centre per minute or so, this is the you know.

So, the enzyme activity can be expressed in three ways. One is you can express it in the terms of unit, you can express it in terms of specific activity which is equivalent to unit divided by milligrams of protein or you can actually be able to express in terms of the catalytic centre activity so, catalytic centre activity where you are actually going to say that unit of an enzyme divided by the number of active sites ok.

So, number of active site of the group ok and either of these three way you can actually be able to express the enzyme activity. But recently the people have also adopted the new activity units for measuring the enzyme activity.

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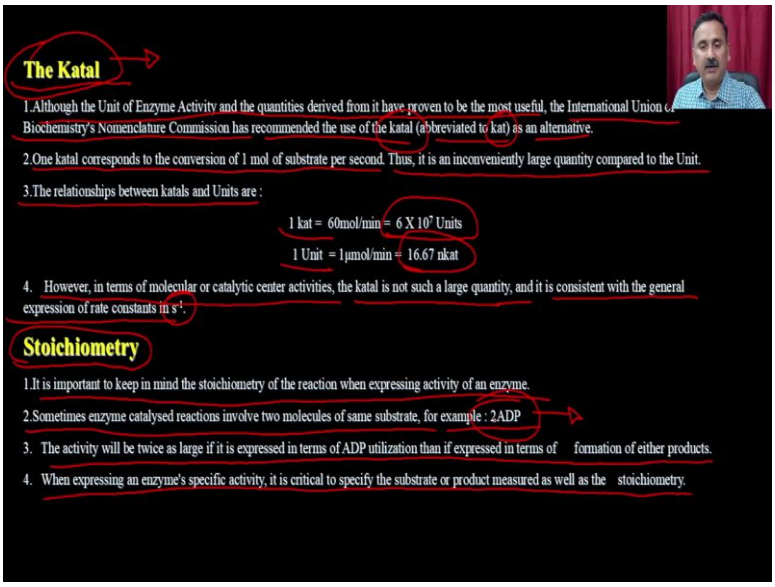

So, one of the that unit is katal, although the unit of enzyme activity and the quantity derived from it proven to be most useful the International Union of Biochemistry and Nomenclature Commission IUBNC has recommended the use of katal abbreviated to kat as an alternative.

So, one katal correspond to the conversion of one mole of substrate per second thus it is in conveniently large quantity compared to unit. So, the relationship between the katals and the units are one katal is equivalent to 60 moles per minute or 6 into 10 to the power 7 units. So, you can actually be able to use this unit which is a recent unit and the relationship between the katal and unit is that one katal is equivalent to 6 into 10 to the power 7 units or 1 unit is equivalent to 16.67 nano katals.

However, in terms of the molar or catalytic centre activity the katal is not such a large quantity and it is consistent with the general expression of the rate constant in the per second. While you are doing this activity measurement stoichiometry is very important. So, it is important to keep in mind stoichiometry of the reaction when expressing the activity of the enzyme.

Sometimes the enzyme catalysed reaction two moles of the same substrates for example, the 2 ADP right. So, in that case you might have to you know change the concentrations accordingly. So, the activity will be twice as large as it is expressed in terms of ADP utilizes then if it is expressed in terms of the formation of either product. When expressing an enzyme activity, it is critical to specify the substrate or the product as well as the stoichiometry.

Now, there are multiple way or multiple conditions what you have to consider before you are going to design and design an assays and you are going to use that for measuring the activity.

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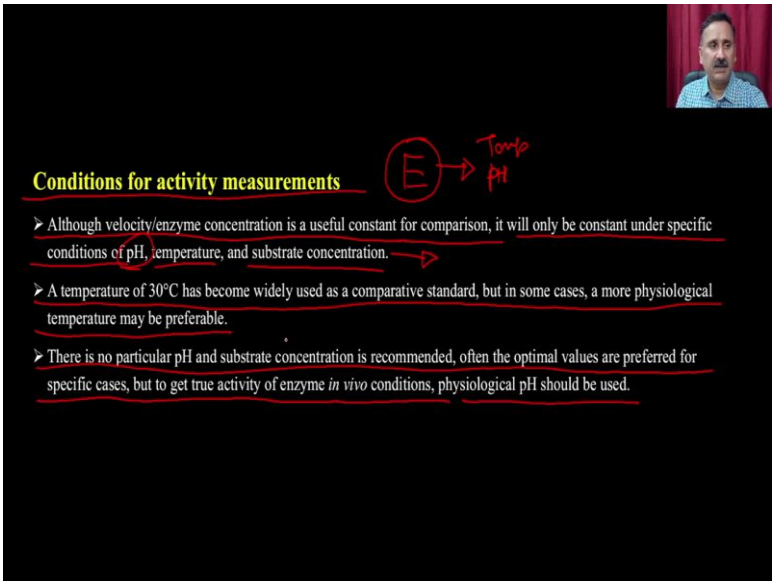

So, conditions for the activity measurements. So, although the velocity or the enzyme concentration is a useful constant for comparison, it also be constant under the specific conditions for example, the pH, temperature and the substrate concentration because many of these parameters are actually going to change the enzyme activity.

And that is why it is important that you should also define that at what pH, at what temperature and at what initial concentration of the substrate you are getting this activity because it is not that the enzyme is actually going to show you the same activity even if you are doing the reaction at 4 degree or 27 degree or something like that.

So, that is why the enzyme activity you are actually going to show, but you also have to show the activity conditions in which what temperature you have used or what pH you have measured and all that, because even if you do like pH measurements and if you do the activity at a pH where the enzyme is not very active it is not going to show you the activity.

The temperature of 30 degree Celsius has become widely used as a comparative standard, but in some cases, a more physiological temperature may be preferable. There is no particular pH and substrate concentration is recommended often the optimal values are preferred for the specific case, but to get the activity of enzyme in vivo conditions physiological pH should be used.

Now, you have we have discussed. So, what we have discussed we have discussed about the different types of enzyme activities, we have measured how we can be able to express the enzyme activity, we have also discussed how the different parameters are actually going to impact the activity measurements, the initial rate constant and all that. Now, we should discuss how and different ways in which you can be able to measure the enzyme activity.

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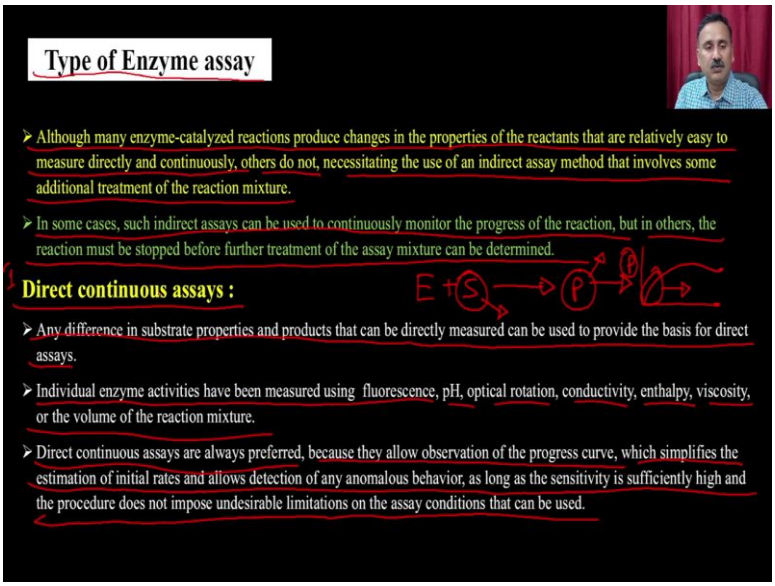

So, type of enzyme assays. So, enzyme assays could be of two types either they will be direct continuous assays or they could be indirect continuous assays. So, although many enzyme-catalyzed reaction products produce change in the property of reactant that has relatively easy to measure directly and continuously others do not, necessitating the use of an indirect method that involves some additional treatment of the reaction mixture.

In some cases, such direct measurement can use to continuously monitor the progress of reactions, but in their cases, reaction must be stopped before further treatment of the assay mixture can be determined. So, you will see that when we are going to discuss some of the assay mixtures and when we are going to discuss about some of the reaction conditions you will see that how the some of the assays are continuous direct measurements and some are continuous indirect measurements.

So, one is the continuous direct measurements. So, any difference in the substrate property than the product can be used directly measure can be used to provide the basis for direct assays. Direct assay means the enzyme is making a substrate and it is forming the products right and you can easily measure this product directly which means either the product is colored or the substrate is colored and you can actually be able to measure how much the substrate left right. So, either of these cases you can actually be able to do direct measurements.

Individual assay, individual activity has been measured using fluorescence, pH, optical rotations, conductivity, enthalpy, viscosity or the volume of the reaction mixture. Direct continuous assay are always preferred because they allow the observation of progress curve which simplifies the estimation of the initial rates and allow the detection of any anomalous behaviour as long as the sensitivity is sufficiently high and the procedure does not impose undesirable limitations on the assay condition that can be used.

So, it is very easy and it is straightforward. That straightforward you see that the product is forming and that is how what you can do is you can just plot the product with time ok and that is how you can be able to calculate the initial rate constant. You can actually be able to use that for calculating the different types of other kinds of kinetic parameters whether the KM, K kAT and all that kind of thing.

So, that is desirable, but many of the cases what happen is that neither the product is very good in terms of unique properties or substrate is also not good in terms of unique properties. So, that you can use that to measure and there is an interference. So, when you are having that kind of assay you are actually going to use the indirect methods to measure the assays.

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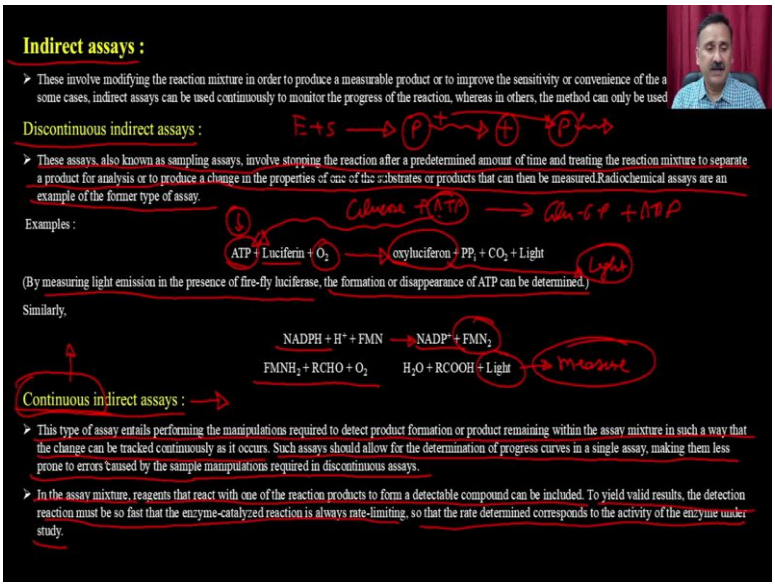

So, indirect assays or indirect measures so, indirect you can have discontinuous indirect assays you can have the continuous indirect assays. So, discontinuous assay where you are actually going to have like enzyme plus substrate forming a product and then you are

actually going to recover or you can actually be able to purify this product and then you are going to put it into another reactions ok and that is how it is actually going to be the discontinuous indirect assay.

So, these assays also known as sampling assay involve stopping the reaction of our pre-driven amount of time and treating the reaction mixture to separate a product for analysis or produce a change in the property of one other substrate or product that can then be measured. Radio chemical assays are the examples of the these kind of assays.

So, for example, you can have the ATP, plus luciferin plus oxygen and it is actually going to give you the oxyluciferin ok. And these are the this is one of the assay which actually can be used to measure the ATP for example. So, suppose this is the assay what I am doing right glucose plus ATP giving glucose 6 phosphate plus ADP.

So, if I want to measure this it is very difficult. So, what I am going to do is I am going to recover the ATP and I am going to put that into this assay and that is how it can be used for measuring the ATP concentrations because the oxyluciferin is actually going to give you the light and that light can be measured.

By measuring the light emission in the presence of firefly luciferase the formation or the disappearance of ATP can be determined. Similarly, you can have the NADPH FMN and it is actually going to give you the NADP plus, and here also you are going to have the FMN 2 is formed and that can be put into this reaction and that is how it is actually going to produce the light and that light can be measured. So, this is actually going to be indirect method because after some time you have to take out these reactions and then you have to measure the ATP you cannot do simultaneously.

Then we can have the continuous indirect assays so, continuous indirect method where you are actually going to have the indirect measurements. It means the substrate is actually going to product is actually going to be measured. But you do not have to aliquot. You can actually be able to add something and then the product is actually going to form the p-prime and that is actually going to have the exclusive property which can be measured.

So, this type of assay entail forming the manipulating manipulations required to detect forward formation or product remaining within the assay mixture in such a way that the

change can be tracked continuously as it occur. Such assays should allow for the determination of the progress curve in a single assay making them the less prone to errors caused by the sample manipulation required in the discontinuous assays.

So, the continuous assays are much better because they are actually going to reduce. First of all they are actually going to make the things faster right because you can actually be able to add everything and then it is actually going to give you the direct measurements.

The second is it is actually going to be less prone to the artefacts because in this particular system when you are aliquoting the reactions, mixtures and you are actually taking out some amount it is actually not going, it is going to interfere with the enzyme kinetics.

In the assay mixture, reagents that react with one of the reaction product to form a detectable compound can be included. To yield the to yield the valid results the detection reaction must be so fast that the reaction catalyzed reaction is always rate limiting, so that the rate determined component to the enzyme activity of the enzyme under assay. This is very important parameters when you are trying to design the continuous indirect assays.

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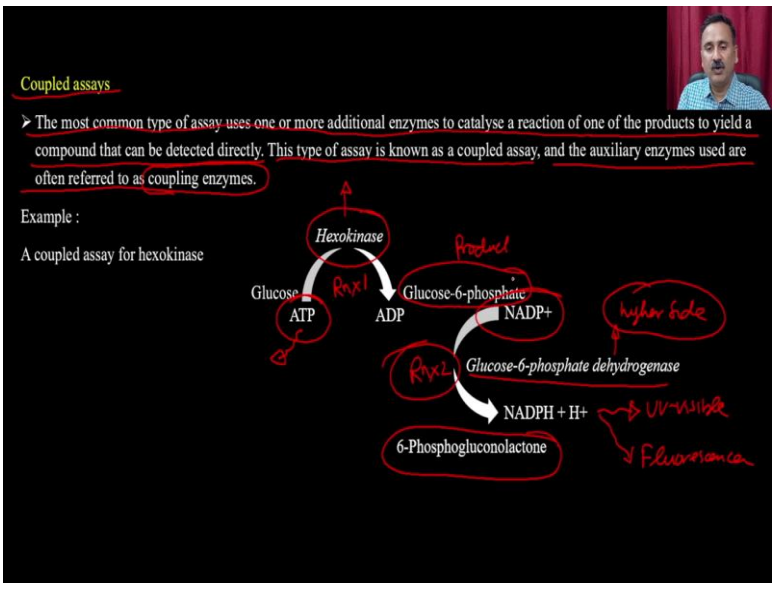

Then we have the coupled assays. So, coupled assays means you are this is the examples of coupled assays where the glucose is getting converted into glucose 6 phosphate with the enzyme of the hexokinase.

So, if you want to measure the activity of hexokinase, we have two options either we couple the formation of ATP to some system or we can actually be able to couple the glucose 6 phosphate to another system right. So, we can actually put the glucose 6 phosphate to glucose 6 phosphate dehydrogenase and that is how it is actually going to show me depletion of NADP plus right.

And that can be monitored both ways that can be monitored with the UV visible spectro photometer or it can also be monitored with the help of the fluorescence because these molecules are fluorescent molecules, so they can actually be able to show you the fluorescence.

So, the most common type of assay uses one or more additional enzyme to catalyze a reaction of one of the product to yield a product compound that can be detected directly. This type of known is this type of assay is known as coupled assay and the auxiliary enzyme used are often referred to as a coupling enzyme. Now, when you want to do a coupling reactions ok it is very important that the activity of this enzyme should be on a higher side compared to this enzyme. Ok.

So, that the conversion of this substrate conversion of this product to this product should be faster because if this enzyme is fast and this enzyme is slow then there will be an accumulation of the product and that is why it is actually not going to give you the direct measurement or it is not going to give you a real image or real situation of the product considerations.

So, it is very important and when you choose the coupled assays or when you try to develop the coupled assay the coupling partners the second enzyme which is actually going to be called as coupled enzyme is should have a higher you know enzymatic activity. So, that there should be no accumulation of the product from the first reactions. So, this is the reaction 1 and this is the reaction 2 right and reaction 2 should be fast so that there should be no accumulation of glucose 6 phosphate.

So, this is all about the enzyme assays what we have discussed, we have discussed about the basics of the enzymes. So, we have discussed about how what are the different enzyme assays system, what you can use what are the precautions and what are the factors which are actually going to impact the enzyme assays.

And then we also discussed about the effect of enzyme concentration on the assay system and the purpose of these discussion is that so that you should be very careful in the when the concentration of the enzyme into the particular enzyme assays system. And then we also discussed about the role of baseline or the blank rates and how it is actually going to impact the enzyme activity.

And lastly, we have also discussed about the units of the enzyme activity and also, we have discussed about the different types of enzyme assays set up what you can actually be able to use to measure the enzyme activity.

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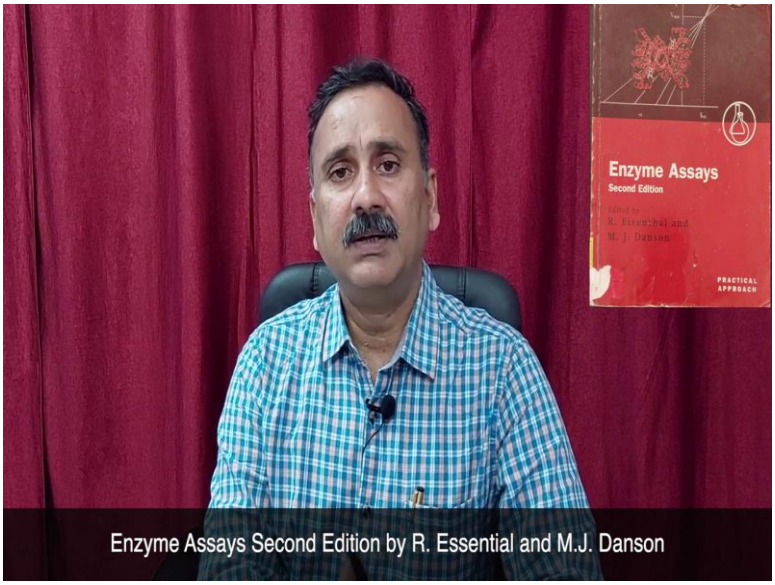

So, with this I would like to conclude my lecture here and if you want to this study or if you want to discuss some more about this aspect what we have discussed today you can actually be able to refer this particular book and I have taken the content from this book. So, it will be easy for you to follow the content. So, with this I would like to conclude my lecture here.

Thank you.