ENZYME SCIENCE AND ENGINEERING

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LECTURE–15

IMMOBILIZED ENZYMES – II

So you would recall that in our previous lecture we had discussed what are immobilized enzymes, why should we immobilize enzymes and what are the requirements of immobilization of enzymes and the three major requirements – a soluble enzyme and the carrier and method of immobilization. We were looking into their characteristics. The soluble enzyme, as we all know, required for immobilization must have certain properties that is it should possess high specific activity and also desirable functional properties that are required for use of immobilization enzyme.

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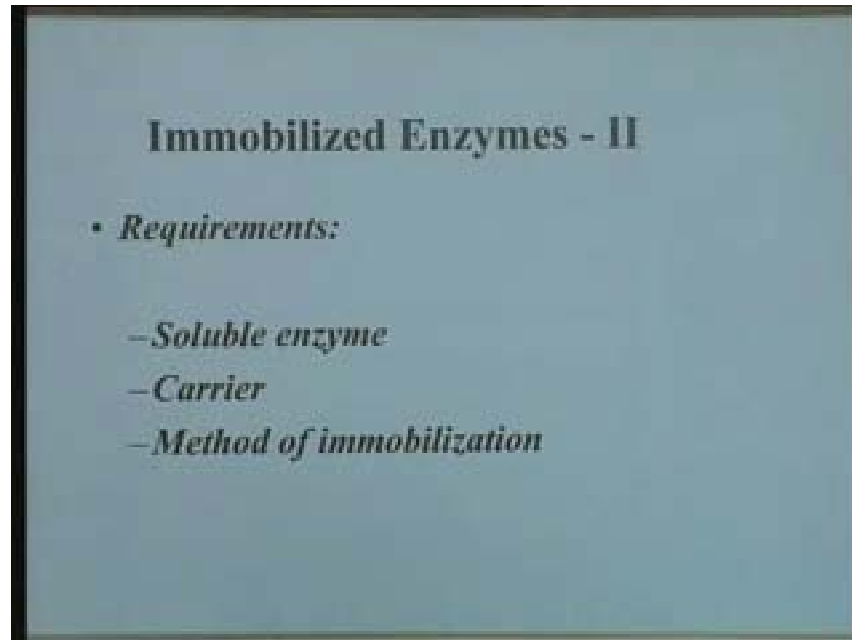

Besides that the carrier must also have certain properties like porosity that means large surface area so that heavy enzyme loading can be given to the immobilized preparation and we were talking about methods of immobilization. A large numbers of methods of immobilization are reported in literature. Most of them can be classified simply into three major categories one is carrier binding methods. In most methods we take either a

prefabricated carrier or a carrier for conditioning either activation or some conditioning and couple that to the enzyme with a physico-chemical interaction.

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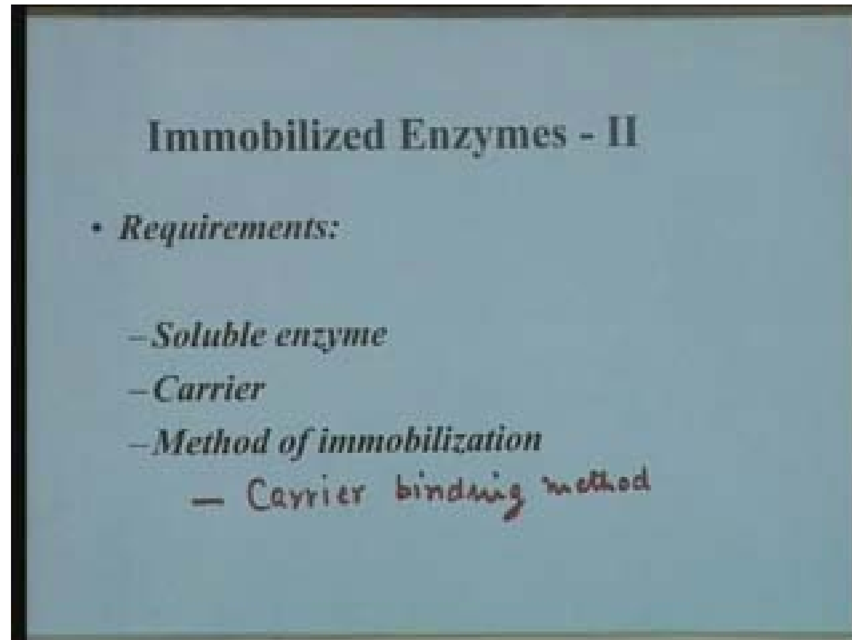

It could be a covalent bond, it could be some non-covalent interactions and those methods fall under carrier binding methods. Typical examples are adsorption; covalent binding and we will be discussing them in detail. The other method is cross linking.

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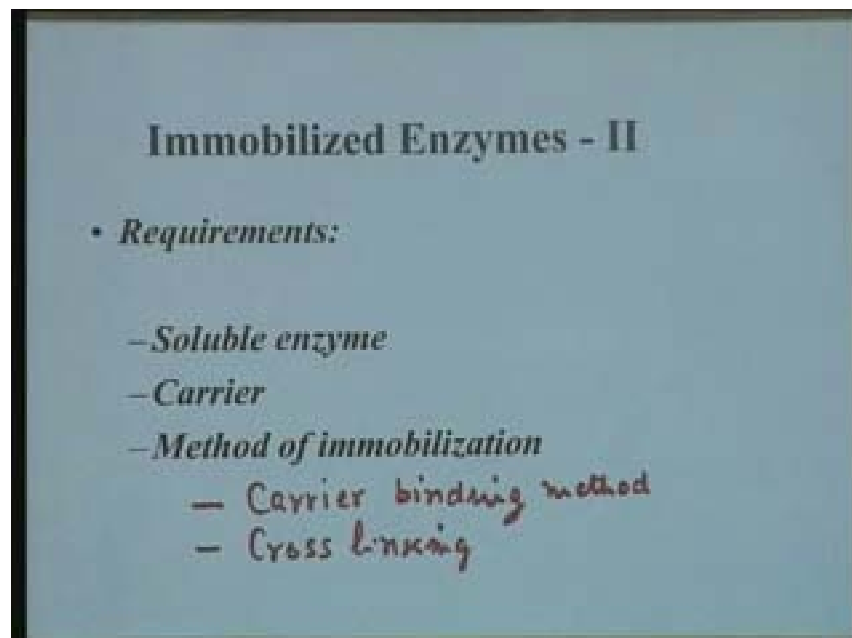

The enzymes themselves posses a large number of functional groups which can be made to react to each other, thereby aggregating the molecules and making them insoluble in water. The aggregates will be insoluble and they can be used as immobilized preparation which can be readily separated from the lipid phase. Just to repeat the cross linking refers to linking more than one enzyme molecule through their functional residues present on their surface. Most of the enzyme molecules will have a large number of functional residues. They may be hydrophobic groups; they may be electrostatic charges or other functional residues. They can be linked under certain conditions, under ambient condition so that their inactivation is not allowed and on those conditions aggregates of the enzymes can be made which can function as immobilized enzymes.

The third type of method is entrapment methods where the enzymes are entrapped within a matrix.

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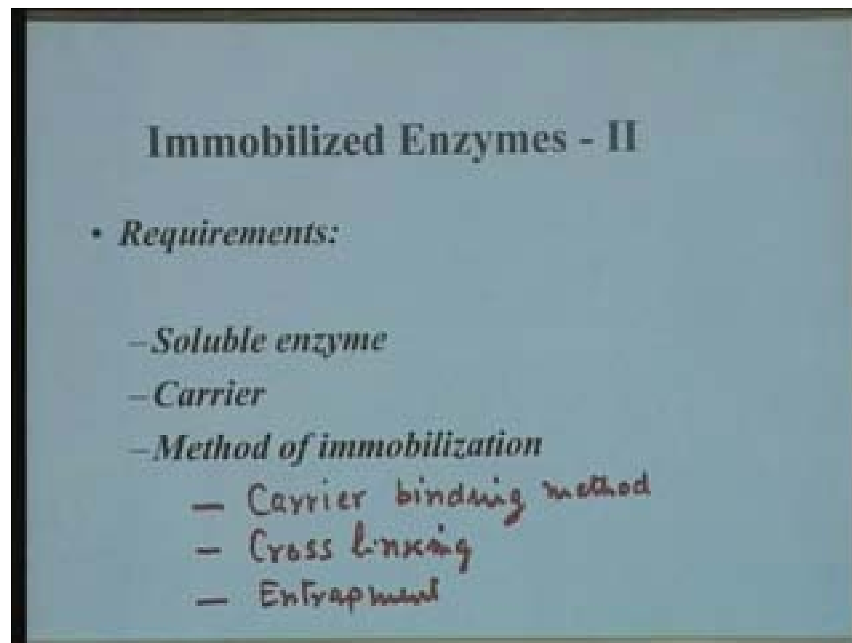

It could be a gel, it could be a micro capsule and there by you could provide them an environment which is very analogues to a living cell and then use them as immobilize preparations. You can see that a large number of methods are available. Under each category also there are number of methods and we need to choose a suitable method for a particular purpose. There are no general rules. It often goes by trial and error and some available information. Out of the methods available, which we will discuss subsequently, if we have to choose an immobilization method to use for a specific application there are four distinct parameters which should be looked into. The first and probably the foremost is yield of immobilization. That means during immobilization procedure what is the loss of enzyme activity because there is no method which can give you hundred percent recovery of the enzyme activity that you have put into the system.

There will be some enzyme which will be deactivated or lost during the immobilization and the loss of enzyme activity has to be kept minimum for all practical purposes. When you consider the yield of an immobilization, it is basically a term which can be considered on the basis of material balance. That means if you take a soluble enzyme, add to a carrier and allow it by any method of immobilization we get two streams - one will be residual enzyme activity in the supernatant which is left uncoupled and immobilized enzyme preparation.

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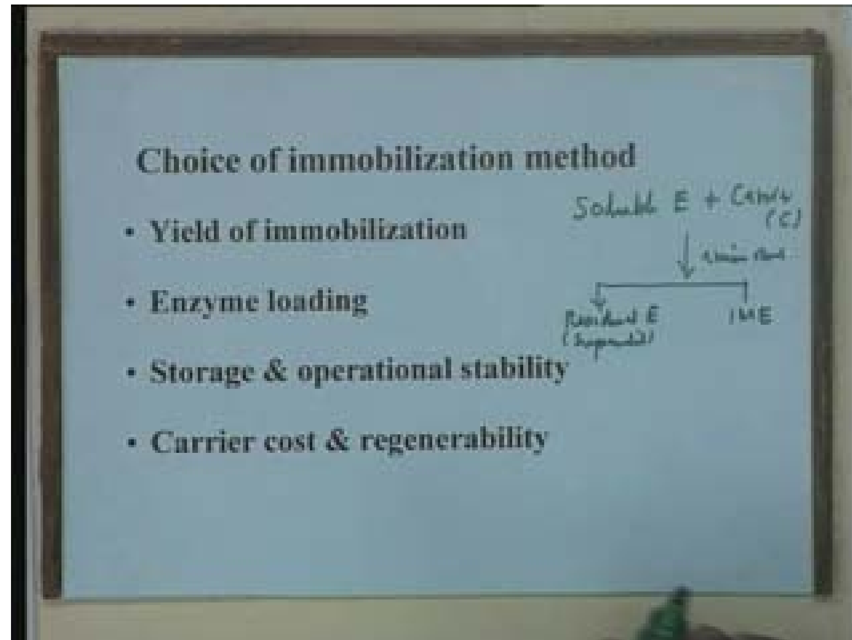

This is the total picture of all the species that will be available and the loss of enzyme activity, soluble enzyme activity let us say as E_0, residual enzyme activity as E_r and the immobilize enzyme activity as E_i, then the loss of enzyme activity is

$$E_0-E_r-E_i/E_0$$

That is the loss or rather fractional loss of enzyme activity.

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Choice of immobilization method

- Yield of immobilization
- Enzyme loading
- Storage & operational stability
- Carrier cost & regenerability

Soluble E (E_0) + Carrier (C) → Residual E (suspended) (E_r), IME E_i

loss: $\left(\frac{E_0 - E_r - E_i}{E_0}\right)$

Of the total enzyme activity, the activity is distributed into two fractions-the immobilized enzyme as well as the uncoupled enzyme in the residue and these two theoretically should combine and form the total value of E_0 but very often at the end of a method there is some balance left and the balance is the loss due to deactivation during immobilization. The yield of the immobilization is defined as

$$r = E_i/E_0\text{-}E_r$$

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Soluble E (E_0) + Carrier (C) → Residual E (suspended) (E_r), IME E_i

loss: $\left(\frac{E_0 - E_r - E_i}{E_0}\right)$

$Y = \frac{E_i}{E_0 - E_r}$

Very often there is confusion in reporting these terms and that's why I have tried to put them in the form of an expression. While we talk of a yield we must keep into mind the residual activity in the supernatant. Its not in the total enzyme because part of the enzyme has been recovered in the supernatant which if desired can be recycled. Therefore the yield will be the activity which is recovered in the immobilized form divided by E_0-E_i in the residue.

When you talk of E_i that is the activity of immobilized enzyme you are measuring in terms of the activity only. So that means it is active. Right in the beginning I made it very clear that when we talk of quantification of enzyme it is just not a physical quantity like mass or concentration. It is the functional property. Its quantity is functional property and the activity represents functional activity. We are not measuring E_i in terms of protein contents. I am measuring all these terms E_0, E_r and E_i in terms of catalytic activity. So it is functional. This loss, if it is a let us a physical quantity like protein concentration the balance will be perfect. You cannot account for any loss of protein in the system. It is only the loss of activity because of the conformational changes. The other factor which is important to be considered is enzyme loading.

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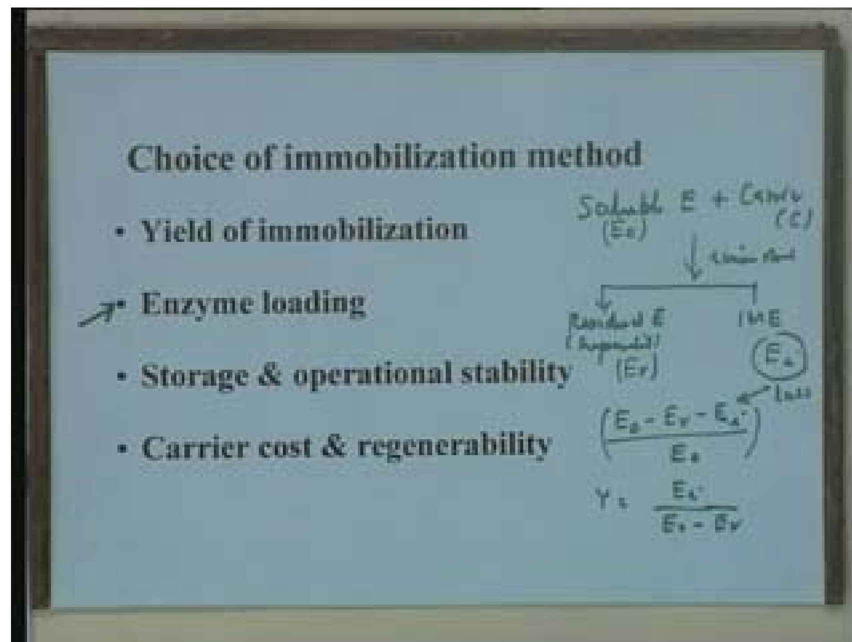

That means that becomes almost similar parameter like what you considered in case of a soluble enzyme that is specific activity. When we talk about specific activity higher the specific activity, enzyme is catalytically better. Similarly in case of enzyme loading we talk of international units per gram of carrier or if you take into account the bulk density of the carrier you can also say international units per ml of carrier bed that means you pack the carrier into a bed, packed bed, and then consider the volume.

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Choice of immobilization method

- Yield of immobilization
- Enzyme loading
- Storage & operational stability
- Carrier cost & regenerability

You can express them in either quantity, either as international units per gram of carrier loading or sometimes we can also refer them in terms of volume and thereby it can be compared directly to the soluble enzyme. So enzyme loading is another factor and we want a preparation with as high loading as possible theoretically. The enzyme loading will also have a direct linkage with the mass transfer limitations. We will see subsequently how mass transfer and catalytic activity are linked. Then the third parameter which is important to look at is storage and operational stability.

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Choice of immobilization method

- Yield of immobilization
- Enzyme loading
- Storage & operational stability
- Carrier cost & regenerability

As I mentioned that we have to look for enzyme and one of the major advantage of immobilized enzyme is that they can be reused, they can be repeatedly used, continuously used and therefore they must possess a much higher stability than the soluble enzyme. This stability is not only in terms of use but also during storage and this parameter is usually expressed in terms of half life.

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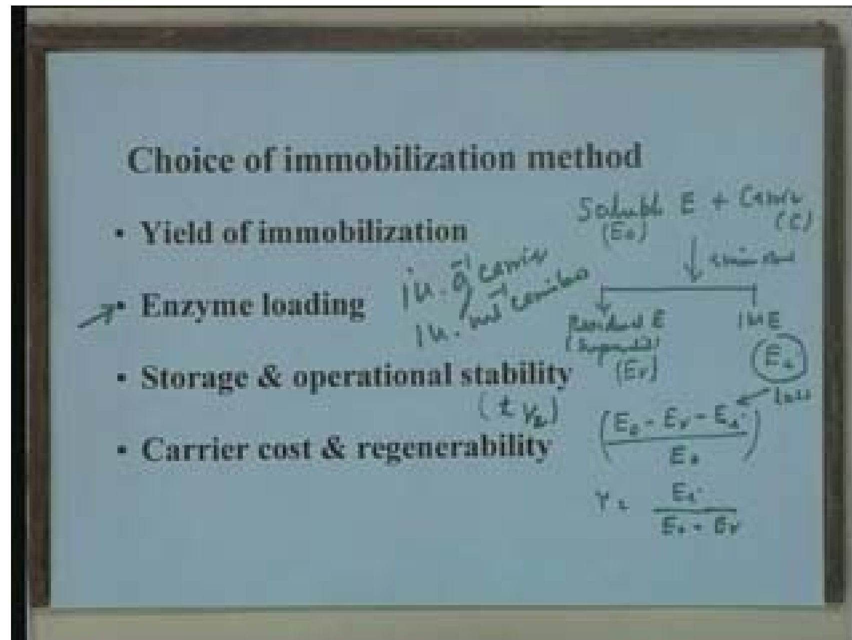

Either half life during storage that means the time in which the activity loses to fifty percent of the original activity or during use in a continuous reactor or in a batch reactor that is in terms of number of cycles. You can express in that term. That will be only restricted when you are talking of a fibre or a surface. The most commonly used matrices are beads- the spherical particles and that’s why gram or volume and they are packed in a packed bed. When I say per ml I am referring to a parameter which is analogues to use in a packed bed. But if you this in a surface, in fibre or in a film you can also express in terms of a surface area. That is always allowed. But for all industrial application the surface excepting for biosensors and things like that which are very specific applications, for industrial catalysis it is used in the form of particles and used with the packed bed. We will discuss those aspects subsequently. Iu/m^2 also is a feasible parameter that we can define.

Then the fourth property which is a very important basis for choosing the immobilization method is carrier cost and regenerability because each method has a limitation of the choice of carrier.

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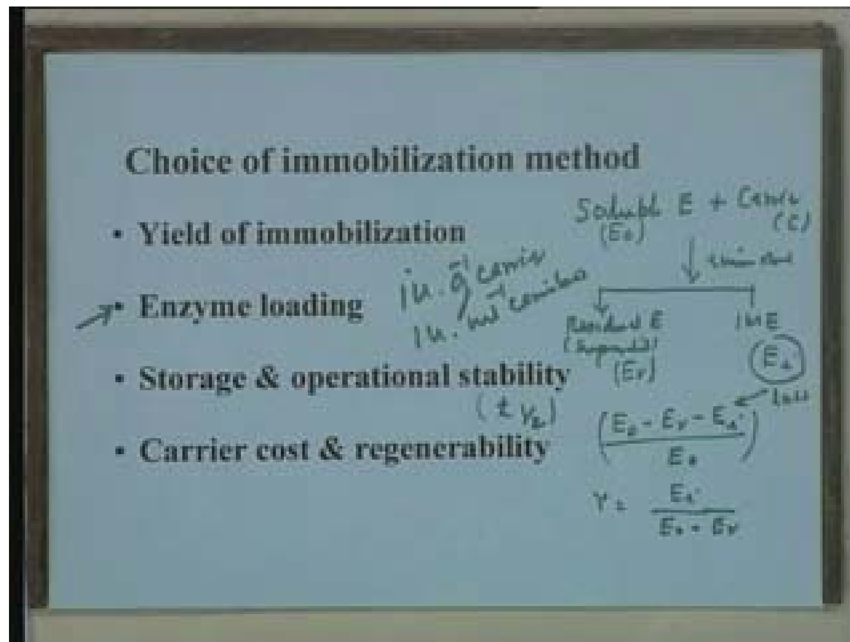

For example in the case of adsorption you require a carrier. If suppose you want to adsorb by ionic binding, you need a carrier which possesses charges. Similarly if you want a covalent binding you need a carrier which possesses certain functional residues which can be coupled to the enzyme. So each method will have some limitations on the choice of carrier and therefore the cost of the carrier. Another parameter which must be taken into account is regenerability. Very often even after the immobilized enzyme preparation has been exhausted during use the carrier can be recovered and reused. So the cost of the carrier must be taken into account in terms of per cycle not the absolute cost. You must differentiate between the absolute cost and cost of the carrier per cycle. Because many of the carriers although expensive, can be used for a long period and in number of repeated cycles. Therefore they are still acceptable and desirable because of other desirable property.

The first and probably the simplest method of immobilization is by adsorption. The adsorption method is a very broad, non-specific method and it involves binding of the enzyme to the surface of an insoluble carrier that has not been specifically functionalized for covalent coupling. I must mark here particularly the phrase “specifically functionalized for covalent coupling”.

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ADSORPTION:

- Binding of the enzyme to the surface of an insoluble carrier that has not been specifically functionalized for covalent coupling.
- An economical method Involving non-specific, multivariant, reversible interactions between enzyme and carrier at mild conditions.
- Usually results in high yields, as it does not cause conformational changes.
- Preparations do not have very high operational stability, but allows regeneration of the carrier.
- Variety of carriers can be used.
- Offers similarity to situations found in biological systems

This probably gives you an idea that this is a very broad non-specific method. If we consider all the carrier binding methods excluding those which involve covalent linkage, rest of all the bindings, interactions between the carrier and the enzyme are covered under the term called adsorption. A very broad based method which involves a number of non-specific, multivariant, reversible interactions between the enzyme and the carrier at mild conditions. The second point also builts in a number of phrases. For example the interactions that are involved are non-specific, very often more than one interaction is involved in a particular method, multivariant that means more than one physical property is employed. In most cases the methods based on adsorption are reversible in nature. That means under certain conditions while you can adsorb the enzyme on to a carrier you can also create condition by which you can desorb the enzyme.

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The basic property of this reversible interaction also makes it a weak binding. Weak binding in the sense while during use, the enzyme can also dissolve slowly over a long period of time and if the carrier characteristics are sturdy they can be regenerated and reused. But enzyme loss can be there big time, over a long period of time. Multivariant means that more than one physico-chemical interaction is involved in binding.

Another feature is mild conditions and that will apply to most of the immobilization methods and also to adsorption.

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That means the environmental conditions required for adsorption must be mild and here I think I like to very specifically define the term mild. Mild refers here not to the value one or two it refers to those conditions under which enzyme is conformationally stable. The definition of mild condition refers to those conditions which are environmentally acceptable to the enzyme. If the enzyme is stable at 70ºC, 70ºC is acceptable and can be considered as mild. But if the enzyme is not stable even at 20ºC you may have to go to 4 degrees to immobilize and the definition of mild will remain at 4 degrees. I have purposely not used the word ambient because you refer it to a well definite range. The advantage of the situation is that usually the methods based on adsorption results in high yields. We are giving the definition of yield that means the total amount of activity, which is resulting in the immobilized preparation is reasonably high, almost in the range of say about 70 to 90 % enzyme activity is recovered in immobilized preparation and because the conditions are mild they don't tend to cause conformational change so that is a very plus point as compared to other methods in which yields will be much lower.

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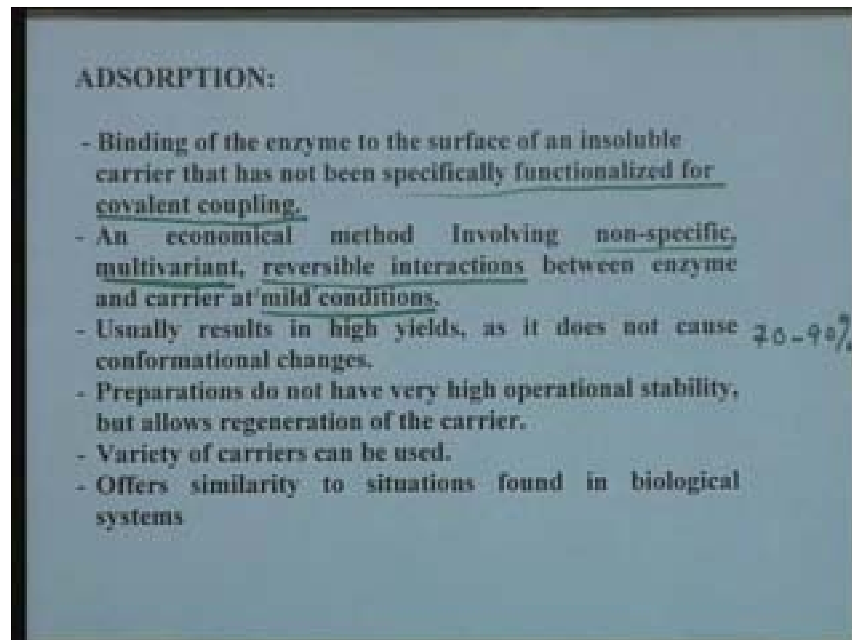

A disadvantage is that the preparations do not have very high operational stability. The method is reversible in nature, the interactions are reversible and so operational stability is usually not very high. Although by various other manipulations like adsorption, coupling with cross linking the operational stability can be improved but intrinsically adsorption based methods are usually operationally high. Again when I say the term high it refers in the comparative form and the comparison here is with covalent binding. If suppose in the case of covalent binding half lives are of the order of few months here it may be of the order of few days. So that is what it refers to but allows the regeneration with carrier. On the other hand although the operational stability is not very high but the advantage is that the carrier can be regenerated and reused. That is the plus point. You have a large variety of carriers that can be used. We will look at some of the examples of

the carriers and finally adsorption based methods have been very often used to study the enzymology of systems that naturally occur in biological system because in case of living cells most of the enzymes are supported on a solid matrix through adsorption only. There are no covalent linkages. Either the enzymes are supported on membrane or they are supported on certain particulate matters and therefore the methods based on adsorption are usually very analogues similar to those that are found in the living system.

Operational stability is defined in terms of half life. Consider the use of immobilized enzyme in a reactor and pack it in a packed bed, feed in and effluent out. Here X-axis is operational time and Y-axis is fraction of conversion. What you notice is that when you start at zero time the fraction of conversion will keep on going down as the enzyme activity is reduced or lost. The fraction of conversion keeping the residence time constant and over operational time, this is operational time not the residence time, residence time is constant here it will go down. If you consider as a first order process then the half life of this that means the time it takes to reach the fifty percent of the original value is half life. If we consider

$$E = E_0 e^{-k_d t}$$

You can simply calculate half life as

$$t_{1/2} = 0.69/k_d$$

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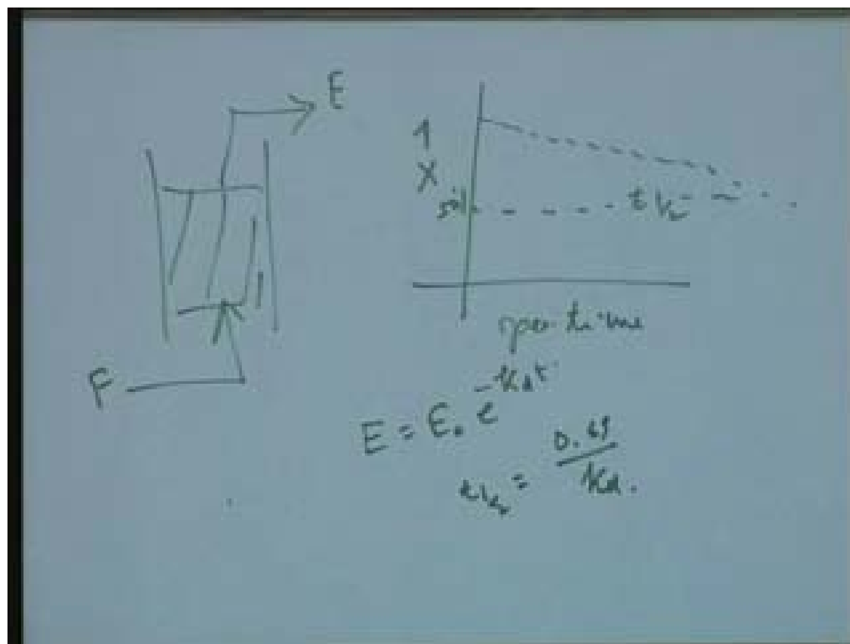

$t_{1/2}$ is less in the case of adsorption. Other way, compared to covalent binding. Right. Because the interaction is reversible in nature so it keeps leaking. A very crude term could be the enzyme keeps leaking values, very small amounts, slowly not in a very fast

rate. This property of leaking of the enzyme from immobilized preparation has been very advantageously used in many cases of the enzyme therapy purposes, where we want to put an immobilized enzyme preparation onsite at a site where the enzyme is required and the enzyme must leak continuously not in a very high concentration and over a long period of time. Some systems like immobilized ……. has been inserted into the blood stream in the immobilized forms - microcapsules and the enzyme leaks slowly and the immobilized system can remain in blood stream over a few months. You have to give another shot to keep the enzyme activity available in the blood stream. So these methods can have an advantage that the reversible nature can also be used in some cases where you want to use the drug or a therapeutic agent with the requirement of slow availability over a long period of time. As far as study of reactor system is concerned, we are not measuring the enzyme activity every time. What we are measuring is the conversion at the effluent. Conversion is related to the enzyme activity. So that's why the graph is in the form of conversion.

Three most common types of adsorption methods are based on physical adsorption which is again a most non-specific type of adsorption where we use hydrogen bonding, electrostatic interactions or Vanderwaal interactions, a variety of interactions where there is no specific reasons and we use a very broad range of carriers like alumina, celite, bentonite, glass etc where which by certain conditions of pH, temperature and salt you can adsorb the enzyme on to the surface and they can be used.

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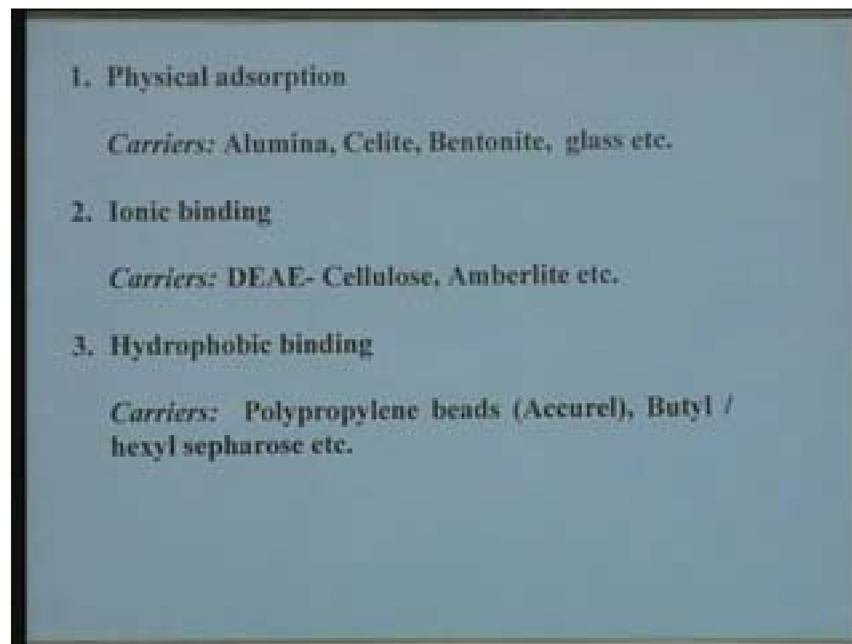

The second method is ionic binding where you take a tailored matrix which has a definite charge on the surface and ion exchange resins are the most commonly used matrices. DEAE-Cellulose, Amberlite etc which are charged particles and these charged particles based on the electrostatic interaction can bind the enzyme molecules at a pH where the

net charge is counter. The enzyme at this stage is counter charged at a particular condition. The third category is hydrophobic binding where we predominantly use hydrophobic interactions on the surface of the carrier and the hydrophobic interactions on the surface of the enzyme protein.

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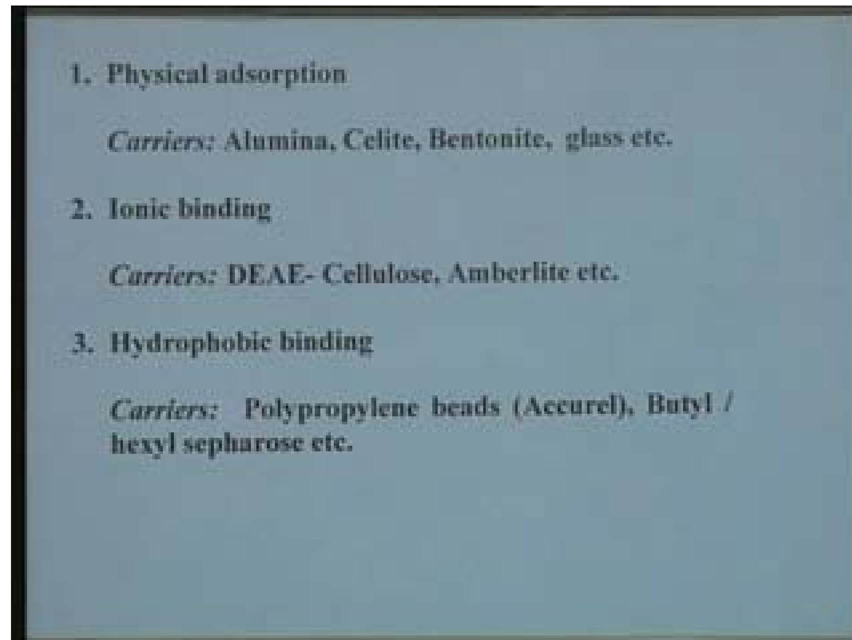

Although bulk of the hydrophobic residues are buried inside the residue of enzyme but still on the surface there are very small hydrophobic patches which can be used here for immobilizing on to the surface of a hydrophobic carrier; carriers like polypropylene beads. There is a commercial product by the name Accurel, which is a microporous carrier consisting of polypropylene, butyl or hexyl sepharose etc. There are number of hydrophobic carriers used and which are available for binding of enzyme. Many of the carriers which are available today for adsorption of enzymes are developed for chromatographic separations. For example all the carriers like hydrophobic binding or ionic binding were originally developed for chromatographic separations where the function is same. That means you are binding through ionic interactions and desorbing it under the conditions of desorption and therefore the same thing applies here. In fact the preparation of immobilized enzyme by adsorption is very much similar to chromatographic separations on ion exchange resin, although we are only interested in the first part of it. Only adsorption part; desorption is not our interest where as in the case of chromatography both the parts are important.

Another important group of adsorption methods is metal salt linkage. Certain metal salts like titanium tetra chloride and ferric chloride form a kind of an inorganic bridge on the surface of the carrier and to which the enzyme molecules can couple. The linkage that is formed, what we have in the literature and what people have used, is the metal salt linkage.

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1. **Physical adsorption**

   Carriers: Alumina, Celite, Bentonite, glass etc.

2. **Ionic binding**

   Carriers: DEAE- Cellulose, Amberlite etc.

3. **Hydrophobic binding**

   Carriers: Polypropylene beads (Accurel), Butyl / hexyl sepharose etc.

4. Metal salt linkage.

It is not a covalent linkage; it is a physical interaction and there by the enzyme is held but has been very successful method from the practical point of view. We will discuss that in detail. Most of the adsorption methods can be characterized by the classical adsorption isotherms. Depending on the nature of the carrier and nature of the enzyme that we are talking about, the isotherms may vary. Three general types of isotherms are known.

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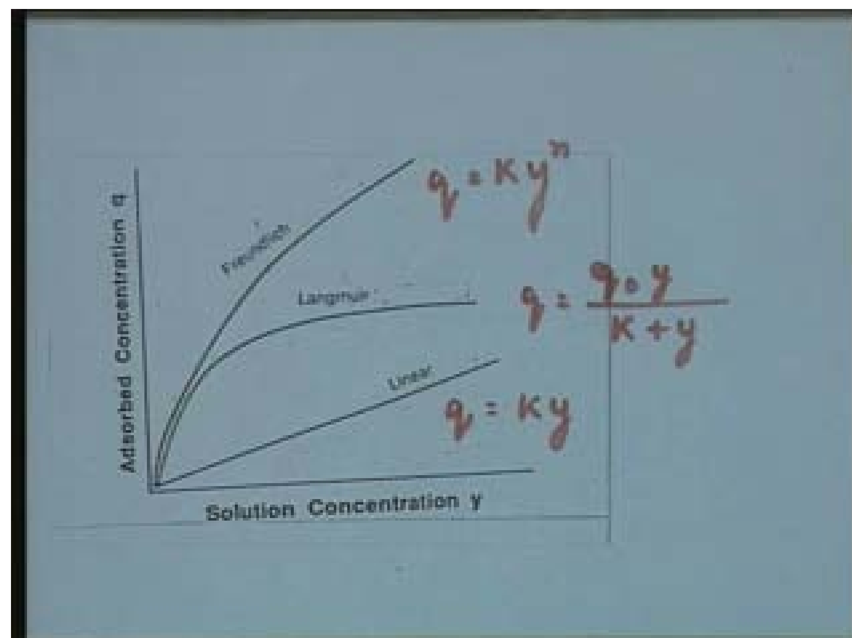

One is linear where q is the concentration of the enzyme protein on to the surface of the carrier; y is the corresponding concentration in the solution at equilibrium. If you take a combination of enzyme in carrier concentration and at equilibrium measure the concentration in the solid phase and the liquid and you make an isotherm, then you may end up sometime in some cases a linear relationship where q is equal to k.y

To make the isotherm, take different ratios of the carrier and the enzyme activity. That means enzyme activity to carrier ratio is varied and at different combinations you allow equilibrium to be formed and under equilibrium condition you measure the concentration of the enzyme in the liquid phase and also in the solid phase. Each point represents a particular enzyme to carrier ratio. You can find three different types of isotherms: linear is simple; Freundlich is more general in the sense that it does not fit into any hypothesis but in general you can vary curve fitting. What ever kind of isotherm you get, you just raise to a factor of n and by analysis of your data you will calculate the value of k and n. You could make a log log plot and you can get the value of k and n.

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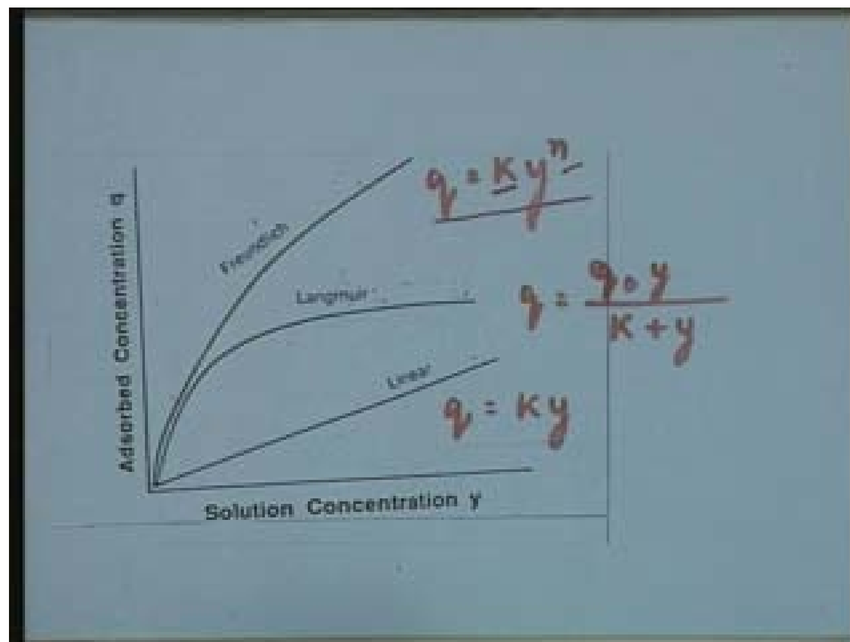

The most established and analytically proven method is Langmuir isotherm. Here q is the concentration of the enzyme protein on to the solid matrix and q_0 is the maximum capacity of the matrix. If you take a very high enzyme concentration compared to carrier, allow equilibrium to be established that means the concentration of the enzyme on to the immobilized preparation which will represent the saturation behavior is q_0. You will notice that it is analogues to your Michaelis Menten kinetics. These three expressions the Langmuir isotherm, Michaelis Menten kinetics, hyperbolic expression and also the Mannose equation for cell growth are almost analogues expressions and that indicates that there is some inter relationship or some similarity in their basic physical interpretation.

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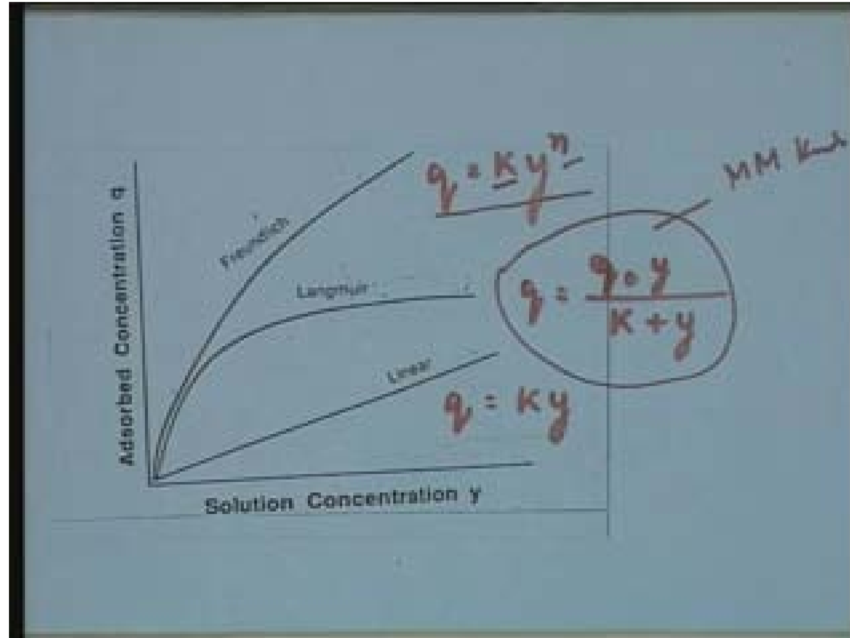

Langmuir isotherm is based on a theoretical development. One can develop on the basis of number of binding sites on the carrier molecule for enzyme. Here just like we consider the enzyme substrate binding where the enzyme acts as the carrier and the substrate is ligand here the carrier will be the matrix and the protein will be the ligand and on those conditions you can develop it or you can analyze it by a double reciprocal plot.

The analogy is that the three expressions, the Langmuir adsorption isotherm, the Michaelis Menten kinetics and Mannose kinetics for cell growth they are analogues terms. You have to develop an analogy and understand the relationship of the corresponding terms. In the case of enzyme we are talking of enzyme substrate complex formation. That means enzyme is the matrix, surface is a ligand. If we talk in terms of adsorption, the substrate gets adsorbed on to the enzyme surface. On the other hand here in the case of adsorption the enzyme is itself a ligand and the matrix or the carrier is the surface on which the ligand adsorbs. In the third case, in the case of cell growth, the Mannose model of microbial cell growth it is a substrate which is a ligand just like enzymes but the matrix is the cell mass itself and it reflects the transport of substrate into the cell. So this is adsorption that is transport and enzyme catalyses enzyme substrate binding. So these three phenomena are intrinsically someway related because they end up in a similar kind of a kinetic expression. As far as the analysis is concerned all the three can also be analyzed by a double reciprocal plot. That is if you plot 1/q versus 1/y you will get a linear plot and you will get the value of q_0 and k.

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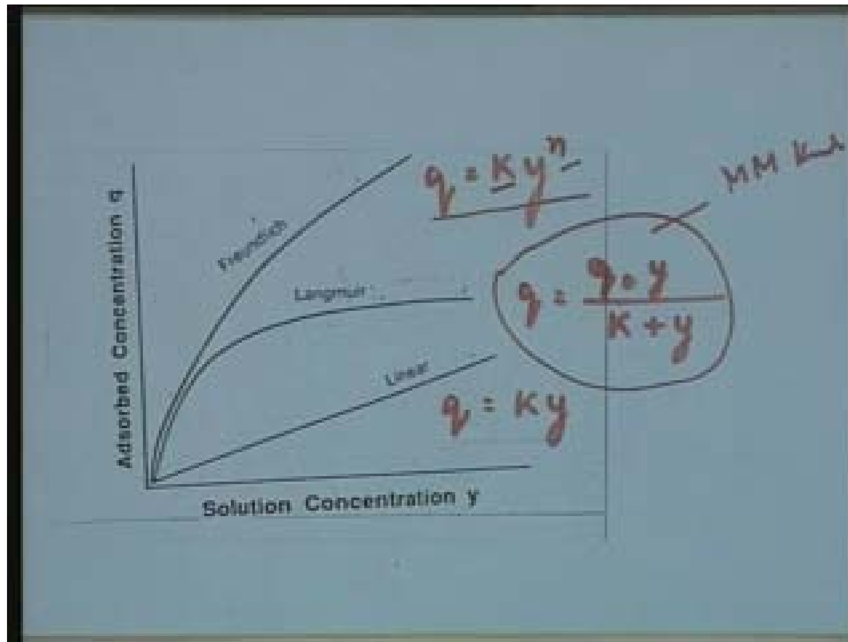

The adsorption process for a particular enzyme and for a particular carrier can be analyzed quantitatively.

Many physical factors influence the adsorption process. I have listed some of them here. The first parameter is pH.

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Factors affecting adsorption:

1. pH
2. Salts
3. Protein concentration
4. Temperature
5. Carrier properties
6. Time

The pH will be a very major parameter which will affect the binding in the case of ionic binding. Even for other forms of binding processes pH plays a key role and very often it

has been noted that the binding by adsorption is maximum at its isoelectric points. Large variety of relationships with respect to pH for the quantity adsorbed has been found. There is no general ruling but in general or a common feature can be considered that at the isoelectric point the pH value will be most preferable for the adsorption processes. No. Net charge is zero. It is not that the proteins are not charged at that pH. The negative and positive charges are equal. Not that there is no charge.

The second parameter is salts.

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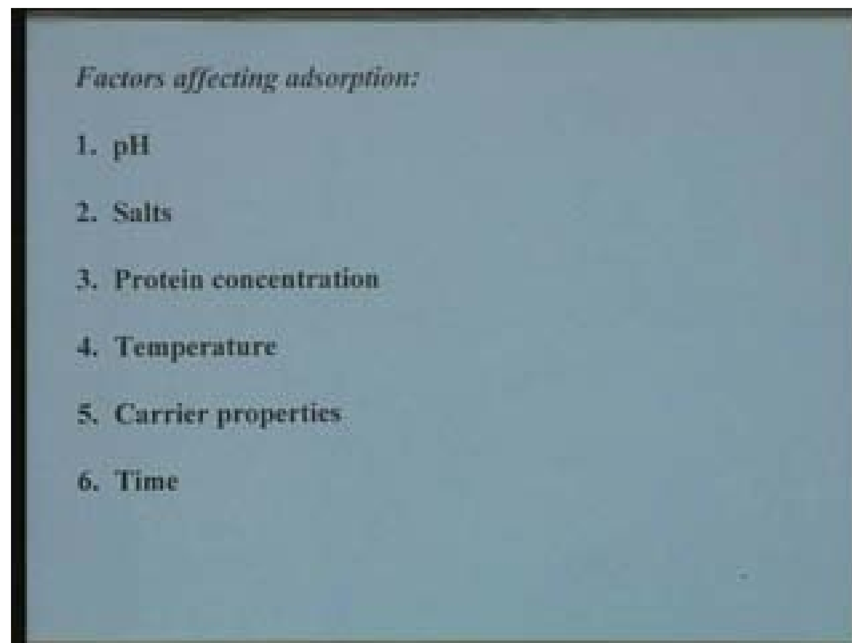

Salts have a very complex behavior. In fact it has almost opposite behavior. You must have studied also in the case of bio separations particularly the concentration of salt is used for increasing as well as decreasing solubility. Salting in and salting out both are feasible and of course the phenomena or the physical interactions are just opposite. But in most cases the salt concentration is required to be kept at a minimum because salts are very good eluents also. In ion exchange chromatography very often we use high ionic strength as a medium for elution of the adsorbed material. Therefore at high salt concentration adsorption is usually poor and minimum salt concentration is required to be kept. Protein concentration will be important and we have seen from the adsorption isotherm itself. The definite ratio of protein to carrier will play a particular equilibrium behavior and higher the protein concentration you can reach nearer the saturation level at equilibrium.

Temperature again is a very significant parameter which must be understood.

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Factors affecting adsorption:

1. pH
2. Salts
3. Protein concentration
4. Temperature
5. Carrier properties
6. Time

If we look at adsorption of a small molecular weight material or a small molecular weight ligands on to a surface the general behavior is that increasing the temperature reduces the adsorption. That means the quantity of ligand adsorbed per unit weight of the carrier for low molecular weight ligands will be less as you increase the temperature and the reason attributed to this is because of the movement of the particles as the temperature increases. That means the movement of particle increases and the tendency is for desorbing rather than adsorption. But in the case of protein adsorption when we are talking about enzymes, we are talking about proteins and macromolecules, adsorption over a narrow range of temperature offers a reverse phenomena. That means adsorption increases with temperature. Mind the word I am using "over a narrow range of temperature increase". If you increase to a higher level desorption also might start. But before desorption even inactivation might start. But we consider the adsorption at …… range of temperature. The primary factors responsible for the increase in adsorption with temperature are when the protein binds to a surface there is some unfolding of the protein molecules, which is an endothermic process and the increase of temperature resulting in enhanced adsorption supports the hypothesis that the binding or adsorption of a protein molecule or a polymeric material to a surface is an endothermic process coupled with unfolding of the confirmation. When I say unfolding I am not referring to total deactivation but minor modification in the conformation which extends its total volume or total surface area.

The other factor which is important, which is probably attributing to this increase of adsorption with the increase in temperature is the diffusional processes because most of time we are talking about the carrier being a porous material and the ligands or the protein here have to diffuse into the surface, into the internal pore surface and the diffusion process gets increased by increase in temperature. So both the unfolding of the protein molecule on the surface of the carrier and the diffusional processes involved in the adsorption in the internal pores ultimately leads to give you a phenomenon which represents increase in adsorption with increase in temperature over a narrow range.

Carrier properties, depending on the property like surface property of the carrier particles which were used as matrix, will also play a role on the adsorption behaviour depending on the nature may be sometime even the carriers are pre-conditioned, swollen to improve the property.

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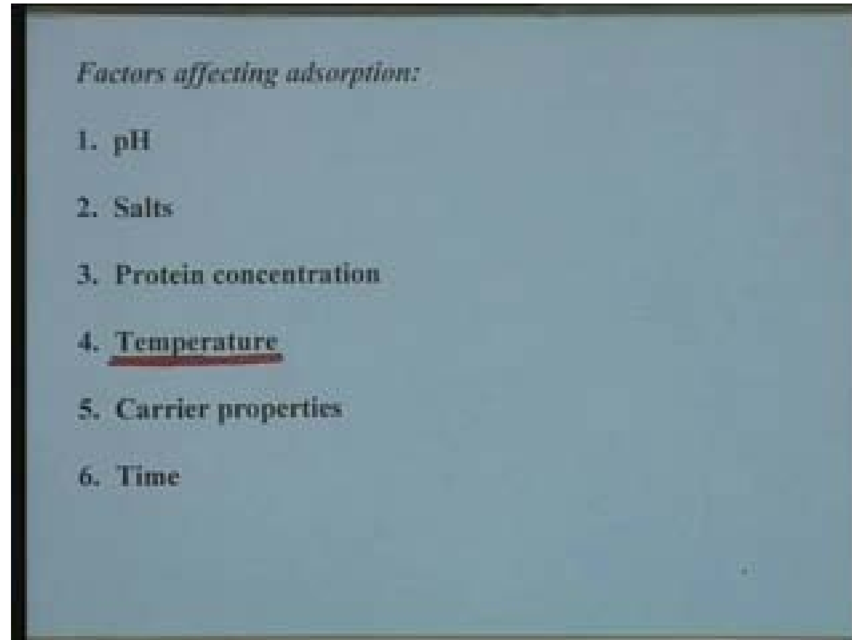

Time of adsorption is another parameter which is significant and important because we are talking about the macromolecular adsorption. So the diffusion is involved and the time required very often is controlled by diffusional limitations and the rate of diffusion controls the time required for adsorption process. This in the case of a small ligand is not a constant.

For adsorption we follow a number of methods. At least four major methods are used for adsorption. The first and probably the most trivial method is the static process.

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Methods for immobilization by adsorption:

(a) The static process

(b) The dynamic batch process

(c) The reactor loading process

(d) The electrodeposition process

That means you bring in an enzyme solution in contact with a carrier and leave it over night or over a long period of time. Adsorption takes place without any hesitation. It takes a very long time and that is the process very often many people use in laboratories. The yields are also much lower particularly for porous carrier because of the diffusional limitations. One of the most disadvantageous situations in the case of static process is that the loading of the enzyme is not uniform over the carrier. Even if you allow for a long period the loading is usually not uniform. Suppose this is the carrier particle. You have lot of enzyme molecules on the surface but on the interior it is very, very rarely distributed and the loading is not uniform.

The most commonly used method in the laboratories is dynamic batch process in which you mix the two components, the enzyme and the carrier under known conditions or under standard conditions for adsorption, which favours adsorption and agitate them, stir them mechanically, under mild stirring so that the carrier particle should not get degraded and also the enzyme molecules should not be influenced. The equilibrium is allowed to be reached over a period of time. It usually leads to a fast method and most common method used in the laboratories.

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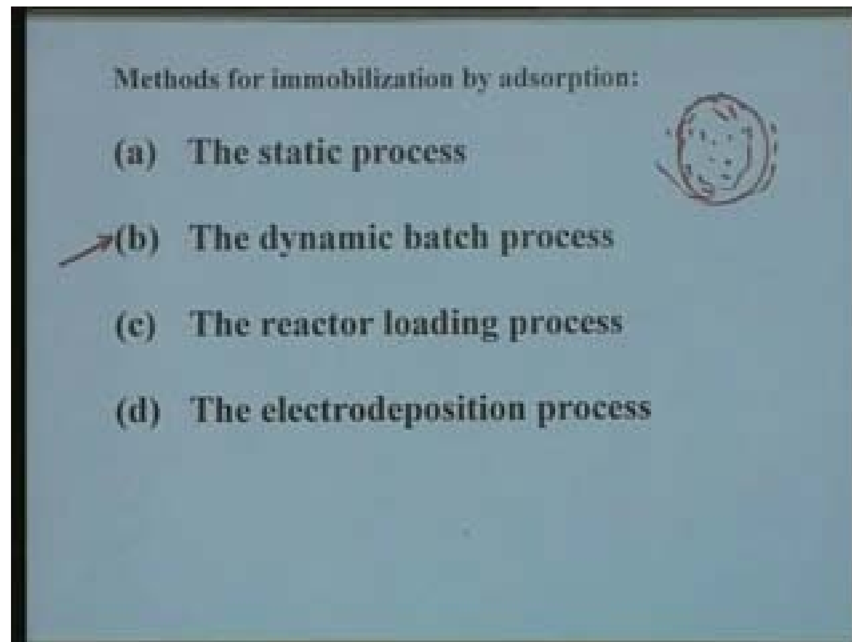

The third method is the reactor loading process. In the commercial establishment when we use adsorption as the method of immobilization we use reactor loading process. That means first of all the reactor which is to be used for bio-conversion of the enzymatic reaction is packed with the carrier itself and then the enzyme solution is recirculated constantly through the carrier bed and over a period of time the enzyme gets adsorbed on the matrix. It could not only be packed bed; it could also be stirred reactor in which the enzyme is added and the enzyme solution is allowed to be stirred along with the carrier and after a period of time when the supernatant enzyme activity has come to minimum value you stop the process.

In all these methods which we are talking about, the final step is washing because very often you will have that some of the enzyme will be adhering to the surface of the particles which is not really immobilized in the proper sense. It is not adsorbed; some adhering along with the liquid and so fresh wash of the buffer at a particular pH should be given so that all the adhering enzyme is washed out and only that which is physically bound by adsorption is retained by the carrier. The final washing in the case of reactor loading process is done with the substrate solution itself because during the operation it has to meet the environmental conditions which are provided by the substrate solution, the feed. Under those conditions it must remain adsorbed and so the final washing in the case of a reactor loading method is provided by the feed substrate itself so that whatever desorption has to take place under feed substrate condition, takes place and the enzyme is in the stable form.

The fourth method is electrodeposition process in which the carrier is placed in proximity to one of the electrodes and the current is applied between the two electrodes and the enzyme particles which are charged usually they get deposited on the carrier particles and at the end the carrier is removed which gets coated by the enzyme particle.

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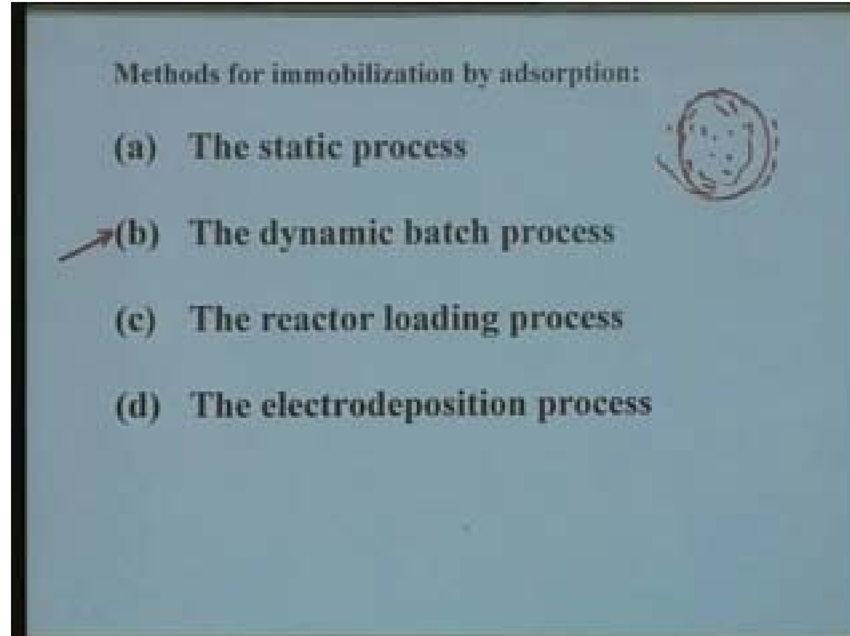

One thing we have to keep in mind, a very important feature that during the electro chemical process there will be certain ions which will be removed from the electrodes or from the process. If the ions are important for enzyme activity then they must be provided in the electrolyte itself. Extra quantity of those will be provided in the electrolyte otherwise you will not be having a functional enzyme preparation in the immobilized form. The last method based on the adsorption process is by inorganic bridge formation as I mentioned or metal salt linkage.

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Immobilization by inorganic bridge formation

(Barker, Emery & Novais, Process Biochem. 5,11,1971)

- Metal salt linkage
- The method employs a transition metal salt ($TiCl_4$, $ZrCl_4$, $FeCl_3$ etc.) for linking an enzyme to the carrier – cellulose, nylon, glass beads etc.
- An activated carrier is prepared by steeping it in a solution of transition metal salt (5-10 min) followed by filtering / drying of the complex at 45^{0}C. Excess unreacted salt is washed with buffer. The activated carrier is stirred with the buffered enzyme solution, followed by washing of the unbound enzyme.

This was originally developed by British group Barker, Emery and Novais, one of the references I have sited here, in seventy one. It involves the metal salt linkage. The basic principle of the method is the method employs transition metal salts like titanium tetrachloride, zirconium tetrachloride or ferric chloride for linking an enzyme to the carrier. Carriers are usually cellulose, nylon, glass beads etc. The usual method is that an activated carrier is prepared by steeping it in a solution of transition metal salt for five to ten minutes and mind it that it is important because longer contact of the matrix in the transient salt solution can result in the degradation because the pH of this solution, like titanium tetra chloride, will be very, very low, almost one to two. If you put cellulose into it, it might get degraded totally. So the time of contact is very important and it is not used mostly more than five minutes and if you take a dilute solution probably you can go higher, followed by filtering and drying of complex at $45^{\circ}C$. Excess unreacted salt is washed with buffer. The activated carrier is then stirred with the buffered enzyme solution followed by washing of the unbound enzyme. The method is very simple and involves two steps, activation of the matrix by steeping it in a transition metal salt solution whereby the transition metal salts form an octahedral complex. The chemistry of that can be considered like this. Titanium tetra chloride will exist in different forms and the complexes will be either dominated by water molecules as the ligand in the octahedral complex form. The titanium tetra chloride solution will exist in different forms complexed with certain ligands. For example in solution water molecule will be present as the ligand and will complex with water and chloride ions.

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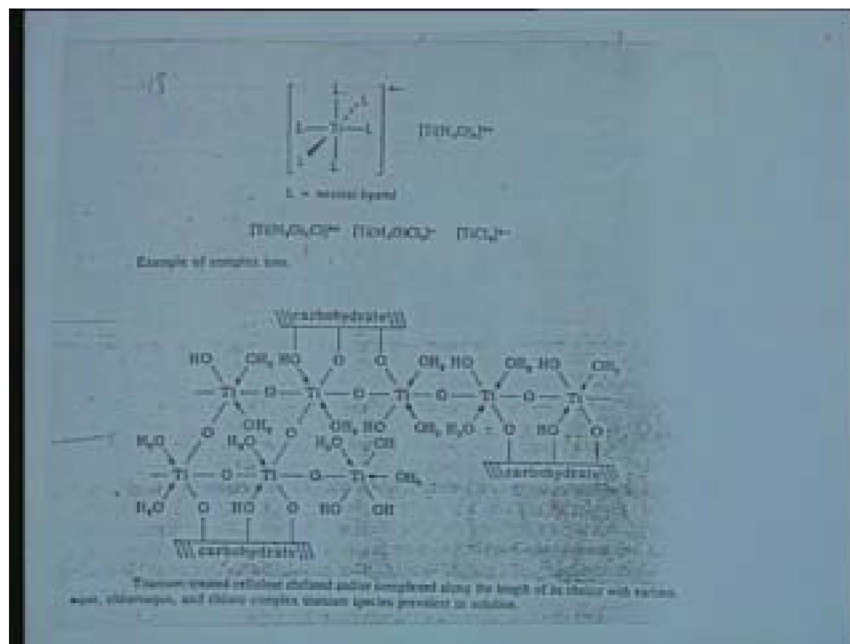

In aqueous form titanium has five water molecules and one chloride. It can also exist as the chloro complex; five chloride ions and one water molecules. It can also exist as total chloride ions in the case of a co-ordination complex. So when this solution is brought in contact with the matrix, any carbohydrate matrix, cellulose has been a very commonly

used matrix, then what happens is that these ligands are replaced by the hydroxyl groups present on the carbohydrate molecule. The ligand particularly the water molecules which are present here are replaced by the hydroxyl groups present on the carbohydrate molecule and you make a network, titanium treated cellulose which is complexed along with the length of the chain on the carbohydrate is formed. When this activated matrix, we consider this as an activated complex, is further brought into contact with the protein or enzyme again some of the water molecules are replaced by the functional groups on the enzyme. Wherever you see water molecules, for example these ones they may be replaced by enzyme molecules. Wherever hydroxyl groups are present they can replace part of them and what ends up is a matrix which has a coordination link of titanium tetra chloride salt along with the enzyme molecule.

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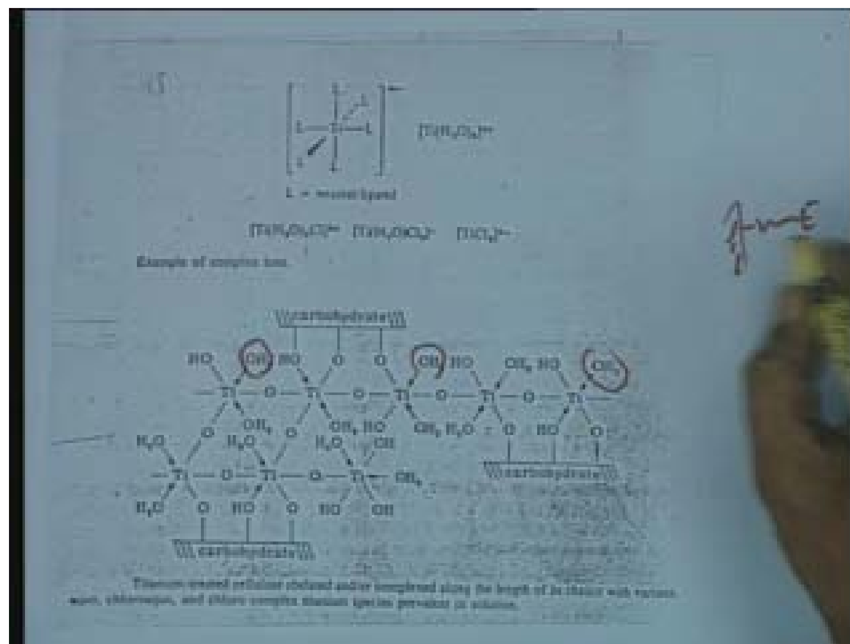

One of the major advantages is that you always end up having a distance between the carrier surface and the enzyme molecules. Therefore enzyme molecules and the carrier surface are separated by these linkages, the metal salt linkages. That provides you a very big advantage of removing the steric hindrance. Many of the enzymes when they are coupled to a solid matrix may not be able to interact with the substrate because of steric hindrance. But in this case because there is distance between the matrix surface and the enzyme, steric hindrance is minimized.

The second major advantage which has been noted in these cases is that if you use a regenerated carrier after the enzyme has been leached out, you again immobilize the enzyme on the regenerated carrier. It gives you a much increased activity and that is the major advantage. The major reason has been that the distance from surface has increased. So steric hindrances are further decreased and therefore particularly with those enzymes which are known to have steric hindrances during immobilization, the ones which use

much larger polymers as the substrate, for those systems the method is very significant. So these are the various methods that we have discussed today. The various methods that involve the coupling of enzyme by adsorption to a matrix and their characteristic features and as I mentioned that the immobilization methods must be evaluated on the basis of their yield, operational stability and carrier regenerability. So we stop here.