ENZYME SCIENCE AND ENGINEERING

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LECTURE–14

IMMOBILIZED ENZYMES (IME)

Hello! So today we will be talking about immobilized enzymes.

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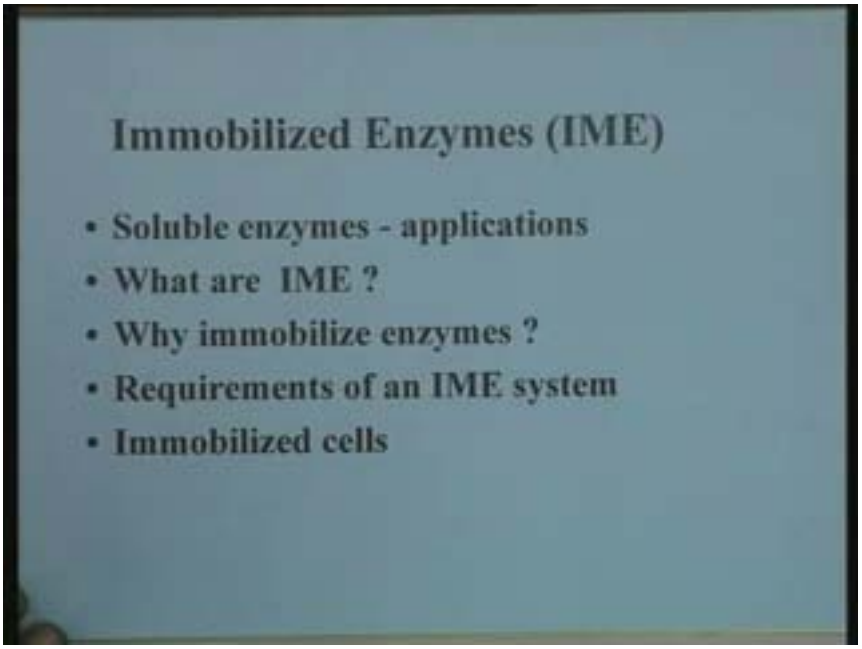

So for we have seen the structure and function of enzymes as they are available in the living cells. We have also seen that functionally they are very efficient catalyst in the sense that they provide a very high degree of specificity, a very high degree of turn over number as far as their function is concerned. They also can catalyze a number of stereospecific chemical transformations that are otherwise difficult to carry out in a single step by other chemical routes and also a major advantage of mild operating conditions which make them very attractive industrial catalyst.

But the fact is that these catalysts have not found such a wide spread use as far as industry is concerned excepting in some conventional applications in foods or pharmaceuticals which are the natural processing agent in the case of industrial applications. Bulk of the other industries has not made use of these biocatalysts at least up to the beginning of the last two decades. If you look at the industrial applications until sixties there was not a very significant application of enzymes in industrial processing.

The major limitation, you also noticed during our understanding of the structure-function relationship of the enzyme, is that they are usually very unstable and that probably is one of the major limitations as far as enzymes are concerned with respect to their application. They cannot be used conventionally in organic solvent. That is the perception that means their structural confirmation in which they are catalytically active is functional only in aqueous medium. Their activity or their function in the normal temperature range also put sometimes a disadvantageous situation in the sense that at those temperatures many of the microbial activities are also optimum and therefore they lead to contamination of products and therefore their industrial application becomes restricted.

Also a major factor in the limitations of the application of soluble enzymes in industry has also been the soluble nature of enzymes that means they have to be used in water soluble form. When we have to use them in water soluble form it only means that the process has to be in the batch mode. That means you take an enzyme solution or a substrate solution, add a quantity of soluble enzyme in a batch mode, provide the optimum temperature and pH, carry out the reaction and terminate the reaction by changing one of the operational parameter either by heating it or by drastic change of pH so that the reaction is terminated and then extract the product.

In this whole process the major problem is that one has to discard the enzyme, one has to deactivate the enzyme and the enzyme is usually not possible to be recovered at the end of the process. Because recovering the enzyme from reaction mixture will be as difficult or as tedious if not more as production of the original enzyme itself and in most cases enzyme being produced by living systems are usually expensive commodities and therefore their deactivation at the end of one batch operation is not a desirable feature and that has been a major limitation. The two issues of unstability and possibility of their use repeatedly or in a continuous mode has been the major driving force of looking at immobilization as an approach to provide a vector platform for the use of enzyme in industrial application.

A major question that will come is what do we mean by immobilization? What is immobilization? If you look at for a very broad based and operational definition of immobilization we can say that enzymes are physically confined or localized in a defined region of a space. That is a definition of immobilized enzymes. The enzymes that are physically confined or localized in a defined region of a space with retention of their catalytic activity and which can be used repeatedly or continuously in a process are usually considered as the immobilization enzyme preparations.

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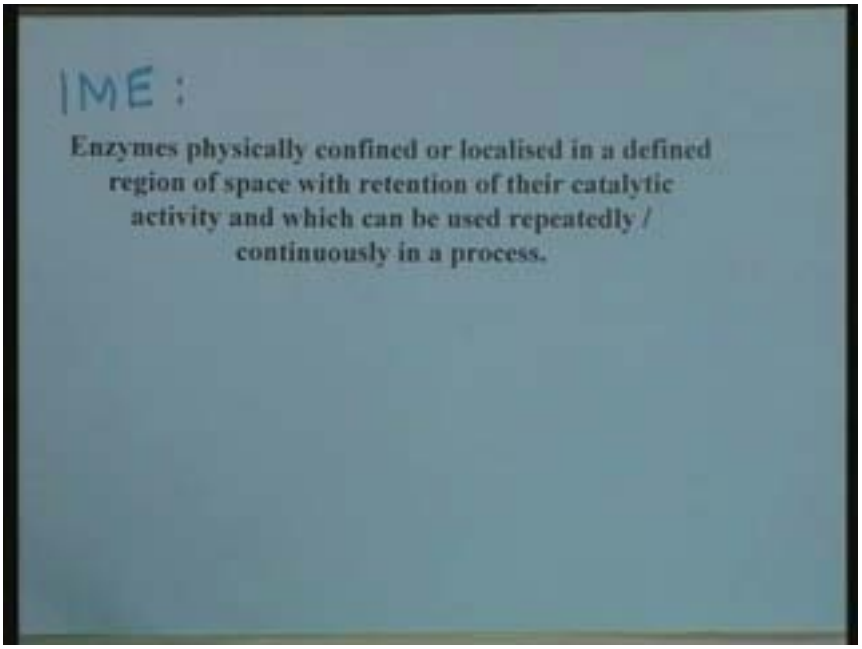

This is basically an operational definition. I like you to concentrate on at least three major phrases built in, in this definition. One is physically confined or localized in a defined region of space.

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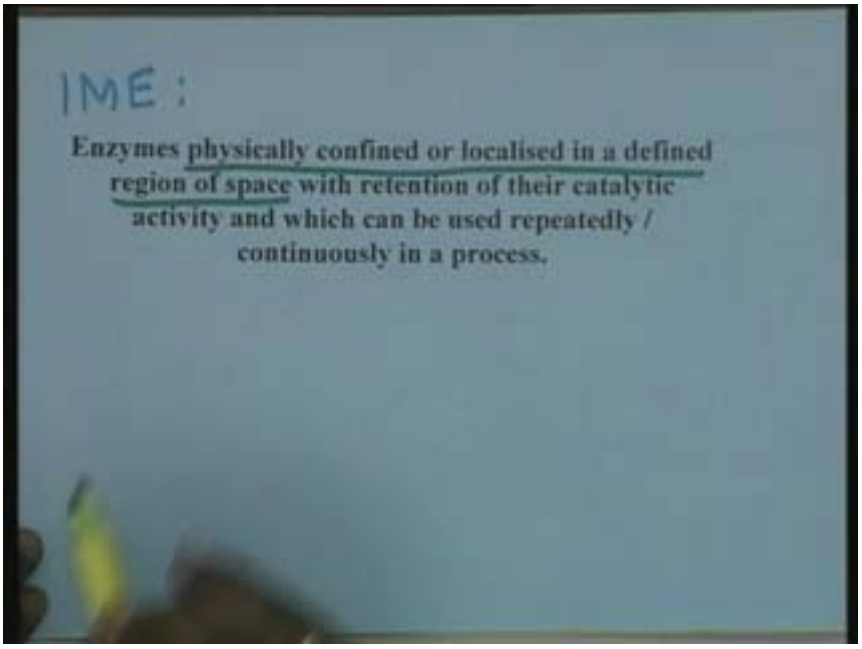

That means the enzyme molecule which is homogenously suspended in a soluble preparation has to be then localized in a narrow region of space and this is no longer uniformly, homogenously distributed in a reaction mixture. An analogy can be made to a heterogeneous catalysis. In the case of chemical catalysis many of the catalyst are used in

a heterogeneous phase, while the use of soluble enzyme will provide a homogenous catalysis. That means the distribution or composition of every species in the reaction mixture whether it is catalyst or a substrate or a product is uniform throughout the reaction mixture. The use of immobilization enzymes will lead to a heterogeneous catalysis. That means while substrate and the product might be homogenously distributed the enzyme will be localized in some area of the space. It will not be homogenously distributed at every point in the reaction mixture. The second phrase which is very important is the retention of their catalytic activity. That means if you do any modification so as to arrive at an immobilization enzyme preparation it should require the retention of their catalytic activity.

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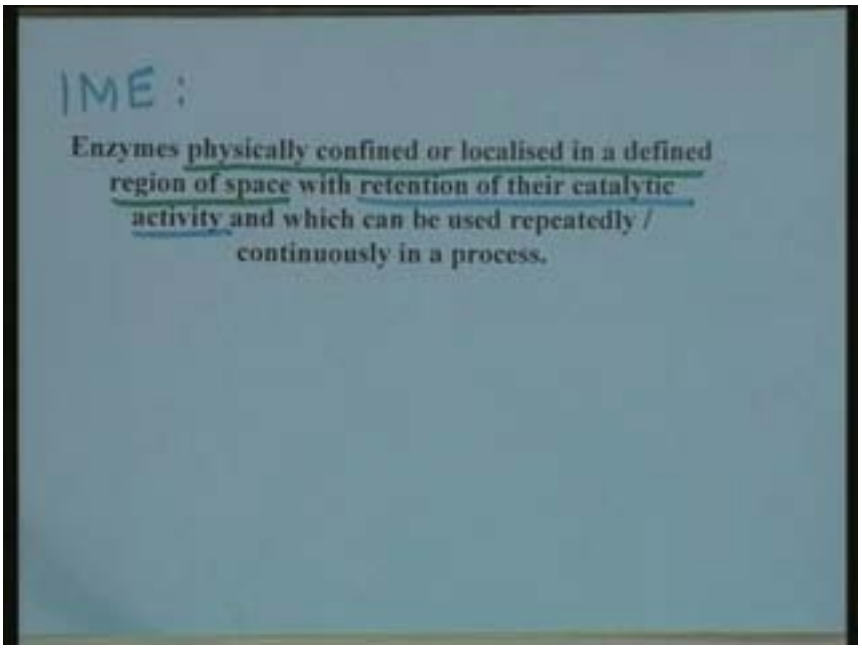

Otherwise it doesn't have any meaning to have immobilized enzyme preparation. At the end of the immobilization enzyme preparation retention of catalytic activity is a major requirement. Retention of catalytic activity is a very relative term. Relative in the sense that hundred percent retention of catalytic activity at the end of any processing step is not feasible. So when we say retention of catalytic activity you are implying that a major fraction of the activity must be retained. The degree of retention will vary from enzyme to enzyme; also the more unstable the enzyme the poor will be retention or method of immobilization; more harsher the method that means if the enzyme is subjected to more harsh treatment in terms of pH, chemicals or temperature the inactivation may be more. Ultimately a major portion of enzyme activity must be retained. If the enzyme activity leads to 0 or 5% or 10% at the end of the immobilization I don't think we should logically call it an immobilization enzyme preparation.

The third issue and which is also operationally very important is which can be used repeatedly or continuously in a process.

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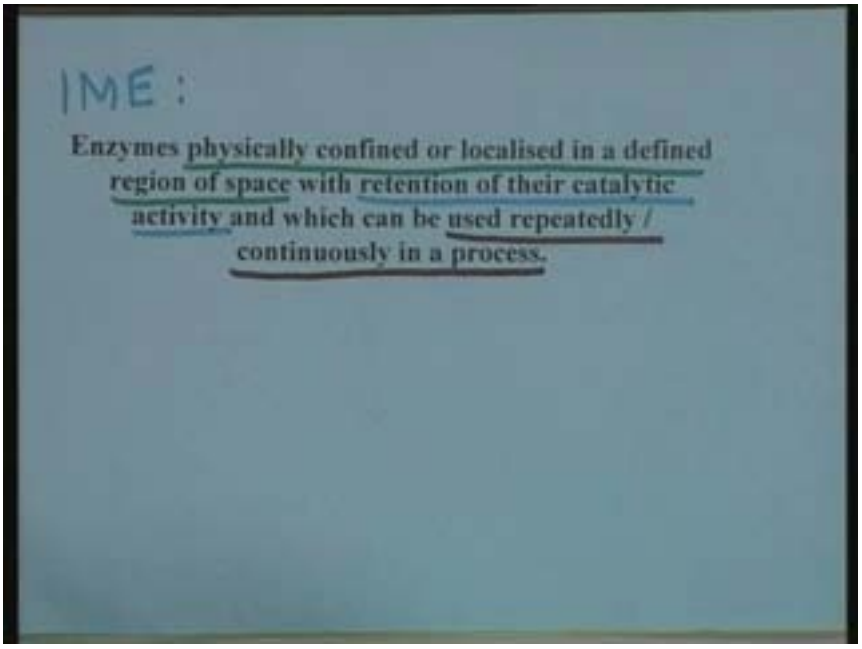

Ultimate objective of immobilization is to use it either repeatedly in a batch mode so that the same enzyme preparation is used in repeated batches one after another or it can be used in a continuous reactor like a plug flow reactor or a continuous stir tank reactor so that you can process some substrate over a long period of time using a same amount of enzyme and therefore decreasing the cost of enzyme per unit quantity of the product produced.

The major objective, from the industrial point of view, of immobilization enzyme would be to reduce the cost of enzyme input into any processing per unit quantity of the product to a significant level and that is what is often achieved. This definition takes care of the three major factors one is the localization of the enzyme or connectivity over a major substrate and such an immobilization can be achieved by different means. There are number of methods. If you can couple an enzyme preparation to a matrix, an insoluble matrix, which is not soluble anyway couple whereas the enzyme activity is retained and this being an insoluble matrix, a particulate matrix, one can pack it into a column or one can use it in a stirred reactor and give a feed continuously and take all the product stream continuously. Both the things are feasible.

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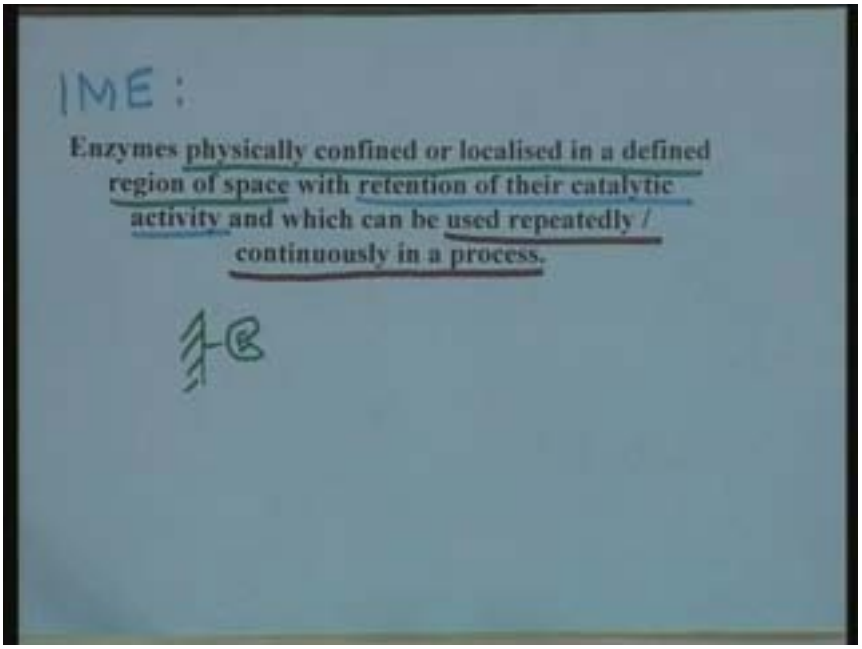

Alternatively instead of coupling to a matrix it could also be feasible that you entrap the enzyme into a matrix. For example you take a permeable micro capsule in which the enzyme molecule can be entrapped such that the enzyme molecules cannot come out but the substrate or the product can freely diffuse in and out and such a system will also be called immobilized system.

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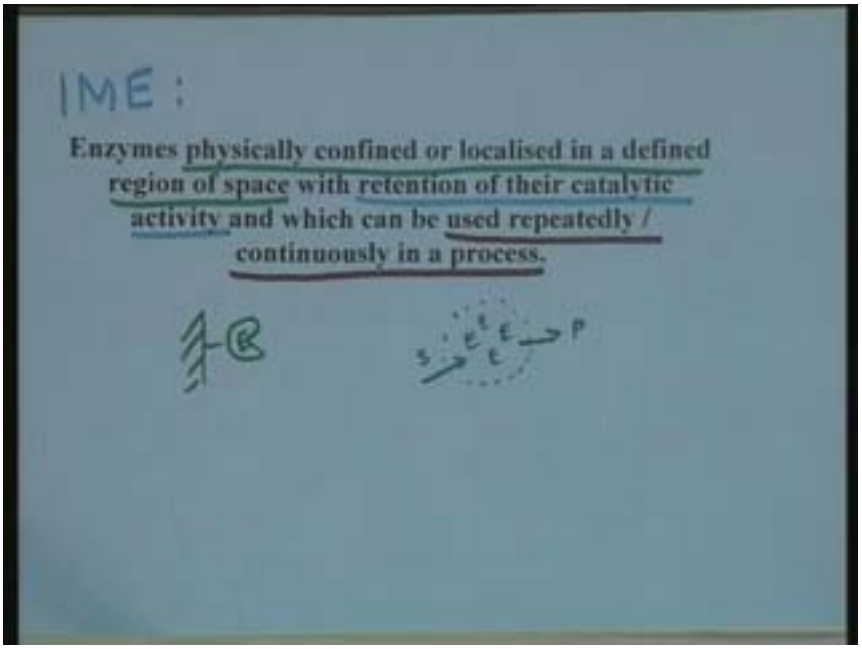

A very extreme situation can be considered of immobilization where we are not really having a physical preparation in the immobilized form but if you use a soluble enzyme in a membrane reactor, a typical membrane reactor, and this is a ultra filtration reactor in which the enzyme molecules are put in and this membrane is able to restrict the flow of enzyme and you can put in the substrate here, the product can go out and the enzyme can be retained in the membrane reactor.

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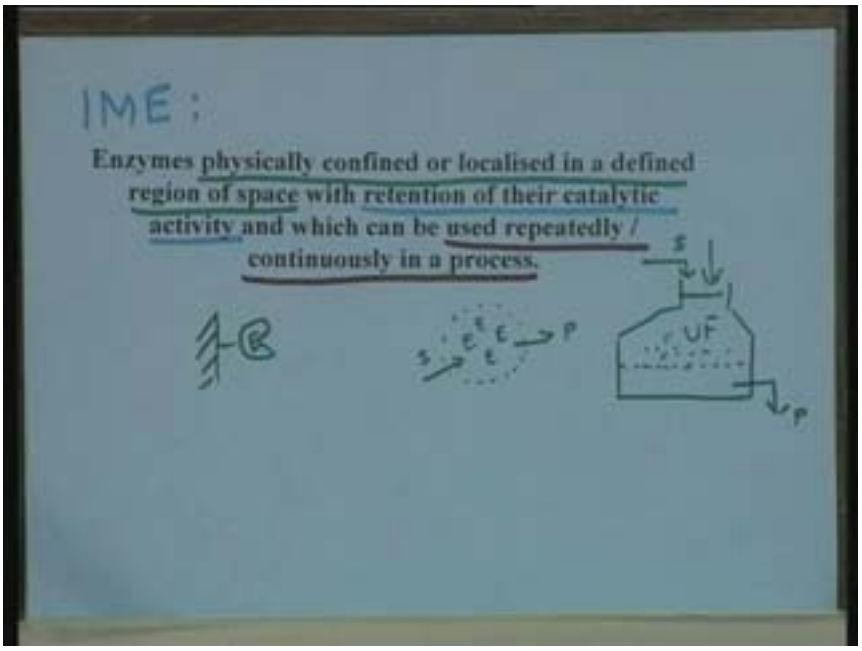

Let us say an ultra filtration cell in which the molecular weight cut off of the membrane is such that the enzyme is not allowed to pass through and retained as the retentate whereas the unconverted substrate and the product formed is passed through a membrane and can be collected. In such a case also as a system we will call as an immobilized enzyme system although the preparation will no longer be immobilized in the physical form, in the form of like what we considered as the bound preparation or entrapped preparation but still functional because in such a system you can use the enzyme continuously or repeatedly without loosing in the product stream. So all these systems will be considered in the format of immobilized enzyme preparation and the enzyme preparation may be in the coupled form in the insoluble matrix, it could be entrapped into a semi permeable cell or entrapped in the gel because gel also provides you a porous network in which the enzyme can be entrapped and they will not be allowed to pass out through the gel pores because of the size limitation once they are entrapped and a similar thing applies to a ultra filtration reaction.

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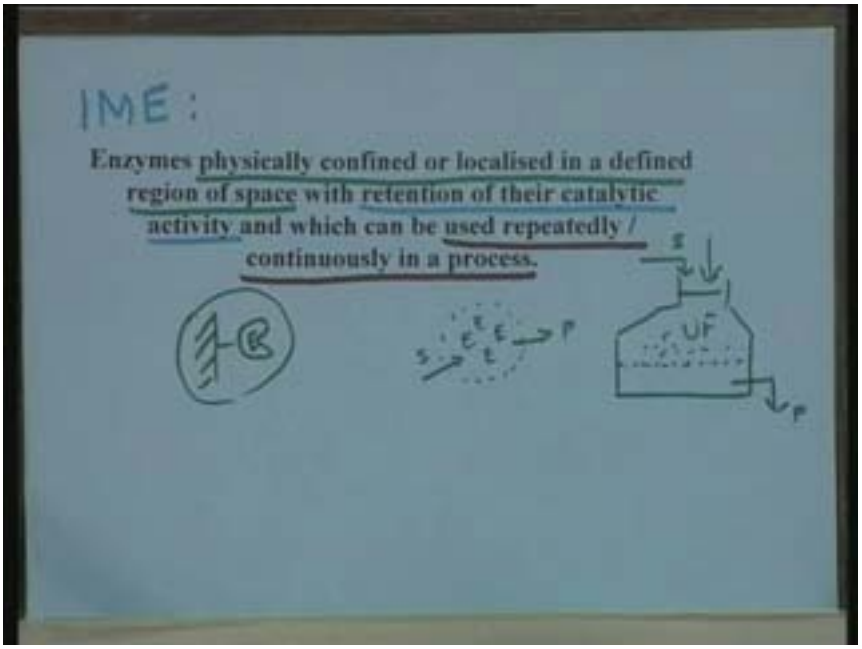

We often consider that if you couple a soluble enzyme molecule on to a solid matrix whether it is an inorganic matrix or an organic matrix or a gel that is the conventional form of immobilization. Unconventionally one can also make all these conditions feasible that means you can localize the enzyme in a defined area of space that is in an ultra filtration cell. I presume that you understand what ultra filtration cell is. You have a stern (15:14) vessel in which at the bottom there is a membrane with a narrow molecular weight cut off. That means it will allow only substances below a certain molecular weight to pass through it. The molecules with higher molecular weight will be retained. In such a reactor if you use an enzyme by putting substrate in and taking out the product which are low molecular weight compounds and enzyme being high molecular weight molecule will be retained in the reactor during use. Such systems also will be considered as immobilized enzyme systems.

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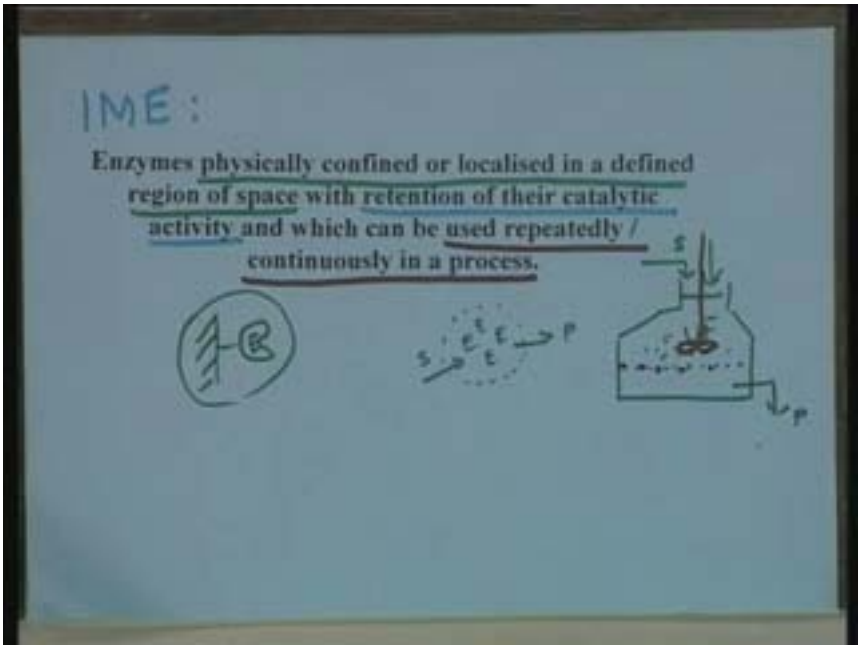

In fact although industrially such systems are not very commonly employed as far as the academic study is concerned these systems have been applied. One of the early systems which were applied was way back in late sixties and early seventies to hydrolysis of cellulose and starch. Because the starch product is low molecular weight glucose similarly cellular product is also glucose. This comes out of the product and the enzyme and the unconverted substrate remains in the retentate.

One limitation will be that the product has to be a low molecular product. That's why it has not industrially become very attractive. There are problems still as far as the cost of the membrane is concerned the following (16.58) operational problems. There are still problems. Today in the literature we will find probably a few thousand methods of immobilization and not that all of them have been commercially viable, because commercially viability is an issue which is linked directly to economics, the power of economics. So it does not restrict to but what we are talking of the basic definition of immobilized enzyme that you can do it in that matter.

As a matter of fact this is an artificial cell as you can consider let us say living cell itself. In living cell also the enzyme is entrapped through a semi permeable membrane which does not allow the enzyme molecules to pass through and some of the product molecules from the cell also are usually ripped off. The cell membrane is much more specific and has quiet distinct features compared to an ordinary ultra filtration cell which only functions based on molecular weight. The cell membrane has still more specific functions and more elaborate functions but here the function is only based on the molecular weight cut off.

The second point is that what are the major advantages or what are the major features and why should we immobilize enzymes?

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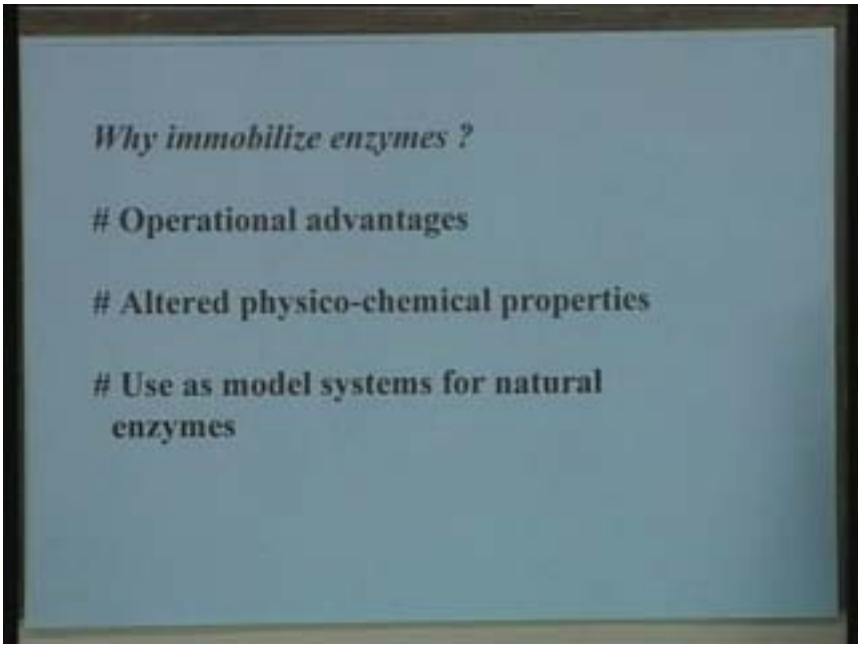

There are mainly two motivating factors. I would like to give you a brief about the historical perspectives. The whole concept of immobilization of enzymes started sometime in mid sixties and not before that mainly for two reasons. One is that the scientist wanted to apply some of the enzymes for industrial processing and obviously for industrial processing you need certain operational advantages The major disadvantages in the case of the enzymes is the restriction in the best mode of processing, the stability, some of the physico chemical properties in respect of pH optimum, temperature optimum, substrate specificity and they could all be taken care for industrial processing.

The second motivating factor was many people also wanted to study the enzymes in their natural habitat or at least as close to the natural habitat. When we talk about the enzyme, structure and functional aspects, we are missing one thing that whatever studies have been made in those aspects they have been made after isolating the enzyme from a cell. It has been de linked from its natural habitat and when we take an enzyme sample in a buffer, its natural structure function may not be identical as it happens in the living cell and that has been recognized very well. If you notice the half life of an enzyme in a living cell you can make certain indirect calculations. You can notice that in a living cell the enzyme is not as unstable as we notice it in the laboratory or pharmaceutical applications. The turn over of the enzyme molecule itself in a living cell is quite low. The cell does not synthesize because protein synthesis in a living cell is quite an energy intensive process and cells often will like to economise the energy consumption as far the protein synthesis is concerned.

Therefore the half life of the enzyme in the living cell, the stability is much higher compared to a free state. If they can try to bring as close to the living environment and the process which they adopted was immobilization. Because most of the enzymes which

people understood very well were the membrane bound enzymes and they noticed that in the membrane bound enzyme there was some kind of a physical coupling between the enzyme and the insoluble membrane as a matrix. Therefore they thought that coupling of an enzyme to an insoluble matrix would be a desirable feature to study enzymes or immobilized enzymes as a model system for natural enzymes and then one can really look into the various properties in terms of their operational properties, in terms of their kinetic properties and also in terms of their substrate specificity and other aspects.

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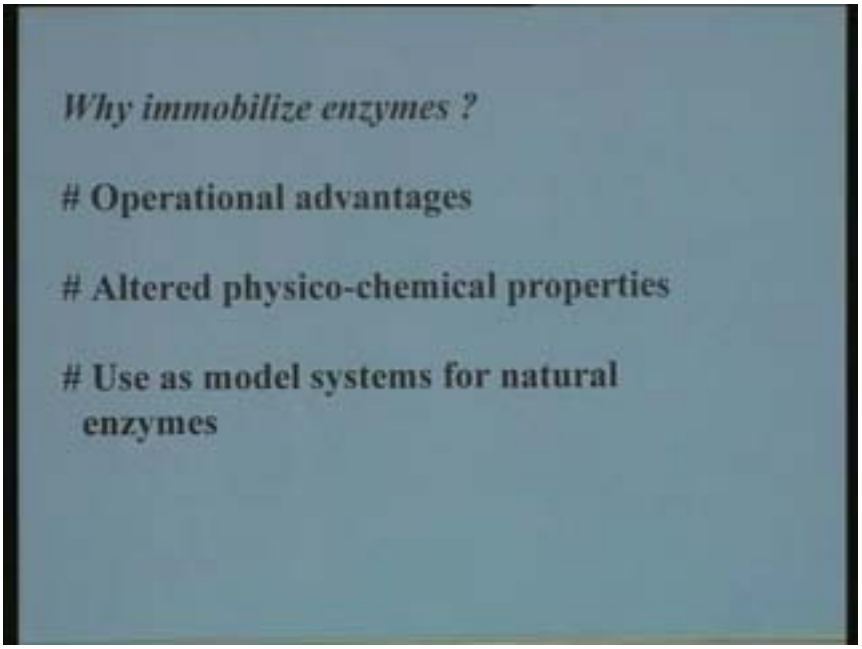

After isolation from the cell you are trying to bring back because you see when they are in a living cell you have no means to study them excepting indirect calculation that this might be happening and this is the rate of flux. In fact most of the metabolic pathways although we understand qualitatively much better but still the quantitative fluxes of the individual enzyme catalysed reactions in a metabolic pathway is still a very difficult aspect. So the only way out left to a scientist is that one isolates and tries to mimic the conditions of a cell so that one can probably try to interpret the functioning in a cell; how it is happening and the immobilization provided isolation of the enzyme. You don't keep it in a cell; you isolate but then again revert back, kind of a pseudo cell for example you entrap that into a micro capsule which is continuous semi-permeable membrane or take a very fine porous film attach an enzyme molecule to this and then this mimics as much as probably your enzyme was supported on a membrane in the living cell and study its properties.

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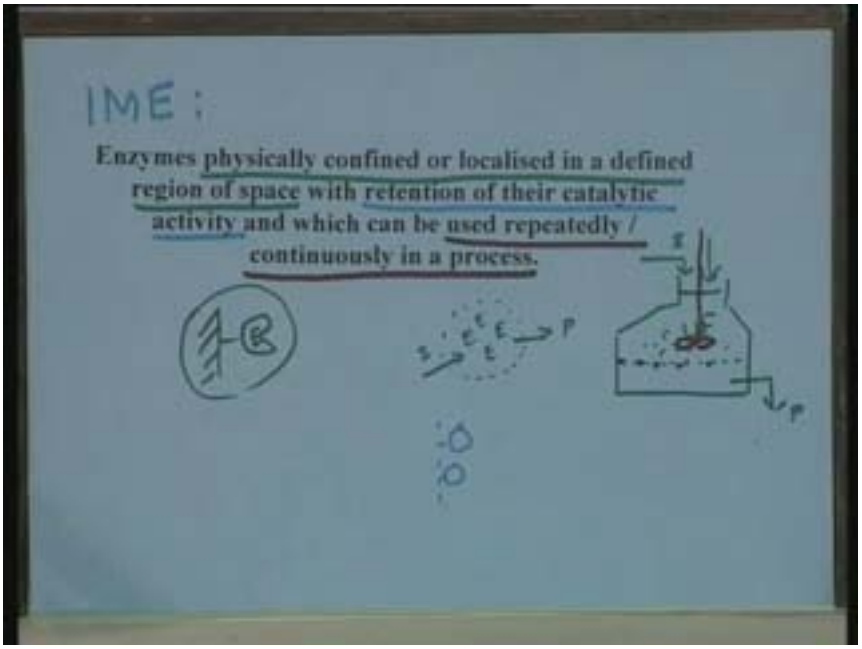

So they were the two motivations and when we talk about the operational advantages because of our major interest we will look at enzymes as an industrial catalyst for use in industrial reactors for conversion of substrates into products. The immobilization offers n number of advantages and one of the major advantages which we have already talked about in the definition itself is its reusability. That means the cost of the enzyme per unit product is significantly reduced by the reusability. Instead of the possibility of using only in soluble mode in a batch reactor you can use in a variety of application modes. A variety of reactor types can be used for carrying out the transformation or reactions instead of using only a batch stirred reactor as we can afford only for a soluble enzyme. Another major advantage is the rapid termination of reaction.

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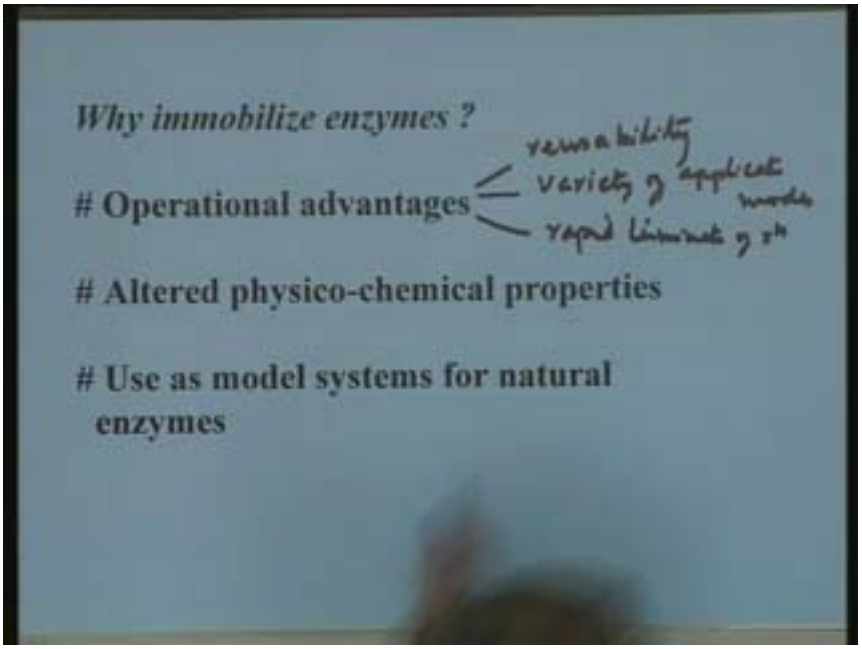

In the case of the soluble enzyme the only means of terminating a reaction is to deactivate the enzyme either by drastic change of pH, heating, boiling in a water bath or adding some kind of chemical, irreversible inhibitors of an enzyme, which can inactivate the enzyme. But in the case of immobilized enzyme you can repeatedly terminate by physically separating it. Even if you use in a batch mode by physically filtering off the enzyme preparation, the reaction is stopped. There is no enzyme present and the reaction is stopped. That gives you a very great operational significance and the use of an immobilized enzyme also leads to relatively purer product. In the case of a batch mode the enzyme is going along a product stream and in some cases the discharge of proteins into the product stream may cause purification problems. Therefore the use of immobilized preparation in those cases will lead to a much purer product or significant convenience as far as the downstream processing of the product stream is concerned.

In fact many of the products which have been commercialized using immobilized enzyme preparations are for application in food and food constituents. In the case of food constituent we don't want the enzyme protein to go as a product in the product stream. In the case of immobilized enzyme that problem is not there because the enzyme is already separated and the product stream as it is can be used as a food constituent directly in the product form. In most cases the immobilization has also resulted in some altered physico-chemical properties. When I say physico-chemical properties the most important properties probably for which enzymes are considered as poor candidates are their stability. In most cases the stability is enhanced and therefore it becomes a desirable feature. The other altered physico-chemical properties are optimum pH and specificity. In many cases the altered optimum pH will result only on the basis of the nature of the carrier you are using.

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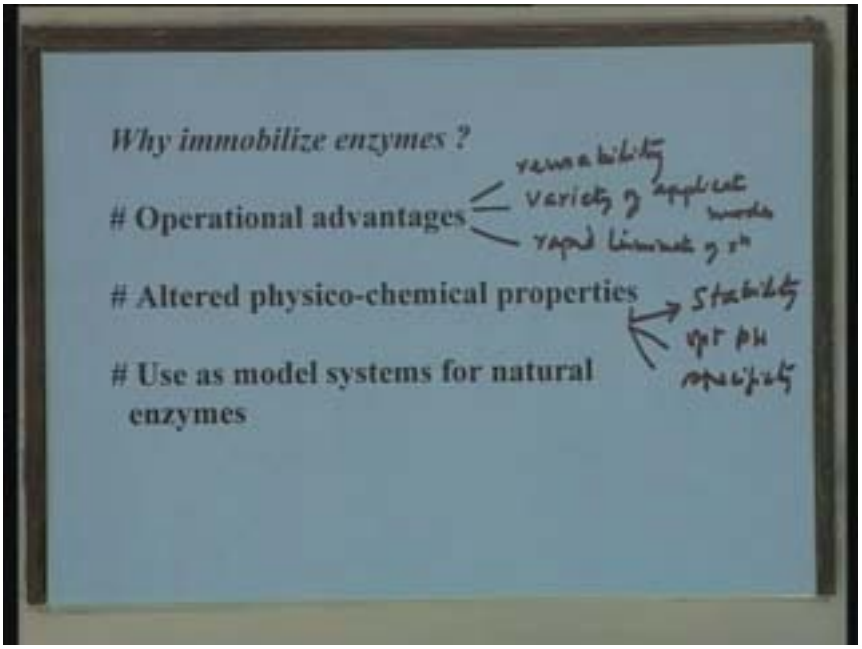

If it is an ionizable carrier, charged carrier then only it will lead to a change in optimum pH as we will see subsequently. But specificity also has been a factor. In fact even one can have an altered k_m for an immobilized enzyme as compared to soluble enzyme which again depending upon the same feature as optimum pH that is the matrix is charged and the substrate is also charged where the partitioning effects, the apparent k_m might also change. The third motivating factor was the use of the model system for natural enzymes that we discussed and which also has been a very significant reason for immobilizing enzymes.

Now let us look at the requirements of an immobilized system. In fact any immobilized enzyme system has three major constituents, three major players

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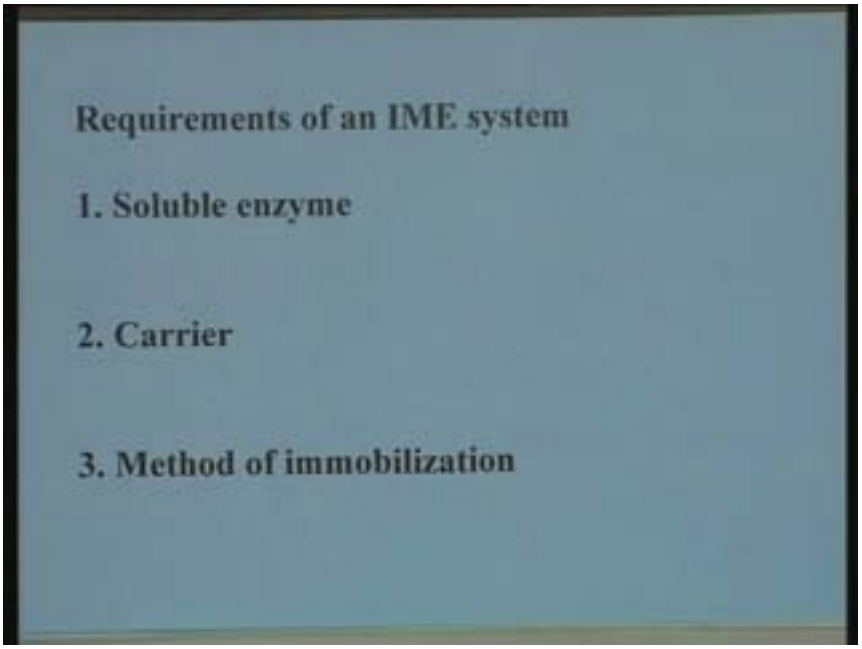

The first player is the soluble enzyme. We have to start with a good preparation of an enzyme isolated from a living system produced by fermentation or isolated from animals or plant cells. You need a soluble enzyme. The other is carrier. You need a matrix to which the enzyme can be immobilized in general. There might be some methods which may be carrier free. But in most cases, carrier is a candidate which is required for a coupling of soluble enzyme onto an insoluble matrix.

The third player is in method of immobilization and the total performance of immobilized enzyme preparation will depend upon the combination of the interactions between these three players. Now what are the properties we look at when we choose a soluble enzyme?

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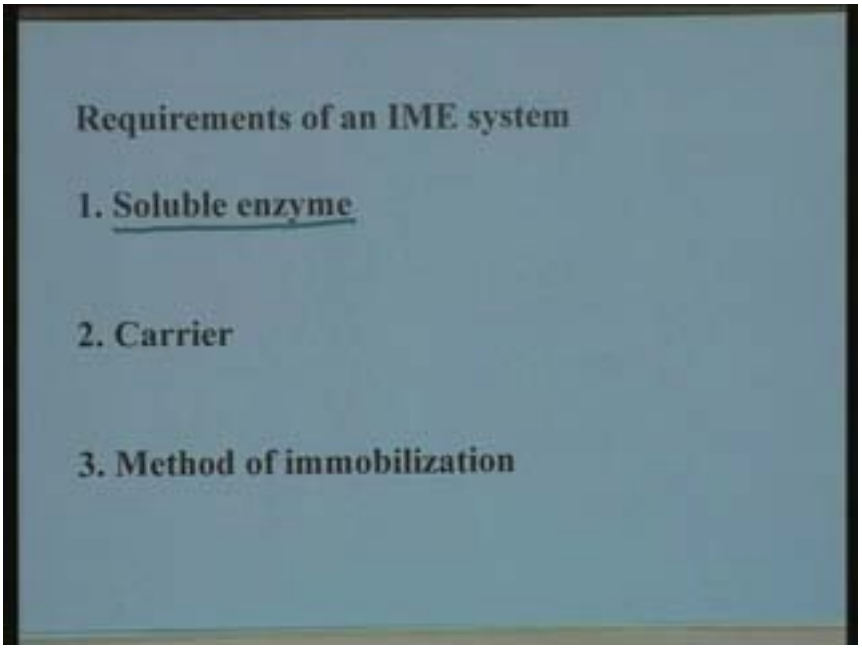

We understand that we require a particular reaction to be catalyzed. So the enzyme is fixed. For the sake of convenience let us assume that we have to isomerise glucose to fructose with enzyme called glucose isomerase.

When we talk of the enzyme for immobilization if we want to ….. (30.05) immobilized enzyme preparation what properties are important for choosing the enzyme source? The major factor is that we will look for an enzyme preparation which has the high specific activity.

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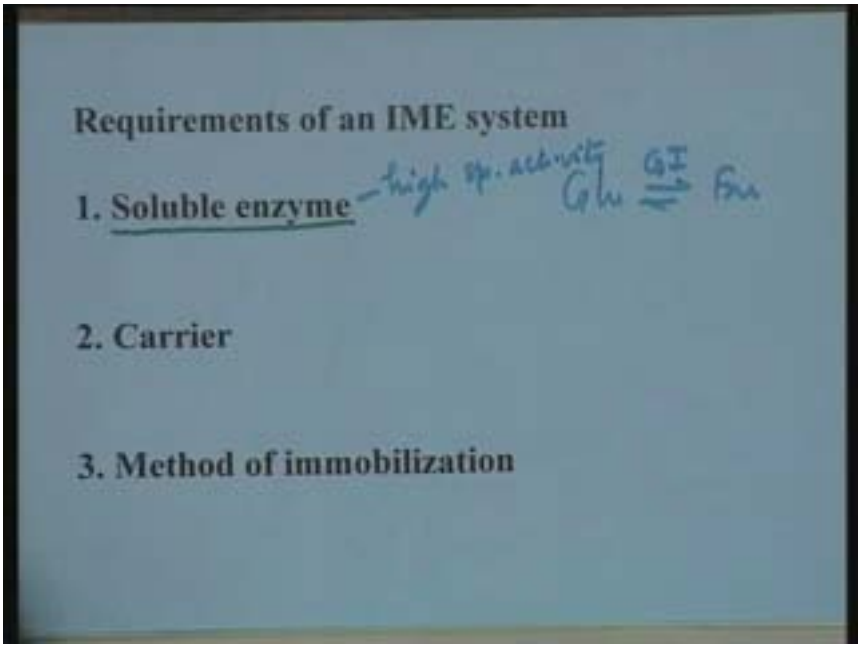

High specific activity means the catalytic power of the enzyme is much high. That means per unit weight of the protein its activity is much higher. This will depend upon two factors one is the source of the enzyme and the purity of the enzyme. Purer the enzyme, the better for immobilization because you can have a very highly active immobilized enzyme preparation or the source will also be important, if you can choose a source which gives you an enzyme which has a much higher specific activity. That means processing cost can be much lesser that is you have to put a much lesser protein for achieving the same catalytic function.

Next we will consider desirable operational parameters.

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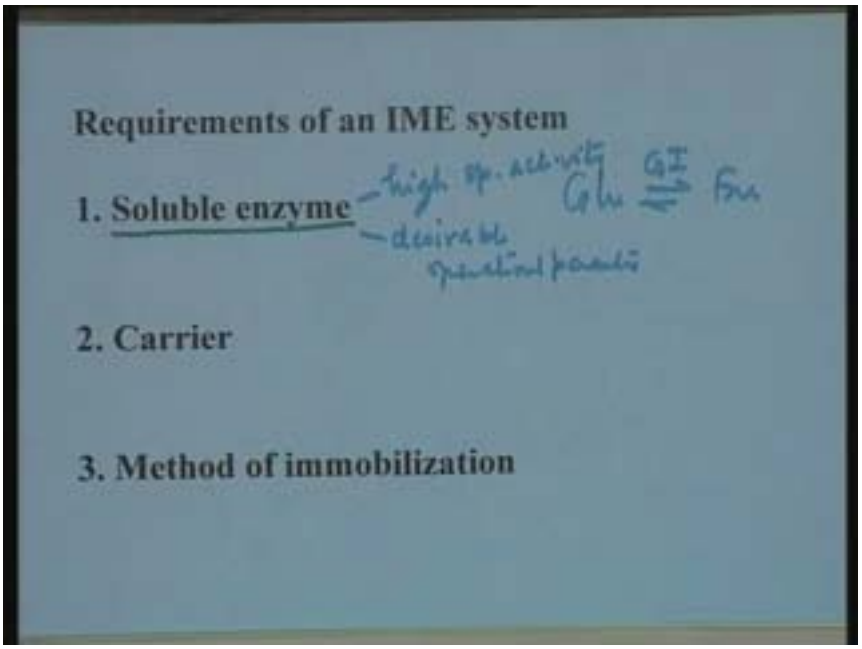

One of the major constraints of operational parameters is for many processes the operational parameters required for an enzymatic catalysis should not induce any side reactions. For example take again the example of glucose isomerisation. In addition to catalytic function by glucose enzyme, this process of glucose isomerisation can also take place in the presence of an alkali. If your environmental pH is alkaline, seven plus, the isomerisation can take place naturally and the isomerisation product will not be only fructose but it will be plus few other hexoses which will be produced and the whole advantage of using an enzymatic process is lost. In such case if you can have an enzyme preparation which has either a neutral or a slightly acidic pH optimum it is desirable. In fact the immobilized enzyme preparation can be tailored so as to have at least minor adjustment of the operational parameters.

The third factor is stability. In the light of specific activity, only your specific activity, again this will also depend on source of the enzyme.

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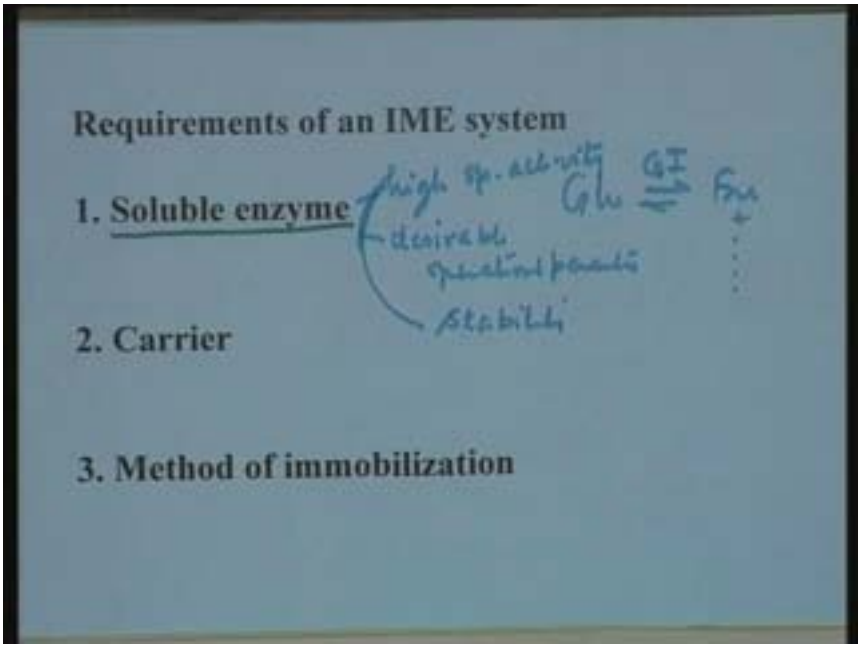

So one needs to choose the enzyme and its further processing for purification so as to have the three properties which are in the positive region and we choose the soluble enzymes on that basis.

The second candidate in the case of immobilized enzyme is carriers. In terms of the total gross weight of the product for example if you look at the total immobilized enzyme preparation almost ninety nine points will be contributed by a carrier. In one gram of immobilized enzyme, the enzyme preparation will be probably only a fraction of milligrams or if not more than one or two milligrams. Bulk of the immobilization enzyme product will be in the form of carrier. We are taking a water insoluble carrier. One of the key parameter here is it has to be insoluble in reaction environment. I am using a general term reaction environment mainly because while most of the reactions are to be carried out in aqueous medium today there are requirements that many of the reactions may be carried out in organic non-polar medium. Therefore the carrier which we choose should be insoluble in the reaction environment and that is a major requirement. It may ultimately depend upon the chemical composition of the carrier.

Logically the carrier should also be non-toxic to the enzymatic reaction and this should not act as an inhibitor or some kind of an irreversible inhibitor to the enzyme catalyzed reaction.

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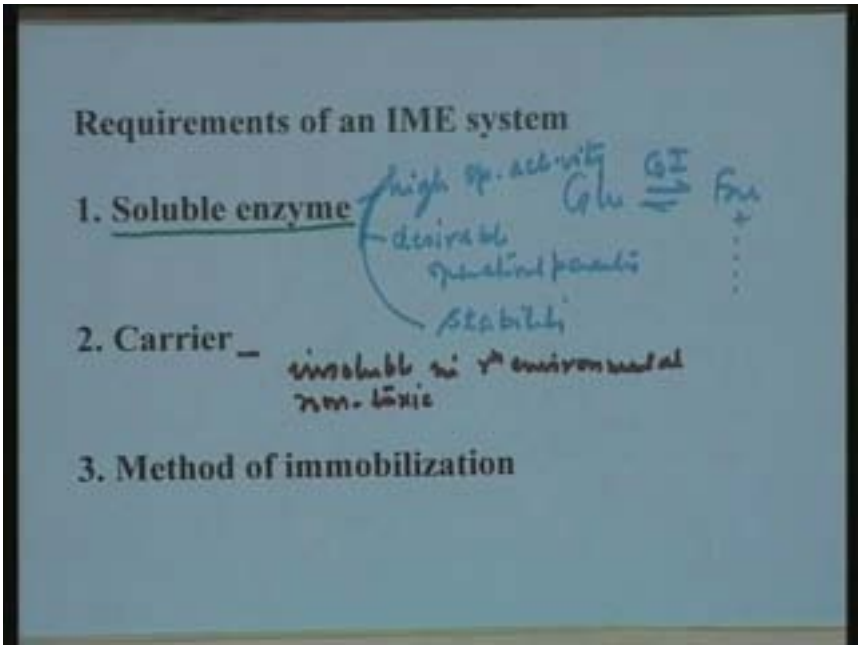

Another very important feature is resistance to microbial, thermal or mechanical degradation because as you recall right in our definition we mentioned that it is required to be used over a long period of time. The use over a long period of time may involve many features which might expose the whole material to many features which may lead to microbial degradation, thermal degradation or mechanical degradation and the carrier should be resistant to them. Therefore carrier itself should be very stable otherwise the stability of the enzyme will not have much of meaning. The carrier should also have a large number of sites for binding of enzyme. The major reason is that ultimately the objective of an immobilized enzyme system will be to have a very high activity.

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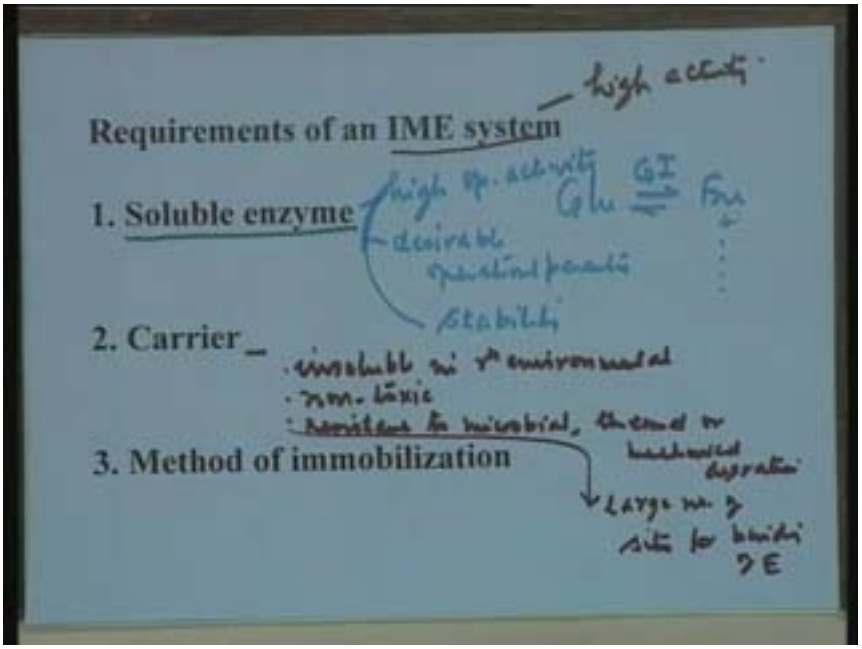

To have a high functional reactivity one need as much of enzyme. The activity or the loading of the enzyme on to the carrier must be very high, the higher you can make and that will require a large number of sites for binding of the enzyme on to the carrier. This will require many of the functional groups to be present on the carrier. It will also depend on the porosity on the carrier. The porosity of the carrier depends on the surface area which will also result from the porosity as well as the particle size. So surface area will also become quite important. Finally if not a major scientific fact but at least the economic feature is the regenerability and cost.

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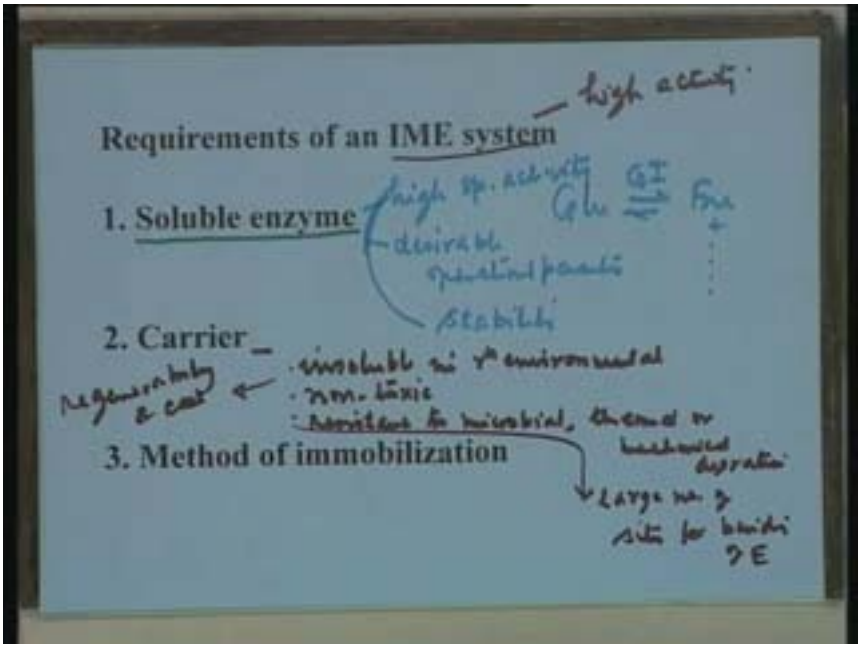

Another criterion which plays a very key role into the carrier matrix. Normally we will prefer a carrier which is not very expensive. In an inexpensive carrier one may say that you may not be able to meet the other desirable properties. One can choose an expensive carrier provided the carrier can be regenerated at the end of the exhaustion of the enzyme. When I say immobilized enzyme should be used continuously or repeatedly again this means that it can be used for couple of days, couple of months, couple of years and not for ever. At the end of the cycle of the operation of an immobilized enzyme preparation, if the carrier can be regenerated it is desirable even if its cost is high. In fact you will notice that in some of the industrially used immobilized enzyme preparations the low cost carriers has been rejected mainly because of some of the undesirable properties that they cannot get you a very high enzyme loading on them and many of the expensive carriers have been used but only under the condition that they can be regenerated. Carriers like organic ion exchange resins like D I cellulose and di Sephadex have been extensively used for immobilization of enzyme by absorption and after use when the whole enzyme is desorbed the carrier is again available for reuse.

To take care of all these desirable features of carrier the properties that you look in a carrier are chemical composition, specific surface area that means surface area per unit weight of the carrier, particle size and ratio of hydrophobic to hydrophilic groups mainly to achieve a very high degree of loading because one has to choose all the functional group of the enzyme molecule for coupling and therefore hydrophobic to hydrophilic groups on the carrier and finally the cost.

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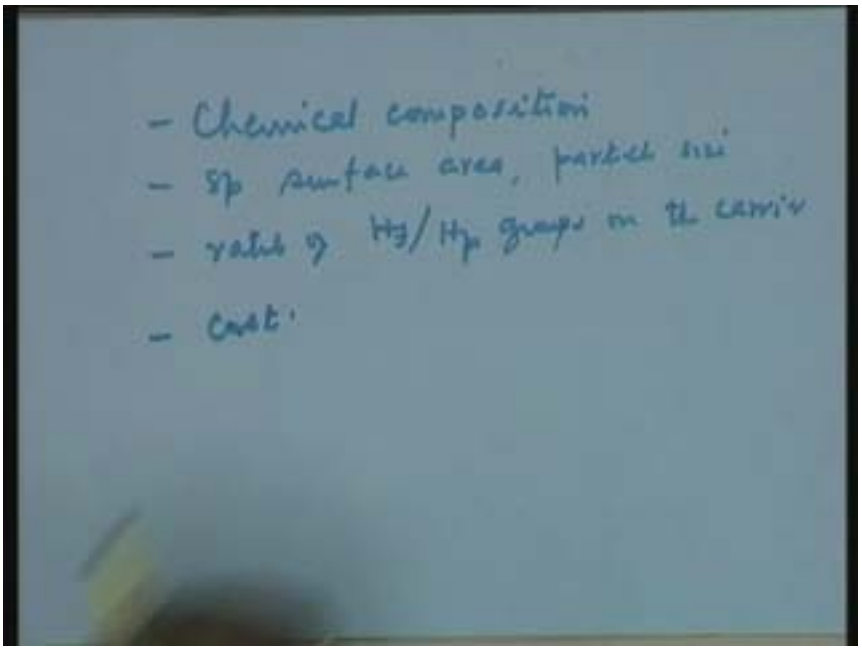

These are the four general features of properties one has to look in the carriers. Large varieties of carriers have been used and are available for use fortunately and these carriers have been classified by various people in different modes, in different forms. A very

generalized scientific approach to classify them will be on the basis of their porosity. Porosity is the basis for classification of carriers. That is they can be non-porous.

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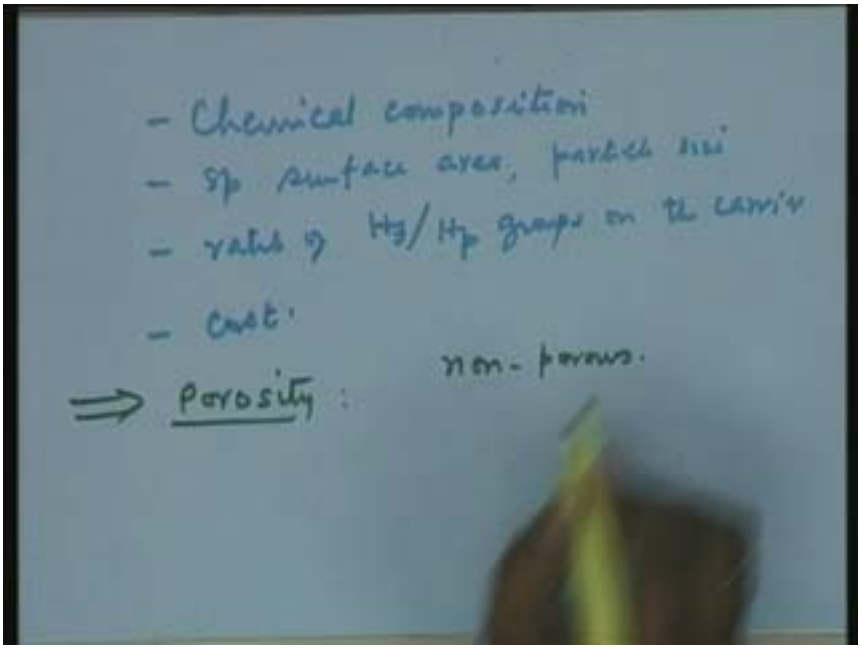

The non porous carriers will have a low specific surface area because the porous matrix will provide lot of internal surface in a matrix. The only surface which is available is the external surface, only part. To get a higher surface area you have to reduce the particle size which also has a great limitation of use because of the smaller the particle size its use in a packed …… (41.18) is difficult because of the high pressure drops. Its use in CSTR is difficult mainly because of its loss through the product stream if it is too fine a particle. Therefore non-porous matrices usually have a limitation of low surface area. As far as the chemical composition, ratio of hydrophobic to hydrophilic group or the cost is concerned it is a general feature.

Non-porous matrix also has an advantage. Mainly they have been used extensively for bio medical applications where an additional desirable feature other than the ones we mentioned in the carriers is the prevention of interfacial immunological responses in the bio-medical system. For example when you want to use an immobilized enzyme preparation for any bio-medical application it will interact with the blood stream and any external material in contact with the blood stream will generate a very vigorous immunological response and it has been noted that the non-porous matrices or the non-porous materials are much softer as far as these responses are concerned compared to porous ones. The typical example of the use of the non-porous matrix in the bio-medical application has been nylon as a non-porous matrix which has been used to immobilize some of the therapeutic enzymes for use in contact with the blood stream.

The other group of matrices is porous matrices, the most preferred kind of material that are used for industrial applications and these matrices are control pore glass, control pore titanium and variety of other synthetic tailored materials which have been produced commercially for generating a high porosity for coupling of enzymes on them. The third kind of material is gel structure. They are porous in nature but they are usually flexible in the pore size or thermoelastic and the pore is flexible and the typical examples which have been used are collagen, poly acrylamide, gelatin and a large number of other gel structures have been used.

In the gel structure you can entrap the enzyme in the gel matrices and the advantage is that the enzyme can experience almost the same kind of water activity or the environment as very close to what is obtained in the case of a living cell. Let us consider the gel matrix and the enzyme entrapped in the lattices. The gel will not allow the enzyme to pass through but the small molecular weight substrate and product can easily be allowed to get in or come out.

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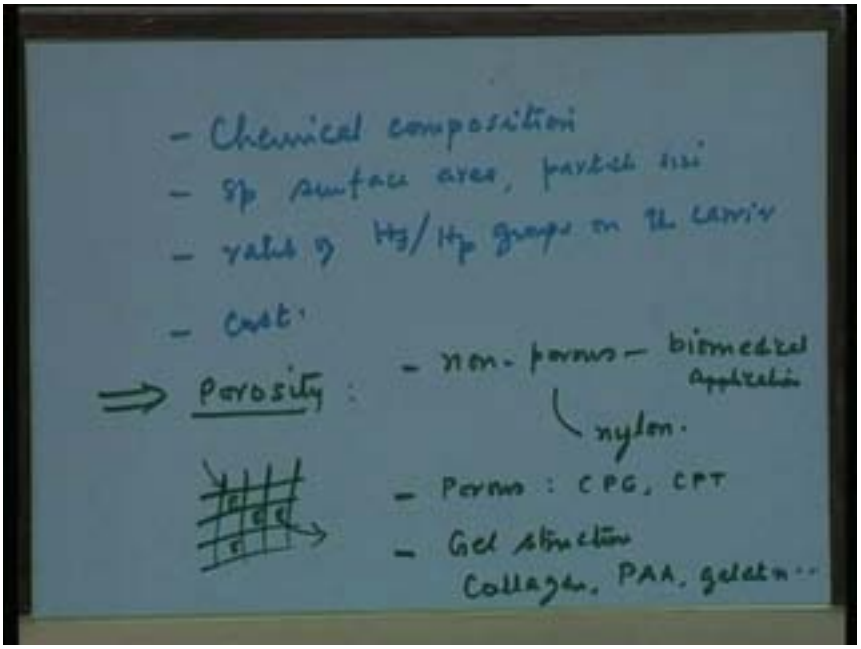

Another way of classifying the carriers will be on the basis of chemical nature. One can have either inorganic carriers or organic carriers. In most cases the inorganic carriers as I mentioned are the whole range of control pore glass, control pore titanium many of the clays which are naturally occurring like bentonite, animal charcoal and things like that fall under inorganic but the organic carriers are usually polysaccharides, proteins and synthetic polymers. Typical example of organic carriers are polysaccharides, cellulose, agarose, even cross linked dextran what we call industrially as sephadex. This is nothing else but cross linked dextran molecules. Then in the case of proteins almost like what we talked about gel structure, collagen and gelatin are typical examples and there is a whole

range of synthetic polymers which have been reported and used for immobilizing enzymes which are usually polystyrene based, vinyl polymers, polyacrylamide and so on.

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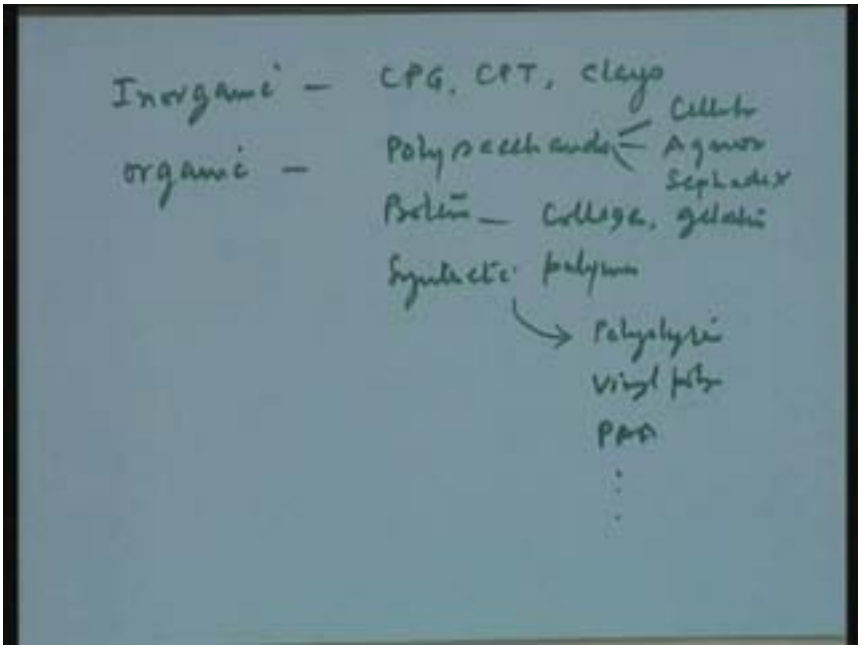

You can classify and a large variety of carriers are available for use for immobilizing enzymes and the choice will depend upon various properties that we have listed.