Metabolic Engineering
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Lecture - 05
Essence of Metabolic Engineering - Part D

Welcome to the last part of our discussion on the introductory topics on metabolic engineering.

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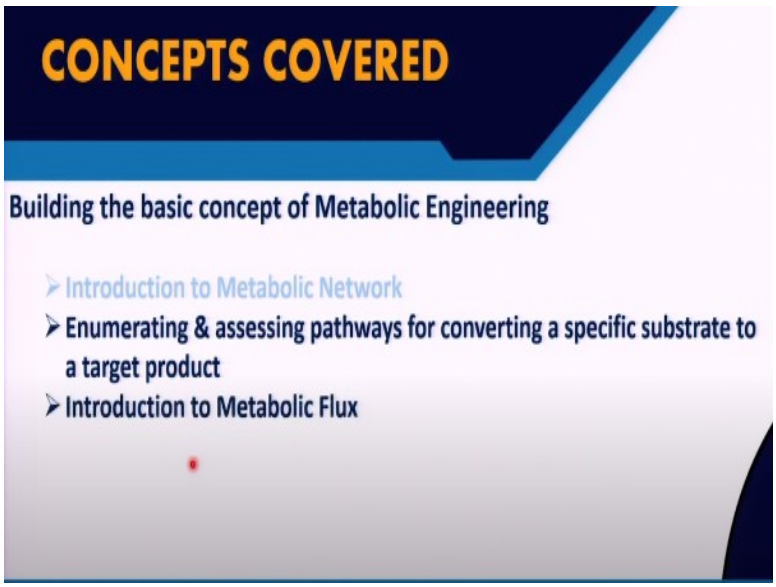

And this is continuation of our earlier discussion where we were trying to build the basic concept of metabolic engineering and particularly the interconnected nature of the metabolic networks that is already discussed. And in this section of this lecture, we are going to learn that the importance of enumeration and assessment of all the pathways for converting a specific substrate to a target product.

And we will be having a brief introduction about metabolic flux and metabolic flux analysis.

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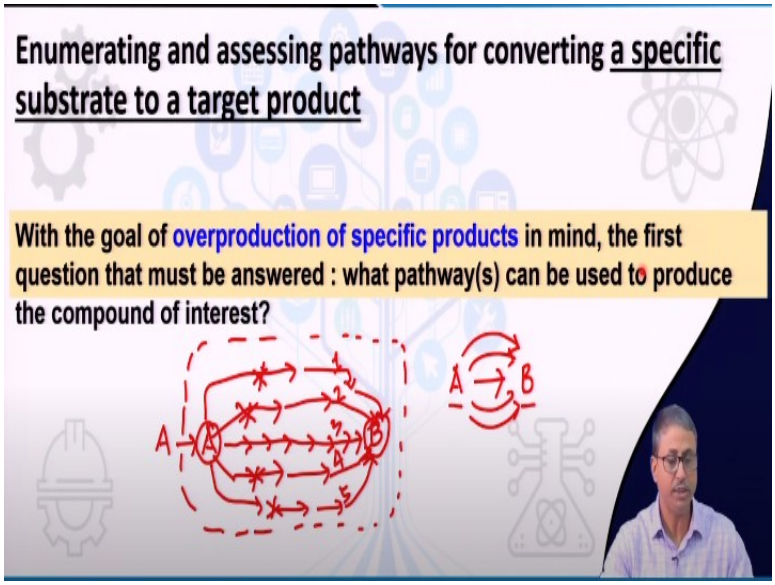

Now enumeration and assessment of all the pathways which are likely to be involved in converting a specific substrate to a target product is one of the major tasks in metabolic engineering. So there are two important aspects. One is the enumeration of the pathways which are likely to be involved and then assessment of these pathways.

So if we want to highlight this particular point, so there could be multiple so let us assume that we have a substrate and the substrate is taken up by the cell and within the cell, so this is the cell boundary if we try to consider it like this. So as the substrate enters inside the cell, so it could be producing the product B, which is actually composed of a number of reactions.

So this is a set of reactions or maybe a particular pathway which is preexisting or we know about this particular pathway. But there could be other several mechanisms by which the same product B is produced. But it might be possible that we are not completely aware about this particular other reactions that which I am now drawing that the alternate reactions like this one, alternate reaction this one, alternate reaction this ones or alternate reaction this ones.

So there could be numbers of such reactions or such pathways, which are potentially capable of converting this particular specific substrate to the targeted product. Now enumeration means that we try to identify all these possible or potential pathways, which are likely to be present in a particular cell or particular cell system and then assess each of these pathway. So there are two points, one is the enumeration.

That is we need to identify that okay, these are the five pathways, so 1, 2, 3, 4 and 5 pathways, which might be converting substrate A or potentially able to convert the substrate A to the product B and then individually we assess each of the pathways. Now this assessment needs certain criteria that based on which we will assess these pathways. So we will briefly talk about that.

So once we identify a specific reaction, so in this case it is the reaction that is the substrate A to product B. Now we will examine all possible reactions or pathways which might be converting A to B and also we will be assessing all those things, all those possible pathways. Now with the goal of overproduction of specific products in mind, when we have in metabolic engineering, we surely will be having a specific goal that we will be producing a particular metabolite or particular product.

Or we will be looking towards improving a particular property of a cell. So when we have a particular property, like particular goal in our mind, like say overproduction of a specific product within using a particular cellular system. The first question that needs to be answered is what pathways can be used to produce the compound of interest. So earlier we were talking about production of ethanol using a simple reaction of pyruvic acid to ethanol.

So if we are interested for example, in producing ethanol in using *E. coli* or other yeast or other systems, so we need to answer the first question that what are the pathways involved in production of ethanol within a *E. coli* or yeast system.

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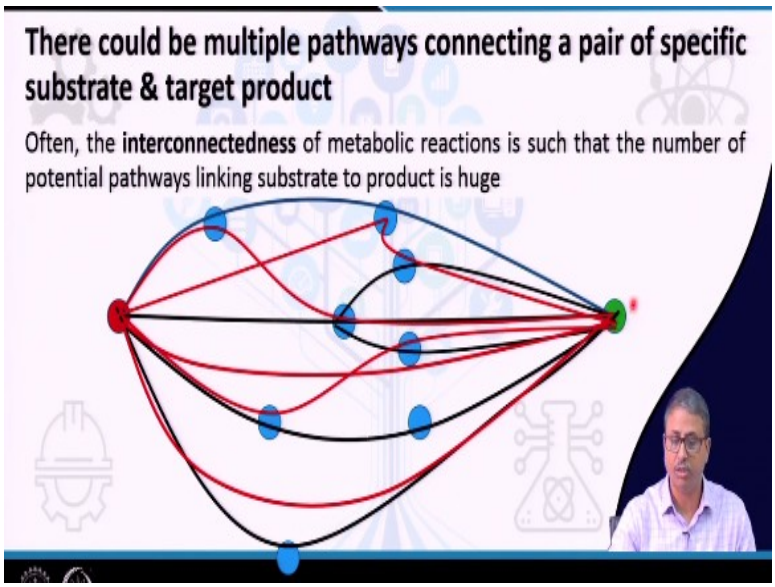

So as I was mentioning, that there could be multiple pathways connecting a pair of specific substrate and target product. Like we have the substrate which is red colored and the product which is green color, and there could be multiple reactions or multiple set of reactions or pathways, which are very clearly evident in case of the glycolysis.

That we were talking about the glycolysis how it is connected to the ED pathway and then pentose phosphate pathway and also with the TCA cycle. So eventually any reaction within these glycolytic pathway is under the influence and under control of multiple reaction. So we need to actually elucidate or enumerate all these multiple pathways which could be responsible for producing ethanol or maybe pyruvic acid if we take for an example.

So the first point is that there could be multiple pathways involved in conversion of a particular substrate to a particular product. And we need to enumerate or identify each of these pathways. Now often the interconnectedness of the metabolic reactions like in the glycolytic reaction we have seen the interconnectedness of metabolic reactions in such that the number of potential pathways linking substrate to the products is actually huge.

So if we want to elucidate all we will be surprised to see that actually from pyruvic acid to ethanol, this could be a very unique reaction, but the flux or the flow of carbon up to pyruvic acid, it is controlled by many reactions within the glycolytic pathway

that ultimately leads to the production of the ethanol. So interconnectedness of the metabolic reactions is also to be included over here.

And there could be actually as we mentioned, there could be huge number of potential pathway. So actually, when we looking we will started looking into the whole genome based data apart from biochemical assessment of different pathways that how a particular yeast or particular bacteria could might be converting substrate like glucose or any pentose sugar or other hexose sugars into ethanol, some number of pathways were elucidated.

But as soon as we started looking into the whole genome sequence of the organism, we were surprised to see there are actually more number of potential pathways which are actually potential pathways that they could actually link the substrate or to the desired product. Now these pathways, first is the all the pathways are need to be identified.

Some of the pathways will be observed chemically that by biochemical assay, by detecting the specific enzymes we can confirm that these pathways are actually indeed present in a particular species or particular cellular system and they are working towards a substrate to product formation.

And some of the pathways are actually potential pathways that under certain circumstances, these enzymes will be expressed or the genes encoding these enzymes will be expressed and these pathways might be working towards the production of the desired product.

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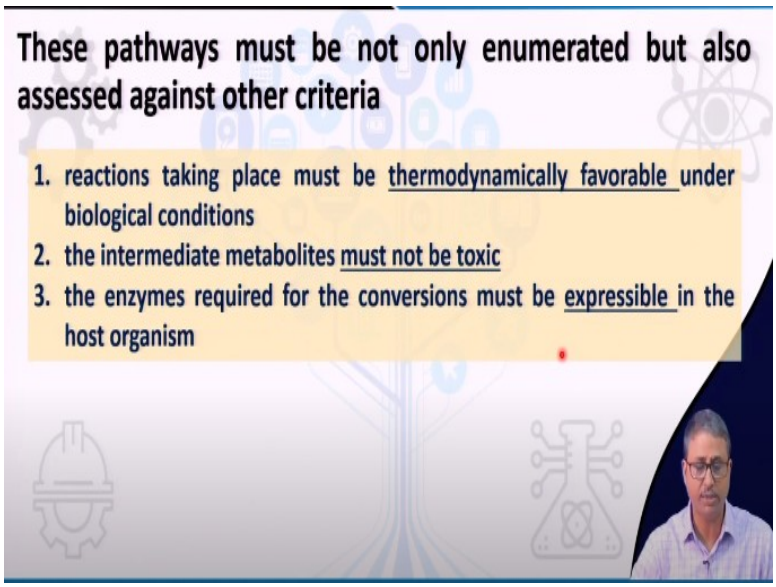

Now these pathways are first to be identified that is called enumeration of these pathways. Okay, these are the pathways, but they are to be assessed against each other. And other criteria means there are multiple criteria that we are going to discuss briefly.

That when we have a number of pathways, which are connecting a specific substrate to a specific product, all these pathways need to be assessed in terms of the thermodynamic feasibility of each of these pathways under biological conditions. Particularly when we integrate the genome annotation data with the mathematical modeling and then try to build the metabolic network and look into the interconnections.

Often we are able to discover or identify a number of novel pathways or potentially new pathways, okay, under which might be working under different environmental conditions or under different structures. Now all these pathways, maybe 5, 6, 10 or maybe 50 pathways are to be assessed in terms of thermodynamic feasibility or whether they are thermodynamically favorable or not that within the cellular system the biological conditions are very precise conditions.

So within the biological conditions whether these all these pathways that we are able to find out are going to work or not. The second point is that the intermediate metabolites, we can actually find out a pathway which is converting our substrate to our desired product. But the intermediates might be toxic as we have noticed and we

will discuss in some of the other classes that intermediates are often found to be toxic to the cell.

So when we assess multiple pathways, which are involved in converting a particular substrate to a particular product, we should also include some assessment about the toxicity of the intermediates. And thirdly, the enzymes required for the conversion because each of the reactions or each of the reactions which are present in a particular pathway or connecting a specific substrate to a specific or a particular targeted product are controlled or catalyzed by enzymes.

Now these enzymes are encoded by specific genes. So the enzymes required for the conversion must be expressible in the host organism. So whatever host we are selecting, it may be the native organism like an *E. coli* gene is being expressed in *E. coli* itself. Or it might be that it is actually a gene or an enzyme encoded by the chromosome of yeast and we are trying to express in *E. coli* system.

So wherever we are expressing these particular gene for producing the over producing the enzymes which are likely to be controlling the process, so that must be expressible. So we may identify theoretically using as theoretically because it is based on the flux analysis and the metabolic networks that we develop, we can actually predict and identify a number of pathways connecting a substrate to a product.

But while evaluating or assessing these pathways, we should also include that whether the genes required for this particular pathway or catalyzing a particular step within this pathway is expressible in the host organism.

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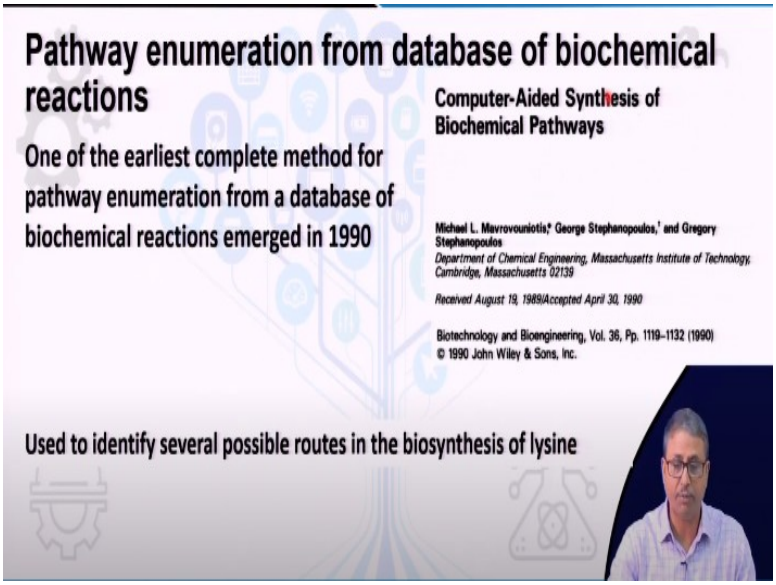

So one of the early studies was in 1990 by the Stephanopoulos group, where pathway enumeration from database of biochemical reactions was attempted using some algorithm. So some computational process was used. And one of this was one of the earliest complete method for pathway enumeration from a database of biochemical reactions that is in way back 1990.

And that was used to identify several possible routes in the biosynthesis of amino acid lysine. So a number of possible pathways were predicted using the database of biochemical reactions.

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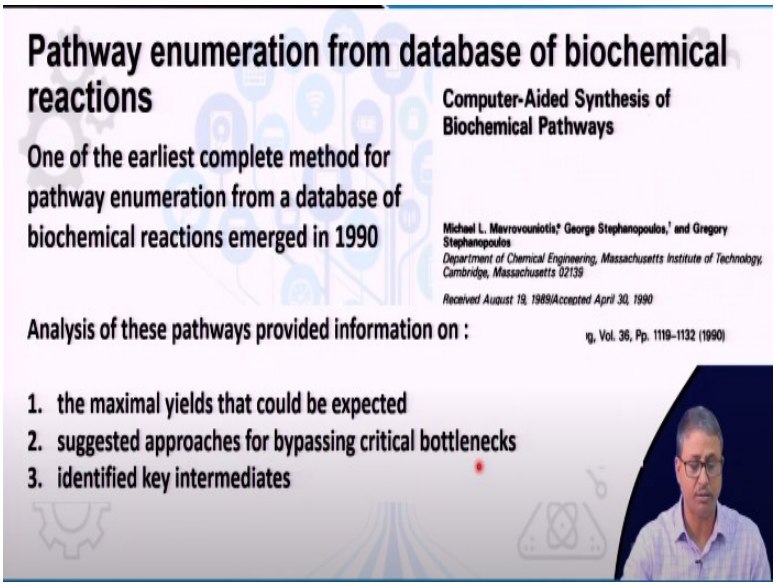

And analysis of this pathway, when multiple pathways were elucidated towards the formation of lysine using this particular algorithm or set of computer program, the

information obtained were the maximum yield that could be expected out of each of the pathways, the suggested approaches for bypassing the critical bottlenecks.

Because many times many a times what we see that the particular biochemical reaction will have some bottlenecks within it, some of the enzymes might be inefficient or lack of enough catalytic abilities. So we need to identify that how can we bypass those bottleneck reactions where actually the flow of molecules or flow of the carbon or flow of the flux is actually reduced or hampered.

And also identify the key intermediates which are produced during the entire stretch of the reaction. So when we do this kind of, started doing this kind of database, use of database of biochemical reactions way back in 1990. So we could identify or this group could successfully identify number of pathways and then evaluated this.

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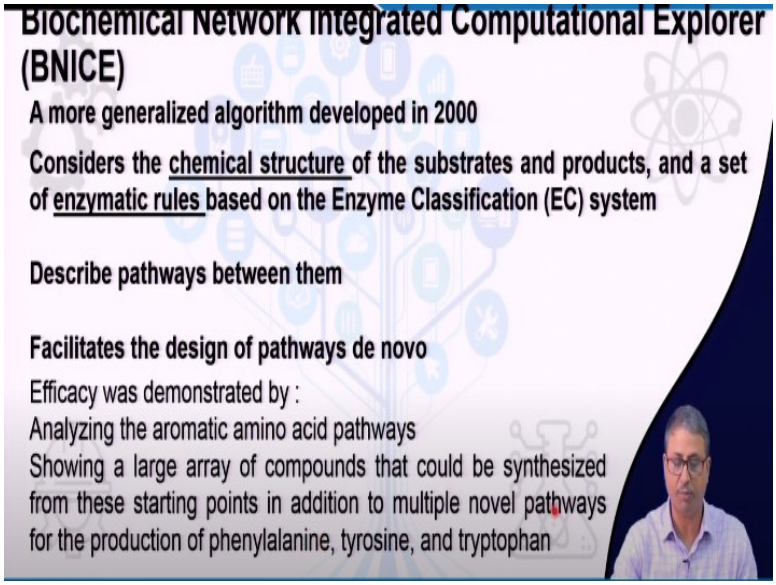

In 2000, a very interesting computational explorer was developed which is biochemical network integrated computational explorer or BNICE. So this is having a more generalized algorithm, which considers the chemical structure of the substrates and the product. So all the structures of the substrates and the products were considered and a set of enzymatic rules based on EC classification system, enzyme classification system was included in that.

And it described the pathway between them. So all these substrates, intermediates, products, the enzymes involved, structures, and the enzyme rules that what type of

reaction it is. Is it a kinase, it is a hydroxylating enzyme, it is a phosphorylating enzyme, it is a dehydrogenase type of reaction, what type of reaction is going on? All these details were included in that.

And that enabled this group or the group who has published this database, computational explorer data, they successfully designed the pathway de novo. And the efficacy of such de novo synthesized pathways were demonstrated by analyzing the aromatic amino acid pathway showing a large compound that would be synthesized from this starting point, in addition to multiple novel pathway. So a number of novel pathways could be discovered.

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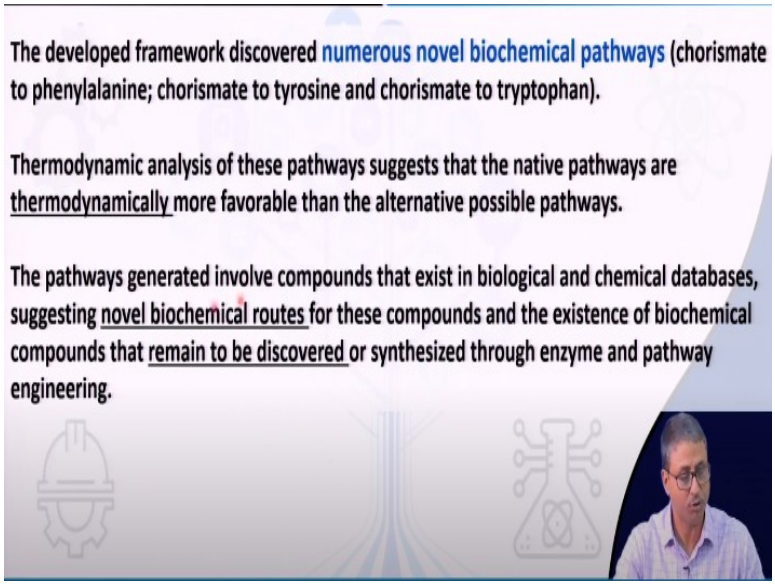

So we could see that, for example, well known substrate for amino acid production with chorismate. So this chorismate could actually lead to the production of phenylalanine to tyrosine and ultimately to tryptophan. So numerous other than these three particular conversion, numerous novel biochemical pathways involved in conversion of the substrate to these products were elucidated.

And the thermodynamic analysis of this pathway suggested that native pathways are thermodynamically more favorable. So as I was mentioned before, that it is not only enumerating or proposing new pathways that we have observed that this pathway could be present in a particular system for converting a substrate to a product, but the thermodynamic favorability or thermodynamic feasibility of such reactions are to be established.

So using this kind of computationally supported database, they were able to show that it is many of the pathways which are proposed, these pathways are not thermodynamically favorable, while the native pathways which are existing or preexisting and known for conversion of these chorismate to tryptophan tyrosine phenylalanine are actually thermodynamically more favorable.

The pathways generated involved compounds that exist in biological and chemical databases. So when they looked into the entire set of data projected or predicted by this computational process, they found many of the intermediates, which are predicted by the system are existing in biological and chemical databases.

That means they are biologically produced and chemically they are relevant, they are already there in the database, suggesting novel biochemical roots for these compounds and the existence of biochemical compounds that remain to be discovered.

So based on their observation, this group, when they studied this, they found that novel pathways and novel reactions to discover such kind of intermediates, which the intermediates are already there, but the pathways are not known, could be discovered or could be identified.

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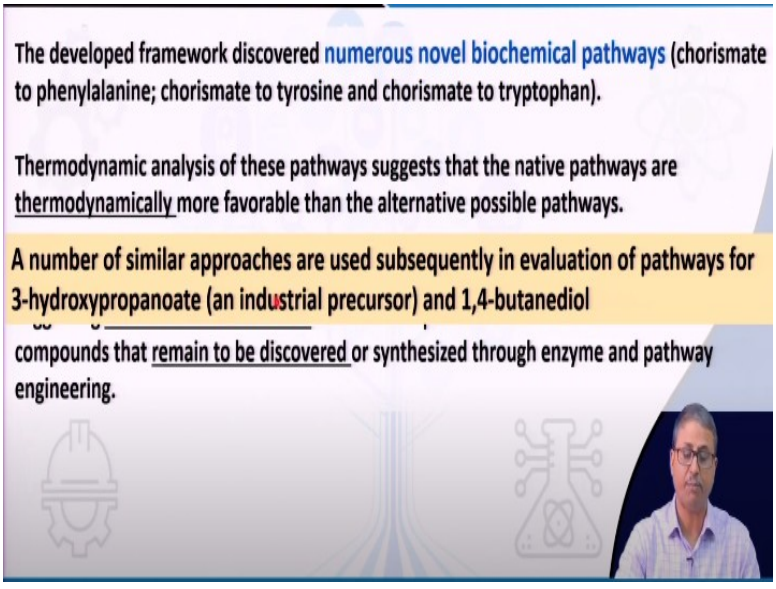

So in number of similar approaches using such kind of systems were used subsequently for evaluating the pathways for 3-hydroxy propionate and 1,4-

butanediol. So all are industrially very important metabolic engineering relevant products.

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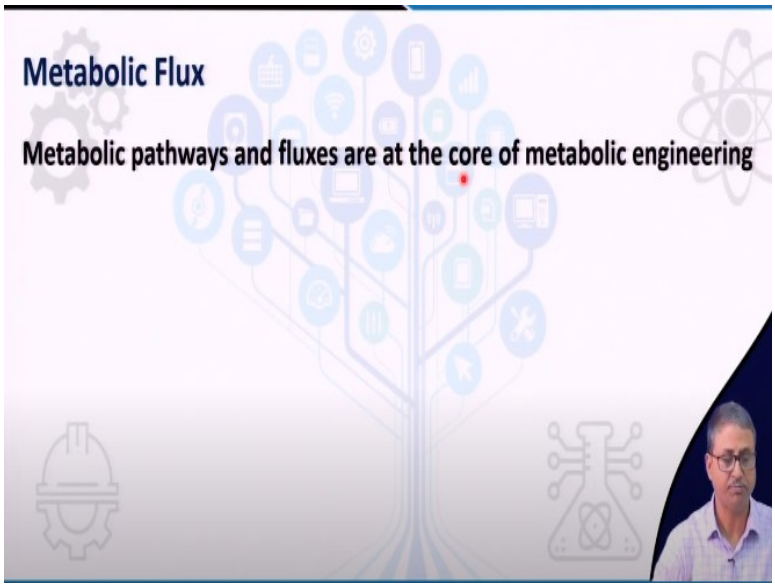

Now we are going to talk briefly about metabolic flux. Metabolic flux, we are going to talk in terms of defining it and metabolic flux analysis. But before entering into these things, we just want to reiterate that metabolic pathways which we are possibly talking for some time now and we have gained some level of understanding because it encompasses a number of reactions and sharing kind of common metabolites within it.

So metabolic pathways and fluxes are at the core of metabolic engineering. So we need to identify and understand the metabolic pathways and also characterize the metabolic fluxes within the studied system and that is identified to be a core of metabolic engineering. So after metabolic network or interconnected systems level understanding, this metabolic flux analysis is another very important aspect.

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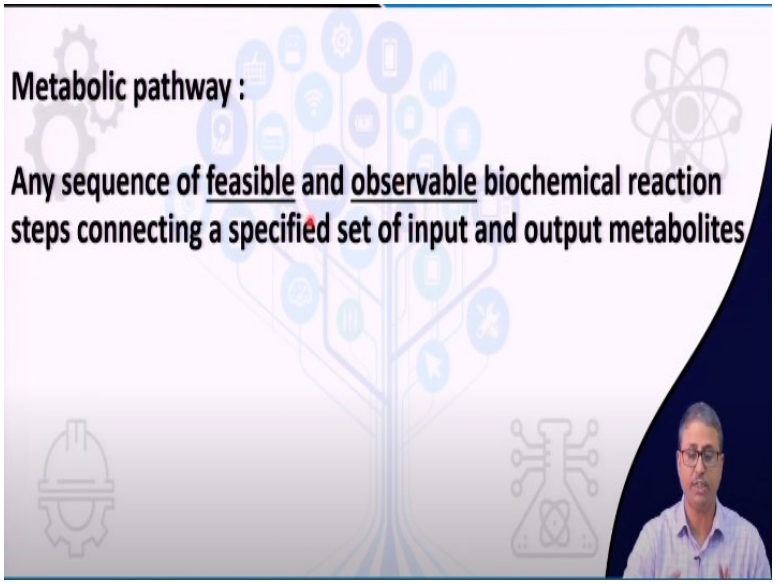

Now here we need to very clearly define metabolic pathway, what is it actually? Metabolic pathway we often use the term in a very general sense that it is producing some kind of product out of a substrate and which is basically catalyzed by a number of enzymes etc. Now it is defined as any sequence of feasible and observable biochemical reaction steps connecting a specified set of input and output metabolites.

So it clearly says that it is basically a sequence of reactions. So it is a pathway. So it is a pathway that means like a glycolytic pathway or tricarboxylic acid cycle, which we called as the TCA cycle, it is a pathway. So that is actually a sequence of reactions. So a number of reactions are there and product of the first reaction is likely to be the substrate for the second reaction and so on and so forth.

And finally, the product is produced. Now this these reactions which are actually representing the pathway must be feasible and observable biochemical reactions. And they must be interconnecting a specific set of input and output.

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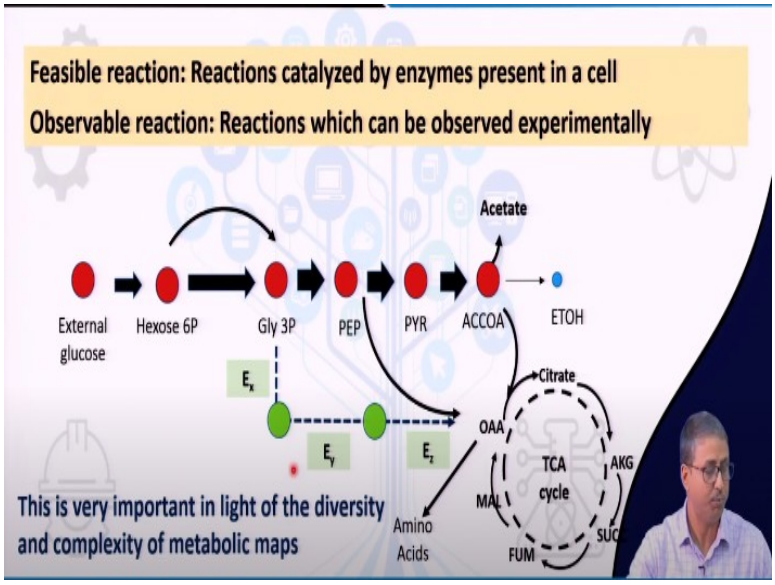

So for example, we are coming back to this picture where we were talking about the glycolytic reaction and along with glycolytic reaction, we are able to see that how acetyl-CoA can be converted to or pyruvate can be converted to ethanol.

And also we see that how the TCA cycle is connected to these acetyl-CoA and even phosphoenolpyruvate can be connected to the TCA cycle by producing oxaloacetate. And the entire set of this TCA cycle is again connected to amino acid biosynthesis. So it represents a number of pathways within it.

Now during our analysis, we can identify a novel pathway or a part of novel set of reactions, where glyceraldehyde 3-phosphate is potentially converted to oxaloacetate. This is kind of an hypothesis that based on certain metabolic or network analysis or metabolic pathway analysis, we can propose that here is a kind of a novel or new pathway. So there are enzymes involved like E x, E y and E z converting this glyceraldehyde 3-phosphate to oxaloacetate.

So this is just a kind of a proposal. This is a hypothetical situation. Now if we propose something like this, then the feasibility of the reaction like in the previous definition of the metabolic pathway, we mentioned about a series of reactions, which are actually feasible and observable. So feasible reaction means the reactions which are catalyzed by enzymes present in a cell.

Now we can propose that maybe it is an E. coli system. So we are studying or working on E. coli. So we may propose based on our modeling and other studies that there are three enzymes which might be capable of converting glyceraldehyde 3-phosphate to oxaloacetate. This is a unique or new pathway we are proposing.

But before we propose or before we go further, we need to understand that whether these enzymes are actually present in E. coli or not. So we may find out that these enzymes are not present in E. coli cells. So these are all purely kind of imaginary kind of enzymes that the model might have predicted, but actually these enzymes are not present inside the cell. So the reactions catalyzed by these enzymes are absolutely not going to be feasible.

So based on this, we possibly will not be able to construct a metabolic pathway. Not only this that feasibility of the reaction, because these enzymes are not present in a cell, but also that whether these reactions can be observed experimentally. That we are able to see that the glyceraldehyde 3-phosphate is converted to this particular product, let us assume this is a kind of product x.

So glyceraldehyde 3-phosphate is getting converted to product x, which can be observed experimentally or not. So we may also find that no this cannot be demonstrated experimentally. So neither the enzyme is found in *E. coli* system nor the conversion of glyceraldehyde 3-phosphate to the product x or even the other products are observed experimentally in a laboratory situation.

Then, perhaps we would not be able to call this particular segment as a new or a pathway for producing oxaloacetate from glyceraldehyde 3-phosphate. But it could be other way around as well, like we may get some pathways which are proposed or indicated by our computational analysis or modeling analysis, during metabolic network studies.

And some of these enzymes or many of these or all of the enzymes could be identified in a particular host system. And also the reactions would be experimentally observed many cases. So it is having a kind of chances that sometimes they are experimentally

observed and they are found to be physical reactions, but sometimes they are not. So it has to be done. So that is the main point over here.

The prediction and all this kind of identification of novel pathways must be coupled with the validation that whether this reaction is going to constitute a metabolic reaction at all. This is very important, particularly in the light of diversity and the complexity of metabolic map.

So when we have different computational tools, because we need to remember one very important fact that the *E. coli* genome is completely sequenced long time ago and we are sequencing more species or variants of E. coli and other suitable host organisms. It is around 4.6 $\times 10^5$ bases are there okay. So there are large amount of sequence bases are there.

And given point of time you will be having some kind of values like 600 to 700 enzymes are expressed in E. coli system. So all these enzymes, some of the enzymes might be predicted. So some genome analyst might be able to predict a couple of new enzymes and might be able to propose a new pathway based on the information that particular scientist have used.

But eventually, these all reactions need to be validated before proposing a new pathway about that particular reaction.

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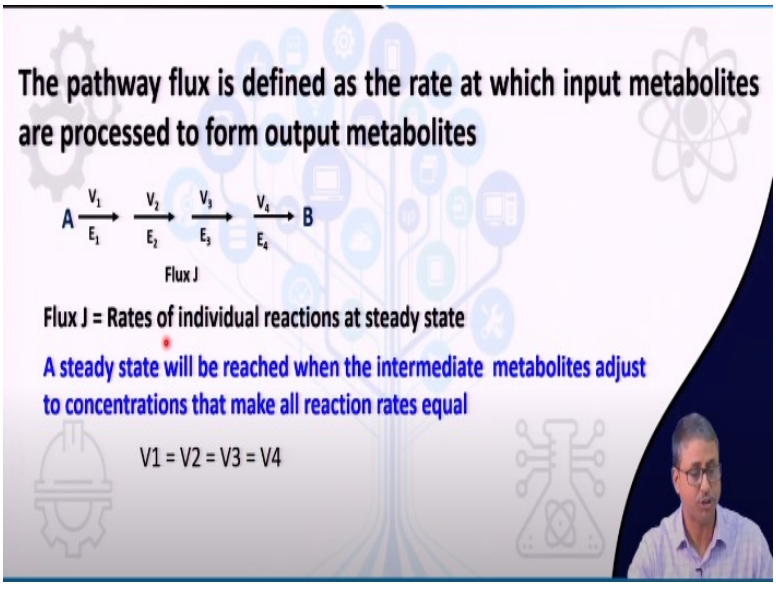
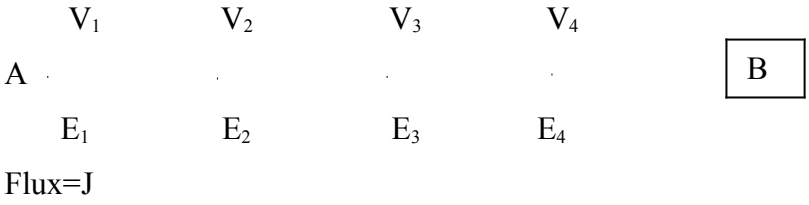


Now the pathway flux. So once the pathway is well defined, the pathway flux can be defined. So pathway flux or metabolic flux of a particular pathway, because it has to be specific for a pathway or specific for a reaction is defined as the rate at which the input metabolites are processed to form the output metabolites.

And if we consider this particular reaction, where you have A to B, is A is the substrate and B is the product in this reaction. And in that case, if we consider flux is J, then the flux J is equal to the rates of individual reactions at steady state.



So whenever the reaction is steady state we will find that the intermediate metabolites are adjusted to concentration that make all the reaction rate equal and therefore J is equal to the rates of the individual reactions, which is V1 = V2 = V3 = V4. Now there may be transient states when the actually transient conditions when the individual reactions are not at steady states. So we have to understand that also.

So the reactions must be reaching into a steady state that is a fundamental consideration before calculating the metabolic flux.

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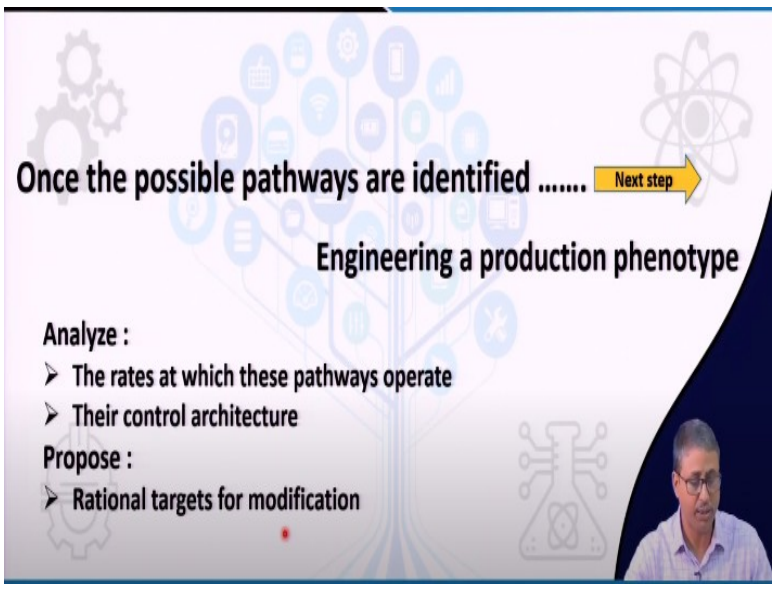

Now once the possible pathways are identified, suppose we have identified what are the possible pathways, we have actually assessed the pathways, found that out of 5 or 10 pathways that we initially enumerated maybe two or three are suitable for further investigation.

The next step towards engineering a production phenotype like before entering into the designing step that how are we going to change the expression pattern or maybe changing the other properties of these enzymes, we need to perform certain other analysis. Analysis with respect to the rates at which these pathways are operating.

That is actually coming out of the flux analysis that the rates at which these pathways are operating, individual reaction pathways and their control architecture. Control architecture means, how this flow of metabolites are getting controlled or being controlled, like in a definition in the definition of the metabolic flux, we have noted that metabolic flux is basically representing the rate of flow of metabolites within a given reaction.

Now this rate of flow of metabolites like a rate of flow of the traffic in a busy metropolitan town or metropolitan city that you may find out the traffic is all through moving in a constant rate or in a kind of a steady rate, steady state, but at some point of time or some point of traffic crossing the flux or the flow of the traffic is abruptly getting disturbed.

There may be some road blockage or there may be some other issues are there. So we need to understand that where are the points that where the flux is actually getting altered? And who are the factors, what are the factors, who are actually controlling this architecture of the flux distribution? I think, if we go further and discuss about the metabolic flux map, then I think this picture will be clearer to us.

And here through this analysis, we propose rational targets for modification. Actually, there may be questions like why are we analyzing the metabolic flux? The metabolic flux are to be analyzed, one fundamental reason is to identify the targets for modification because we are not satisfied with the products that we are currently getting out of a particular cellular system.

So we want that identify the reactions which are actually to be modified or to be altered or to be changed using some recombinant DNA technology methods. So how do we identify the targets? We may identify the target just because we know about these reaction or we have some rational thoughts or rational view on identifying these targets. Once we identify the targets, we are able to reach to a point where we will be able to modify or implement genetic engineering tools.

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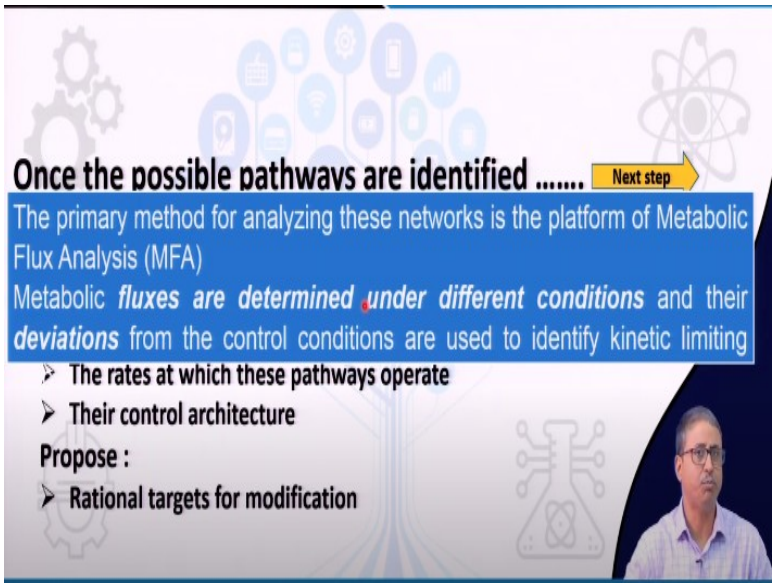

Now metabolic flux analysis is this method, which is enabling us to understand the flux of the entire metabolic pathway. So the primary method for analyzing this network is the platform which is called metabolic flux analysis. So through the metabolic flux analysis or MFA, we are able to analyze the flux through the entire network of the metabolic pathway.

The metabolic fluxes are determined under different conditions. So in MFA, we generally allow the cells to grow under control condition as well as under different altered condition like with varying oxygen concentration, varying substrate like glucose to other hexose sugar or maybe pentose sugar with more change in pH or change in oxygen concentration as I mentioned to.

And then try to find out how the flux of carbon for example, if you are studying the carbon metabolism is changed or altered, when we change the conditions. And by creating which are called actually perturbations by such perturbation studies, we are

able to find out what are the points where actually the flux is being controlled or regulated.

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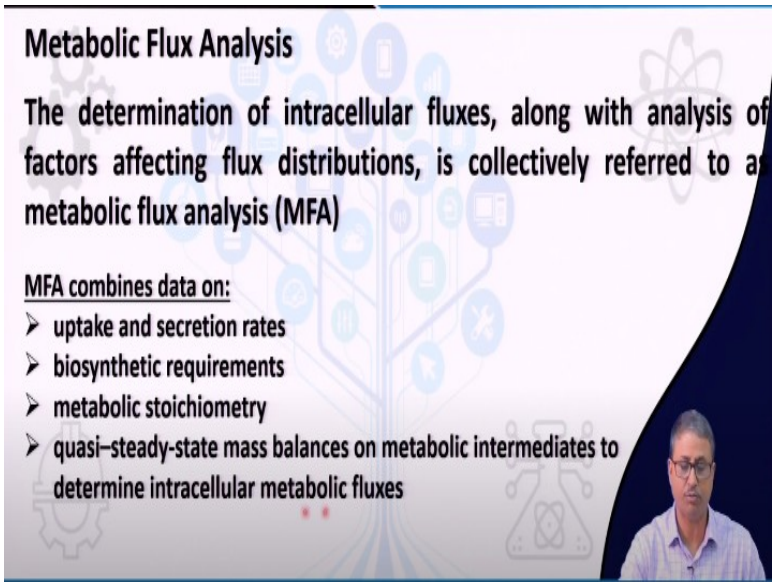

So metabolic flux analysis includes the determination of intracellular fluxes. So after the substrate is taken up, so it is started with historically with 13 C carbon for example. So once the labelled carbon is taken up inside the cell, we are able to measure different metabolites where this label 13 C carbon is there.

So basically we determine the intracellular flux that what is the rate of flow of movement of these labelled substrates for example, along with these analysis of factors affecting the flux distribution. Factors could be indigenous factors like other substrate, other metabolites, cofactors reducing equivalent like NADH, NADPH, FADH2 or give energy sources like ATP or GTP etc.

Or they could be factors which are collected from the external environment or the growth medium. And then collectively, all these data will be represented in terms of the metabolic map that we will be able to figure out that how this metabolic flux is being changed as the reaction proceeds from the starting reaction one to the last reaction, reaction L.

So basically the MFA or metabolic flux analysis combines data on uptake and secretion rates. So how the substrate, initial substrate is taken up and how the products are released or transported out. Biosynthetic requirements, what are the associated

requirements other than the substrates. Metabolic stoichiometry how the reactions are progressing and the stoichiometric characteristics of these reactions.

And quasi-steady-state mass balance on metabolic intermediate to determine the intracellular metabolic fluxes.

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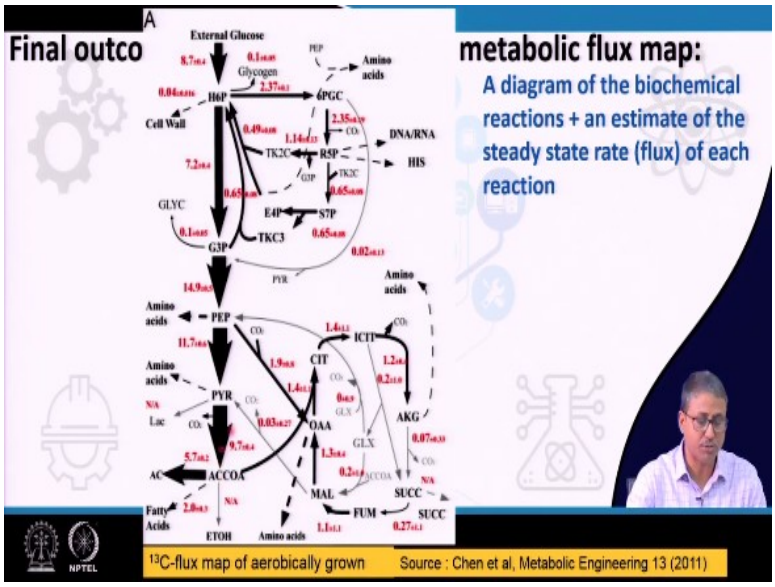

So final outcome of this metabolic flux calculation is a metabolic flux map like as presented over here. And you can see the 13 C flux map of aerobically grown E. coli cell. So similarly, in the same study the Chen et al has also shown how it is changed when the same E. coli cell is grown under anaerobic condition.

As you can see here, that this thickness of the arrow indicates the relative proportion of the, relative proportion of the flux which is actually carried out by this particular stretch of the reaction. So this is the entire glycolytic reaction up to pyruvic acid. So you can see that it is, the flux is relatively higher when glyceraldehyde 3-phosphate is converted to phosphoenolpyruvate and phosphoenolpyruvate to pyruvic acid.

And then finally, under aerobic condition, a strong flux is there that pyruvic acid is going towards acetyl-CoA. Because, why this strong flux is there during aerobic condition, because if we move to anaerobic condition, we will see there is a decline over here. Because this acetyl-CoA is going to enter into TCA cycle. So that is the kind of flux but in E. coli it is very interesting system.

We will discuss about E. coli in detail in the other lectures. That this huge amount of flux is also there towards acetate formation from acetyl-CoA. So pyruvic acid to acetyl-CoA. Acetyl-CoA supposed to feed this entire TCA cycle. But at the same time acetyl-CoA is also responsible largely to produce acetate. So in case of E. coli that is true.

So you can so such kind of flux and we can see the relative importance of each of the reactions and also can identify that suppose we want to improve the flux towards TCA cycle, okay. We can see that relatively less flux is moving towards TCA cycle. So what could be the reason? Because up to this huge amount of flux is coming. Then suddenly the flux is getting reduced over here.

So what could be the limiting factor or what could be the governing factor, controlling factor. Who is responsible for splitting the flux into acetate side or this TCA cycle side. So these are some kind of discussions and some kind of understanding that we actually gain once we identify a particular target.

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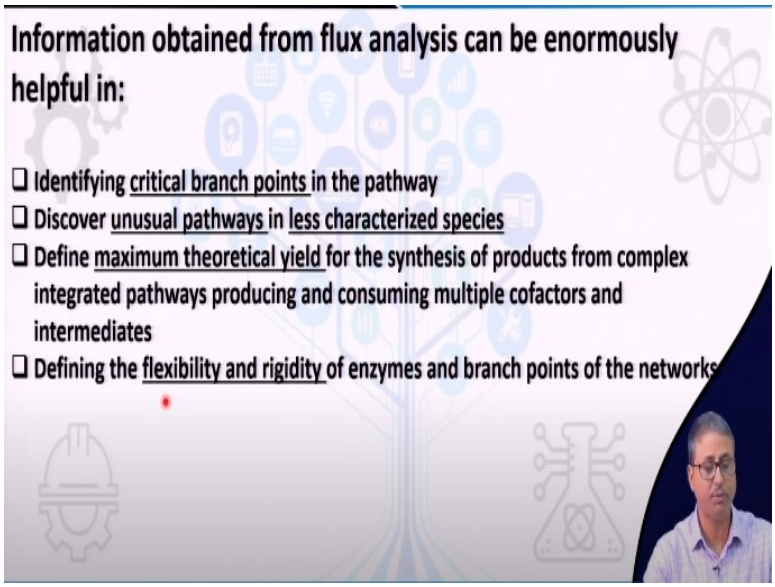

Now information about these metabolic flux can be enormously helpful in identifying the critical branch points in the pathway. Like in the previous slide, we could identify a branch point where the flux coming from pyruvic acid to acetyl-CoA is distributed towards TCA cycle and acetate. In case of E. coli, a large fraction of the flux is going towards acetate rather than in towards the TCA cycle site.

So we can identify the critical branch points towards in this pathway. Discover unusual pathways in less characterized species. So instead of *E. coli* suppose, we are working on some novel bacterial strain or newly isolated organism, we can identify what are the unusual pathways if we study these kind of flux analysis or flux maps.

Define the maximum theoretical yield for the synthesis of products from complex integrated pathway producing and consuming multiple cofactors and intermediates. So what could be the maximum yield out of the given moles of substrate that is provided to the organism that can be calculated based on the flux analysis. And ultimately, this flux analysis defines the flexibility and rigidity of the enzymes and branch point of the networks.

So when we talk about these kinds of metabolic reactions, we see there is a flux going down and there are flux branch points where the flux is being splitted between different part of the pathways. And we are finally able to define or identify the nodes or the branch points, which are flexible or rigid.

And based on the this identification of their flexibility and rigidity of enzymes and branch points, subsequent and further strategies were developed and ultimately those strategies are implemented through genetic engineering tools.

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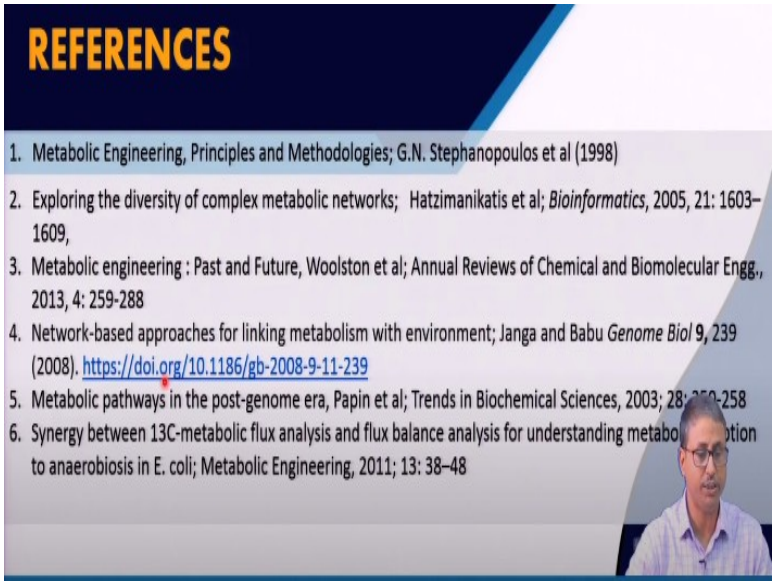

So during this particular part of the lecture, we have used the following references.

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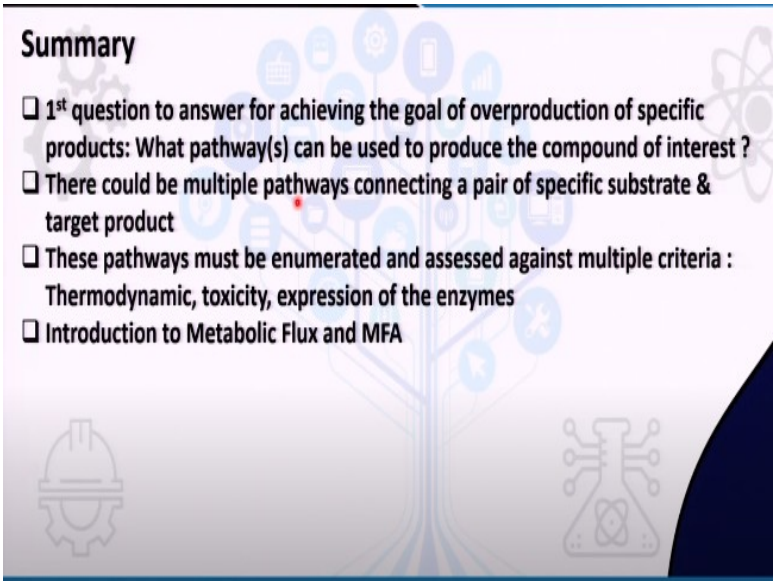

And to summarize this part of the lecture. So basically we have highlighted the first question to answer for achieving the goal of overproduction of a specific product, what pathways can we use to produce a compound of interest, because there could be multiple pathways connecting a particular substrate to a particular product.

Now when we have such kind of multiple pathways, often predicted by different metabolic network analysis, we need to understand this all these pathways must be enumerated and assessed against multiple criteria, including their thermodynamic feasibility, favorability, the toxicity status of the intermediates and expression of the genes and enzymes.

We have also defined or tried to understand the metabolic pathway and what is the usefulness of the feasibility and observability of the individual reactions. And finally, again a brief idea about what is metabolic flux and what is the importance of metabolic flux analysis. Thank you.