Metabolic Engineering
Prof. Amit Ghosh
School of Engineering Science and Engineering
Indian Institute of Technology – Kharagpur

Lecture – 18
Flux Variability Analysis (FVA) and Flux Coupling (FC)

Welcome to the metabolic engineering course. So, today we will learn about flux variability analysis and flux coupling. So, these are the 2 important topics in metabolic network.

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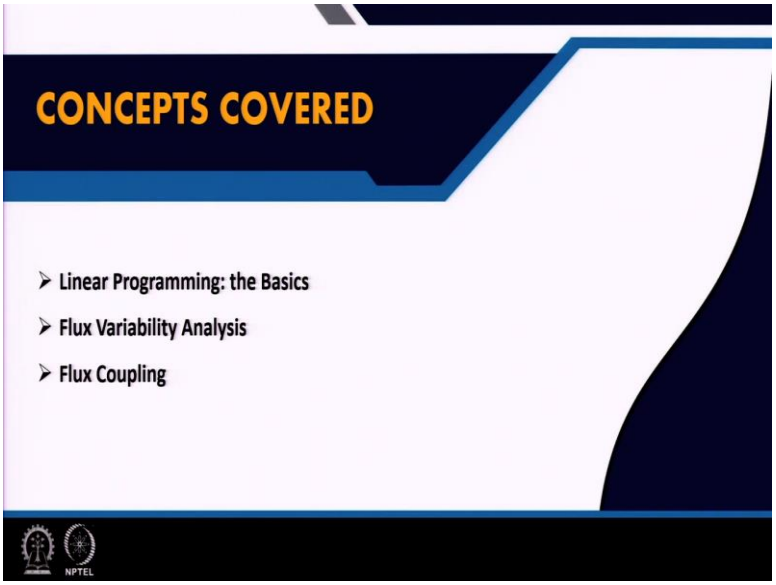

Where you need to know some basic about linear programming we will learn a little bit about linear programming today and then followed by flux variability analysis and flux coupling.

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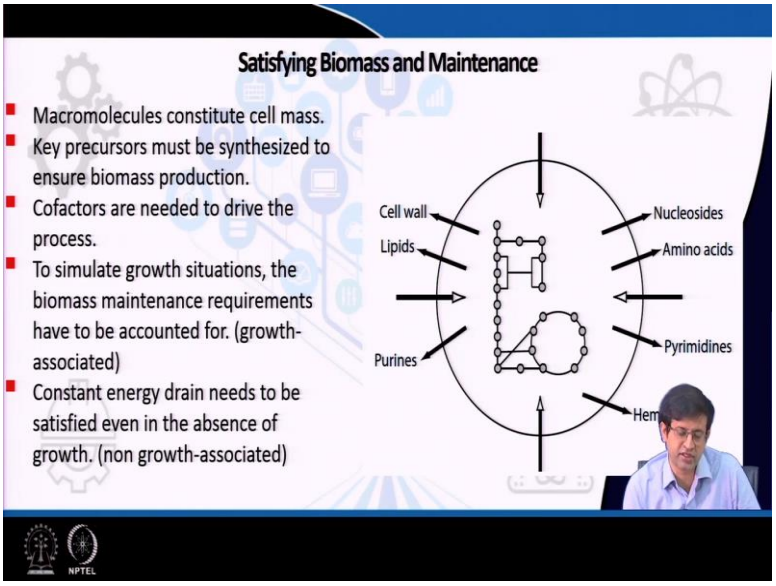

So, before we go to flux variability and flux coupling. In the last class we learned about the biomass and maintenance what is required for the cell. In that the macromolecules actually constitute of cell mass, so we assume that only macromolecules like protein DNA and they constitute the biomass equation and the cofactors are needed to drive the process. So, to simulate growth simulation the biomass maintenance requirements have to be satisfied.

So, it is mathematically the model it will grow only when all the biomass components are synthesized inside the cell in that way you know that all bio synthetic pathway for the biomass components are present inside the cell otherwise it will not grow the model will not grow that requirements should be satisfied. And also apart from biomass equation there will be constant drain in absence of growth.

So, there are carbons which are flowing into secondary metabolite which actually drain the energy as well along with the non growth associated reaction. So, there are growth associated reaction that is growth coupled reaction and then non growth coupled reaction. So that we have already explained how to actually you can determine the growth coupled reaction and non growth coupled reaction.

So, when you want to calculate the growth coupled reaction you maximize that reaction to see whether it is a growth coupled reaction or not. If the maximization of that reaction increases the

growth or the growth reaction there is a flux in the growth reaction then you know that is the biomass equation if there, is a flux in the biomass equation then you know that it is a growth coupled reaction. This is the strategy where you can find out which are the reaction is actually growth coupled.

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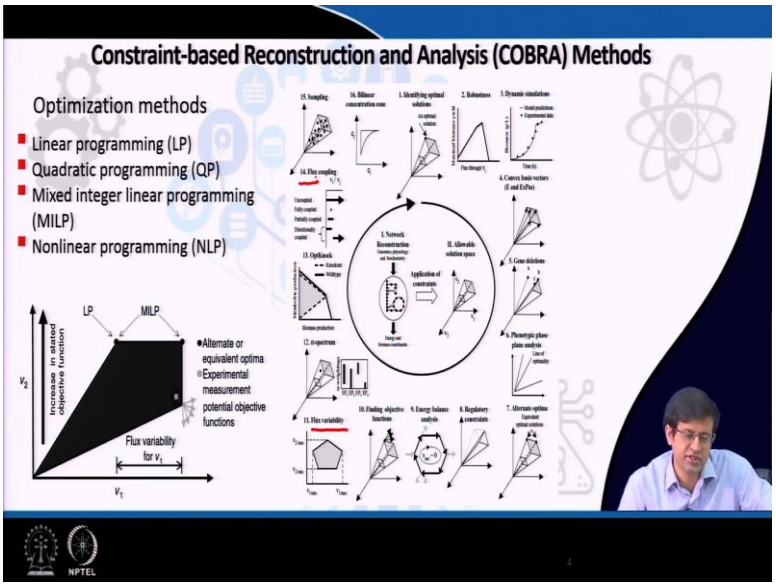

So, the constraint based reconstruction and analysis method that is a COBRA that involves a lot of techniques the difference optimization algorithms is available. As for example we will learn about the flux variability analysis today where this part of the flux variability analyzed, you can see that this is what we are going to learn today and also we learned about flux coupling. So, where is flux coupling so here are these 2.

So, there are many techniques to optimize the network to extract new information. So, out of that 14 and 11 we will focus on it, and these require different types of optimization algorithm, for example linear programming will be used mostly and in rare cases like we will use mixed integer linear programming and nonlinear programming, but mostly it is based on linear programming.

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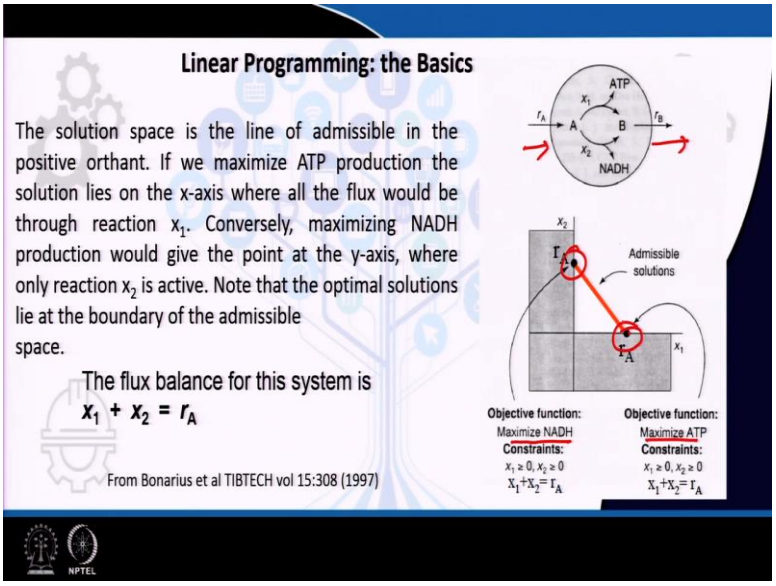

So, lets learn about some linear programming today what is linear programming, the basics of linear programming? Suppose, you have a network that has only 2 metabolites so you have intake a metabolite A is entering the cell and from here and then at the output we have the metabolite B and we have 2 internal reactions. So, the reaction 1 which is x 1 and the reaction 2 as x 2 so, x 1 is producing ATP and x 2 is producing NADH.

So that then how you maximize now this network can be maximized for ATP production and as well as for NADH production. And the optimal solution lies at the admissible solution of the optimal solution if you maximize ATP then you will see that it lies at the x axis. So, here your solution the maximization of ATP you have a constraints of x 1 + x 2 = r A that is the input flux. So, here r A = r B.

So, we have the how much your flux is entering is equal to the amount of flux going out. So, when you maximize ATP then it will reach here at the point the r A it will become the maximum ATP this network will produce can produce r A and also here maximize NADH is also the maximum amount of NADH this network gives is also r A. So, these are the 2 extreme when you maximize NADH you have the x 1 = x 2 = r A.

And when you maximize ATP then x 1 = r A. But the optimal solution lies in only straight lines. So, this is the orange colour straight line you can see that is they have 2 extreme points. So, when

you maximize ATP you come here and when you maximize NADH you are here but any point on this straight line is a solution any point on this is actually a solution which is actually a combination of ATP production and NADH production. So, this is a simple solution network where do you want to optimize the network to see how much ATP and NADH you can produce given the input and output reaction.

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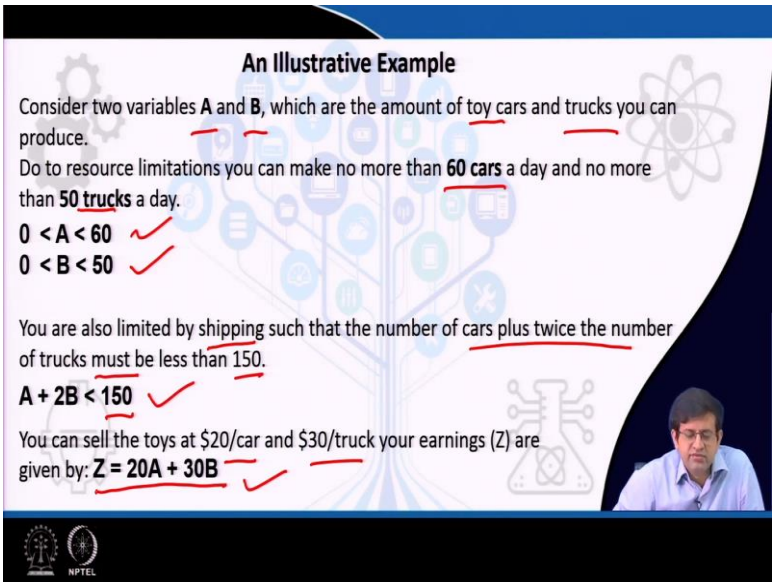

So, now we think about a more complicated problem where you have 2 variables. So, 2 variables in this sense we have metabolite variable A and variable B which are basically amount of toys cars and the trucks you can produce in a workshop. So, you have an industry, you set up an industry where you are producing toy cars and trucks and those the number of toy cars you are producing you can denote it by A and number of cars or trucks you are producing you take it as B.

So, A and B are the variable. Due to resource limitation you can produce no more than 60 cars and no more than 50 trucks, this is the resource limitation you have because you have some infrastructure and based on the infrastructure you have your industry can produce only 60 cars and 50 trucks. So, your variable A and B lies from 0 to 60 anything between 0 to 60 and then B that is number of trucks goes from 0 to 50.

So, any point between these in this range that is the number of trucks you are producing and you are limited by shipping also not just the infrastructure you have shipping facility which has some limitation. So, the number of cars plus the twice the number of trucks must be less than 150. So that constant you have that the number of trucks and the number of cars that you want to see every day is limited by 150.

So, you can now you can sell these toy car and truck in the market at the rate of like 20 dollar per car and 30 dollar per truck. So, the selling price and then you are earning become 20 into A that is A is the number of cars and B is the number of trucks. So, this is your total earnings so that becomes your objective function. So, because you want to maximize your earnings because given a company your main target is to actually how you can earn more for to earn to increase the earnings.

So, you have to define a objective function which is basically the number of cars and number of trucks you want to sell in the market at a price which is given here. So, this is now we can see these are the constraint. You have constraint 1, constraint 2 and constraint 3 and this is the objective function. So, now your problem is defined so, you have a set of equation which act as a constant and you have an objective function. Now you can maximize it you maximize Z, so, what value of A and B you should produce car every day so that you are earning is maximum. So it is a very simple problem that you can maximize and see how much it is how much.

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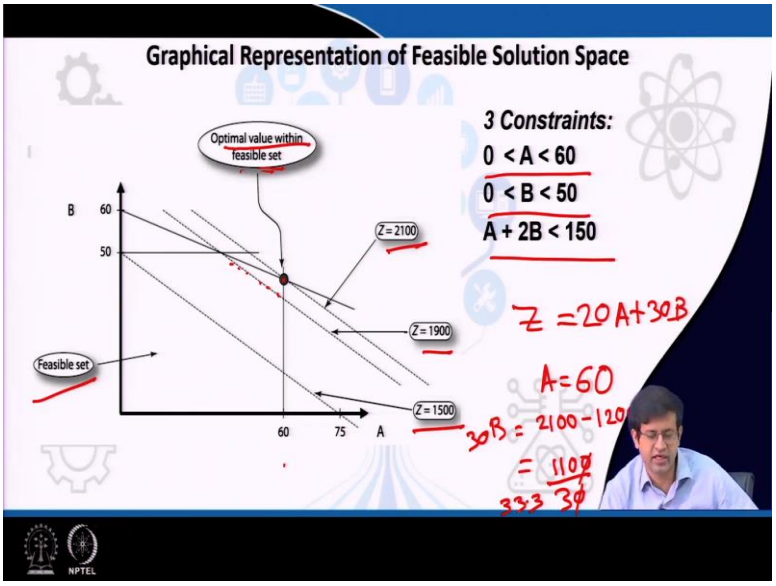

Earning you can produces. So, once you have 3 constraints A greater than 0 less than 60, B greater than 0 less than 50 and we have a shipping constraint, A + 2B equal to less than 150. So these are the 3 constraint you have based on that. Let us see what is the value of Z. So what is Z you have define as that is your earning, Z is defined as 20 A + 30 B that is Z = 20 A + 30 B. So, A is the number of car and B is the number of truck so, you can see if I draw Z = 1500.

So, then your solution lies here so it is passing through first you draw a rectangular box at equal to A goes from 0 to 60. So, you draw a straight line here so you are this is the parallel to the y axis and then parallel to the x axis you have a rectangular box and within this rectangular box you have all the feasible solution sets. So, this rectangular box which you see here is actually any point in this rectangular box is actually a solution that is your earning.

But you want to maximize the earnings, so, why should I draw the line. If I draw Z = 1500 then it lies within the rectangle but the Z is not maximum see Z is 1500, but slowly if I increase the Z = 1500 to 1900 then you will see your earning has increased and also it is lying. So, any point in this line is actually your earning which is fixed by 1900. So, you can have many numbers of points.

So, if you draw a line at Z = 2100 then you can see it this passing through this point that is the optimal value within the feasible set. That is a maximum earning within the feasible set you can

correlate with your metabolic network where you say that maximize your biomass that is your maximum production of biomass as the point where you have the maximum growth. So, similarly here your earning is maximum at this optimal point where the Z is maximum that is 2100.

So, at this point you can see why it is lies on the rectangle and then also it is at the Z is maximum. So, this becomes your optimal value within feasible set. So, you have to choose a point where, your earning is maximum but it also lies in the feasible set also. If it is lying outside the feasible set then it is not a solution. So, what we see is basically the maximum earning in this case is become 2100.

Where at this point you have A = 60 that is if the number of cars are 60 and then what is the value of B. B is basically 2100 - 20 into 60 so how much, 1200. So then how much you are getting the B = 1100 you will get. This way you can actually divide it by 30 we have basically the 30 B basically. So, is basically 33.3 so, it is a fractional number you have almost around 33 trucks you have to produce, 33.33. So, this way you can actually calculate how much how to maximize your earnings such that what combination of car and truck you should produce every day so that your earning become maximum.

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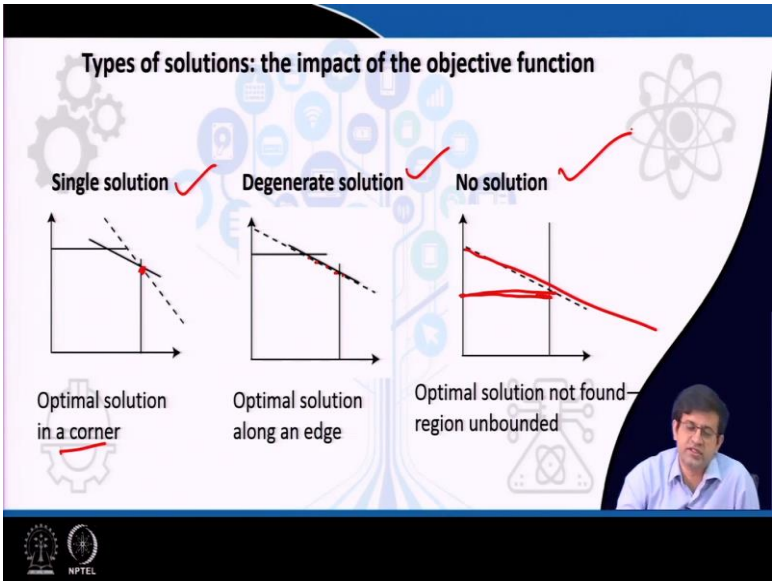

So, now come to the solution the impact of the objective function that is the solution whether we have a single solution or a degenerate solution. So, here you can see that if you choose Z = 1500 then your solution is degenerate, write, you have multiple solution. So, any point on the straight line is basically a solution, if you choose Z = 1900 you have many solution because it passes through the rectangle where any point within the rectangle is a solution.

Because it lies on this from here to here, you can see it lies in the rectangle. That is why any point on this line is a solution and that is why you have a degenerate solution. So, there are multiple solution for a given value object. So, for a given earning you have so many combinations you can play with number of truck, car. So, in that case your solution is degenerate is not a unique when you have a unique solution then it is not degenerate.

So that is what. So, in this case you can see a single solution. So, there is a non degenerate solution but here you can see it can have many solutions. So, it is a degenerate solution and in another case you can see that there is no solution because it is lying outside the box. Suppose the boxes here so it is not going through the box. So that is why you see that there is no solution. So, there are the types of solution you can see that optimal solution is in the corner.

So, here in the corner of the box, the optimal solution is at the edge. So, along an edge and then the optimal solution not found in the region unbound. So, in this case we have a unique solution, in this case we have a degenerate solution, in this case we have no solution. So that is, these are the 3 solution, 3 types of solution you can get when you run an optimization problem.

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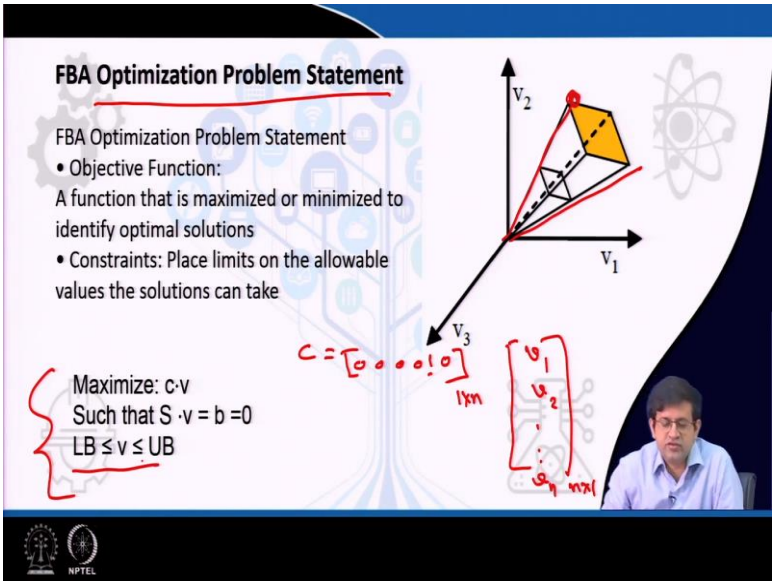

So, now this is the FBA optimization problem which I have already defined in the last class. The FBA optimization is basically maximize the objective function that is c multiplied by v where v is the flux. So, c, the value of c is like a vector whose close components are basically is a row vector which is basically it has 0, 0, 0, 0, 1, 0 only 1 element is non zero. So that is why you can actually you have to choose only one, if you choose the biomass equation as the objective function then you keep it 1 and the flux vector you already know is basically v 1, v 2, v 3 up to v n.

So, this is 1 into n and this is n into 1 so, if you multiply you get only one reaction. So, if you multiply c into v then it will give a one flux value that is flux, it can be a biomass or ATP whichever you want to decide. So, if you decide one to be here then which is a component of the biomass then that becomes the objective function and this you already know that steady state condition S.v = 0 and the lower bound and our upper bound for all the fluxes you are optimizing.

So, this is the FBA scheme that you apply for calculating the flux where you maximize the biomass and when you apply constraints you can see you solution space lies in the cone. So, the cone you can see and when you maximize biomass then the optimal solution lies at the edge at a point where that is the only point where the biomass is maximum. This is a how you can set up the optimization problem in FBA a function that is maximize, you can either maximize or

minimize to identify the optimal solution. And the constraint place limit to the allowed flux the solution can take.

So, we apply all constraint here though to actually allow the flux to be in a certain range. So, this is how you define the FBA optimization problem.

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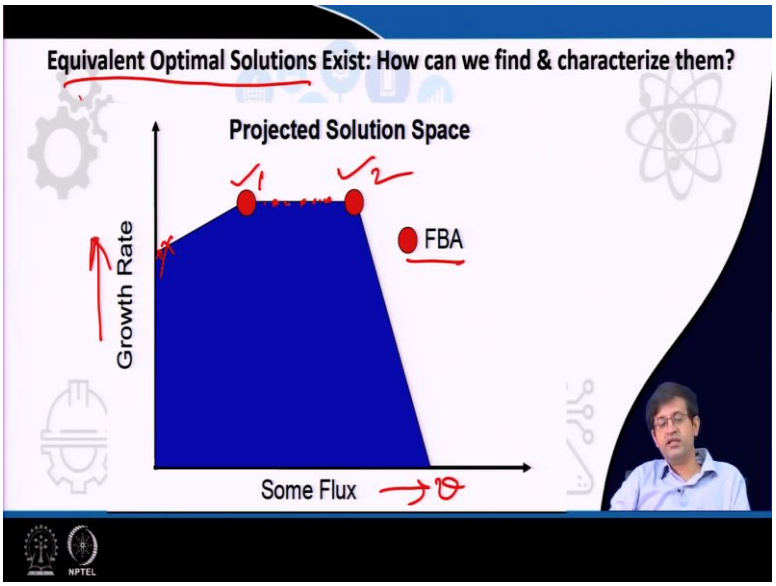

And then you will see that the FBA optimization problem when you maximize the growth rate. So, this is FBA optimization the projected solutions for, so, this is the solution space some flux you are plotting. So, here you have flux v, any arbitrary flux and you have on the y axis the growth rate. So, if you plot that then you will see that the solution lies in this blue region. So, any point on this solution is a solution to your problem but the growth rate is maximum only at this 2 point, point 1, point 2.

The growth is maximum because you see further you go it is going outside the solution space. So, it is you have to the points lies at the edge where the growth rate is maximum if I choose the optimal solution here; here the growth rate is not maximum. So, this is not correct, but here you can see that the growth rate is maximum in point 1 and 2 but they are degenerate. If I choose this point also then also it is growth rate is maximum, this point also you can growth rate is maximum, this point also growth rate is maximum.

So, what you understand is that the maximum growth rate can be projected by many solutions. So, there are many equivalent optimal solution exists in the network. So, there are many equivalent optimal solutions. Because the growth rate is maximum here also, here also, here also, here also all the point along the straight line is actually you will give you the growth rate maximum. But your solution, but the growth rate is fixed but your solution to the fluxes are actually changing. So, there is a range so how can you find this flux range where the growth rate is maximum.

So, if I say the growth rate is this much value then what is the range of the flux that can give rise to maximum growth rate? So, this is the idea that came into in metabolic network.

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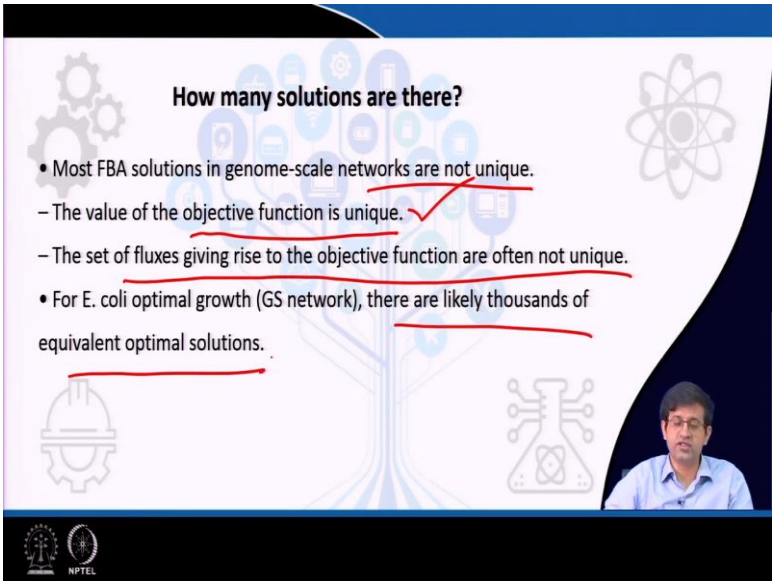

And they call it as a flux variability analysis. How many solutions are there? Most of the solutions in genome scale metabolic network are not unique. So, when you run FBA the genome scale network the FBA solutions are not unique in the sense that flux values are actually changing, if you run FBA 2 time, 3 time in MATLAB then you will see the solution to the fluxes are changing with time because there is no unique solution there is a range.

So, the value of the objective function is unique there is a growth rate is unique but the flux value which give rise to this unique objective function is actually changing and the set of flux giving rise to the object function are also not unique. The flux, you are getting from the objective

function are also not unique. For E. coli, genome scale network they are likely thousands of equivalent optimal solution.

So, now we can think of that given a E. coli a metabolic model you optimize the growth rate the growth rate is unique. So, here the growth rate is unique but what you get is there are 1000 of equivalent optimal solutions. So, the flux values which give rise to unique objective function that is growth rate have many solutions. So, how do you handle this? So, this is one of the problems in the flux value analysis, FBA where you get a unique objective function but the solution for the optimal solution there are many equivalent optimal solution for this objective function. So, how do you characterize them?

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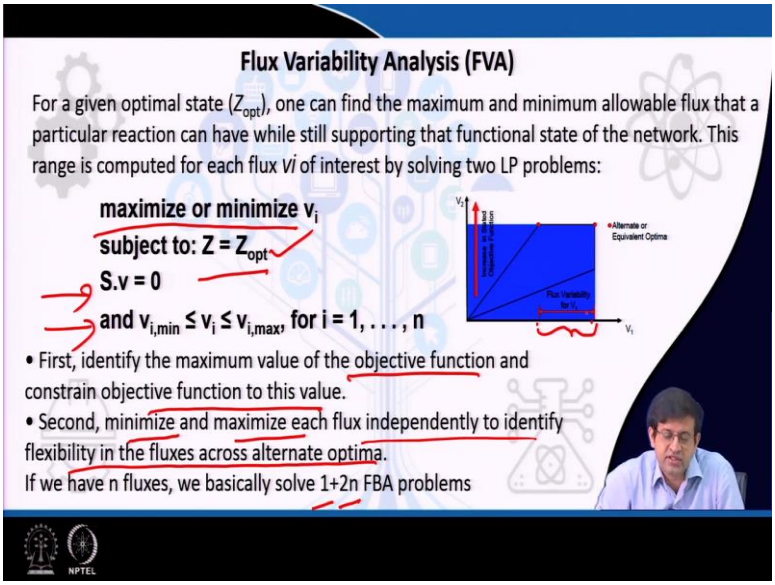

These give rise to flux variability analysis. So, how we want to calculate the range of fluxes for which the growth rate is maximum. So, for a given optimal state that is the biomass objective function how one can find the maximum and minimum allowed flux and that a particular reaction can have, which is support the functional state of the network this can be computed by assuming, by solving 2 LP problem.

So, for each and every reaction you have to run 2 LP problems. So, first optimize for example, biomass and then you keep it as fixed like, Z = Z opt. That is the maximum value of the objective function that is the biomass you can fix that can be one reaction that is the biomass equation and

that you can fix that value. And then you minimize and maximize each and every reaction in the network.

Suppose there are 1000 reaction then how many times you have to actually run the linear programming, 2n times. So, basically, 1 FBA run we have to do for growth maximization that is 1 and 2n times basically you have to run for each and every reaction. Now 1 for maximization and 1 for minimization that is why 2 and the remaining constraints are remain the same that is S.v = 0 and the upper bound, lower bound as it is applied in FBA.

And then first you identify the maximum value of the objective function suppose your objective function is growth rate that you run fast and then you constrain the objective function to this value. When you constrain and here you are constraining using this equation you are constraining that objective function. And then secondly you minimize and maximize each flux independently to identify the flexibility in the fluxes across the alternate optima.

So that, the each reaction has a flexibility here you can see that the flexibility goes from here to here. So, given objective function that is biomass the v 1 ranges from this value to this value so this becomes your range. So, any point on this range is actually give rise to a maximum biomass in this cell. And this is a concept of flux variability analysis where is very important because the optimal solution there are many optimal solution for maximum objective function. So, that need to be taken care by running flux variability analysis that is why flux variability analysis is very important in a metabolic network where you can calculate the range of fluxes for a given objective function.

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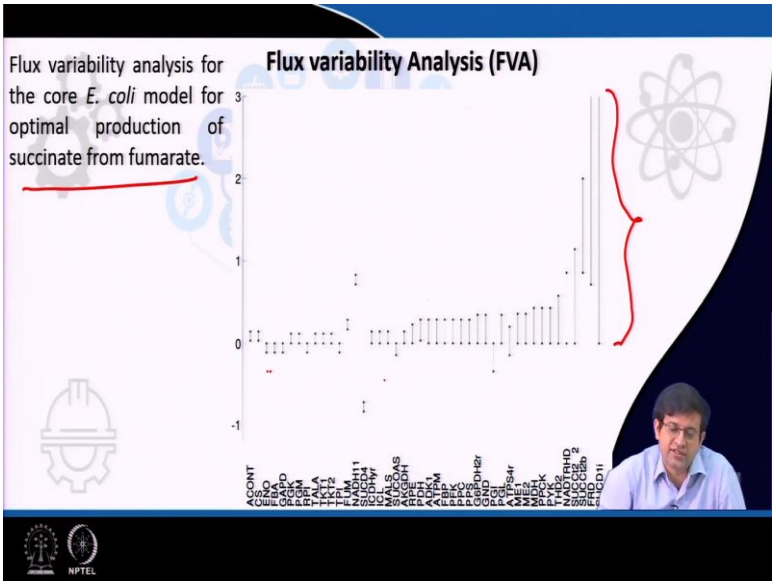

So, this has been calculated for E. coil model for production of succinate from fumarate. So, they produce succinate from E. coli model and they calculated the flux variability analyzing. Now we can see these are the reactions they have plotted in the x axis and the name of the reaction and on the y axis you can see the range. So, the maximum range you can see is coming for SUCD1i this is the maximum value of the range.

So, this is the range it lies where the succinate fumarate is maximum. Then FRD you can see the range is different and then so on it is arranged in the ascending order. So, this is the minimum range ACONT which is present in the TCA cycle and then so on. As you can see they have plotted the range of each of the reaction. This method is actually very powerful when you in metabolic engineering while you want to know given a production of metabolites how much variability exists in the reaction. So, this way you can actually play with the reaction and see how much flexibility the model has, how much capacity the model can take up, so that the production increases?

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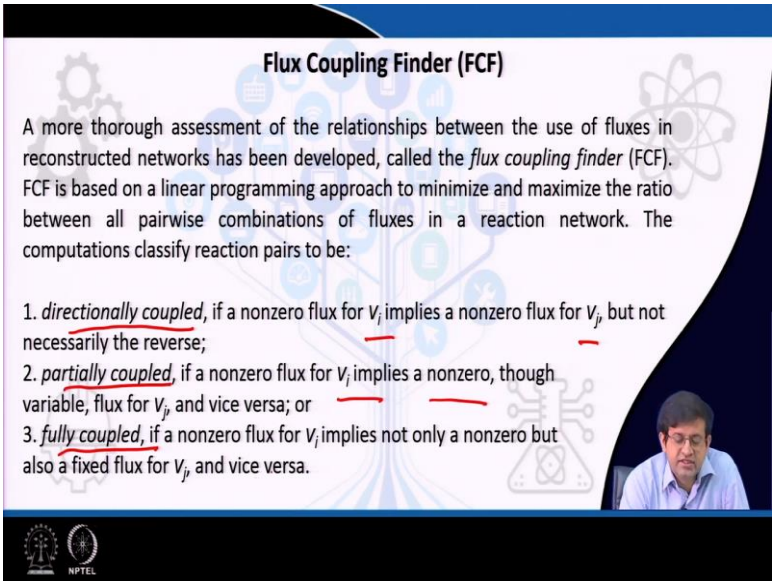

So, next concept we will learn about the flux coupling or flux coupling finder a more thorough assessment of the relationship between the use of fluxes in reconstructed network has been developed and is known as flux coupling finder. So, how do you relate one flux to the other you can characterize and thereafter can characterized by flux coupling finder FCF this is the based on again on linear programming approaches to minimize and maximize the ratio between all pairwise combination of fluxes in a reaction network.

The computation what do you have to do is, actually you have to calculate make a ratio of flux. Like suppose you want to calculate the relationship between v 1 and v 2 and then you make a ratio of v 1 / v 2 and then you do linear optimization. So, through flux coupling finder you can find whether the reaction are directionally coupled or partially coupled or fully coupled.

So, these are the 3 properties you can find like given a reaction how many reactions are actually directionally coupled in a genome scale metabolic network or it is partially coupled or fully coupled that event estimate from the network using this algorithm. So, if a nonzero flux of Vi implies a nonzero flux of Vj suppose I have I choose any pair of reactions. So, in this problem we actually choose only pairwise reaction.

Suppose I choose v i and v j then if v i is nonzero flux and that implies that nonzero flux for v j but the reverse is not true then it is directionally coupled. So, if v 1 actually is nonzero then v j is

also nonzero but the reverse is not true. And therefore partially coupled if we see that nonzero flux v i implies nonzero though variable and flux for v j and vice versa then it is partially coupled.

Similarly for fully coupled if a nonzero fluxes implies for v i implies not only nonzero but also fixed value of v j. So, basically both v i and v j are constant then if they change by some amount then both v i and v j are also changing by same amount then it is known as fully coupled. We will go into more detail.

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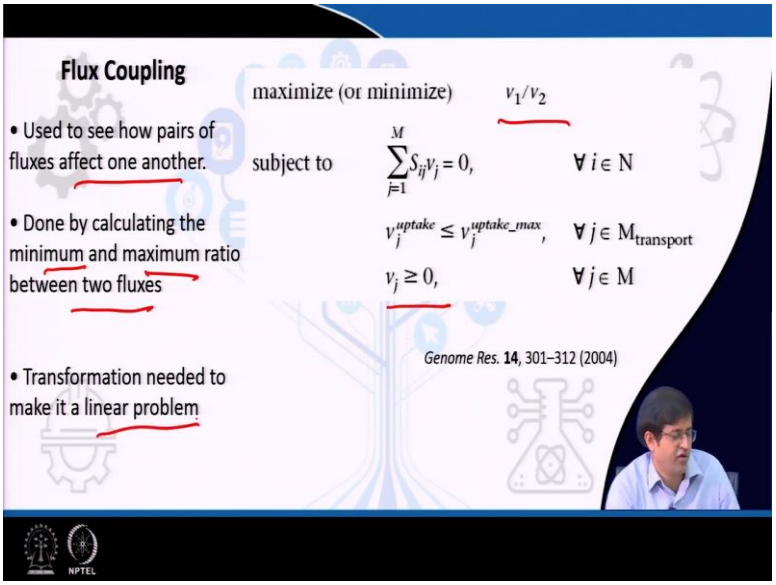

In the subsequent slide, so in order to actually solve this problem they define a optimization problem where they define a maximize v 1 and v 2. So, v 1 divided by v 2 that is the ratio of the flux, they maximize or minimize, subject to you have the steady state condition S.v = 0 and then the uptake rate of the reaction for example the glucose and then we assume that v j greater than 0. So, if that v 2 is 0 then it becomes infinity.

That is why you have to make sure that v 2 should be greater than 0. And then you actually maximize the ratio of v 1 and v 2. So used to see how pairs of fluxes affect each other. So, this is why the method is applied when you want to see that how these pairwise fluxes are affecting each other and done by calculating the minimum and maximum ratio between 2 fluxes. And also you can make this problem into linear problem because right now it is a nonlinear problem that is

why you have to transform this mathematical problem into a linear problem. So, these are the steps which are followed in flux coupling.

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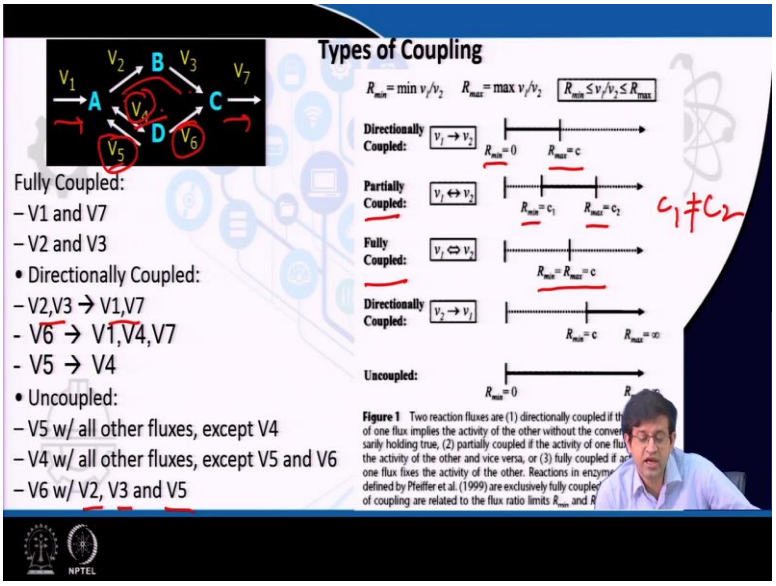

And this is the example of flux coupling what really mean for directionally couple you can see that the R min that is a v 1 / v 2 if the R min is 0 and R max is a constant value then v 1 is actually directionally coupled to v 2. So, v 1 implies v 2. And then for partially coupled you see that both R min and R max are some constant value but they are not equal. So, c 1 is not equal to c 2. For partially couple if c 1 become equal to c 2 then it become fully coupled.

So, the difference between partially and fully coupled is that c 1 and c 2 are not equal when it becomes c 1 = c 2 that is R min = R max then it is fully coupled. And for directionally coupled either R min can be 0 or R min can be a constant. So, for that if R min equal to constant then R max become infinity. So, the directionally coupled are the coupled reactions are actually if the activity of one flux implies the activity of other without converse necessary holding to so, if we say that v 1 is a directionally couple with the v 2.

But if this is true v 1 implies v 2 but v 2 does not mean it is directionally coupled with v 1 the reverse is not true and not always true. So, these are the example of a small network where you can see that v 1 and v 7 are actually fully coupled and v 2, v 3 are actually fully coupled they are

actually the same amount of flux are going into v 2 and v 3 because they are linear pathway. And also the input and output fluxes also remain the same.

That is why v 1 and v 7 are actually fully coupled and the directionally coupled reactions are v 2, v 3 and v 1, v 7 they are actually directionally coupled. Directionally coupled means is that the amount of flux going through these reactions are not actually same but they are somehow directionally coupled and uncoupled reactions are basically v 5 with all other fluxes which actually uncoupled flux and except v 4 and v 4 with all other fluxes.

So, v 4 here is also uncoupled with all other fluxes because they are not at all connected to each other and v 6 with fluxes v 2, v 3, v 5, v 6. So, it is uncoupled with v 2, v 3, v 5; and v 5 and v 4 are actually directionally coupled. In this way you can bring about interesting property of the network while we can characterize how the network are coupled how the reactions are coupled? And based on that you can design metabolic engineering where you can improve the production of sudden compound provided that it is not influencing other reaction.

So, many reactions are actually directionally coupled partially coupled but make sure that if you perturb a reaction it may actually disturb the whole network. So, because the; types of coupling which exists in the network should be very careful.

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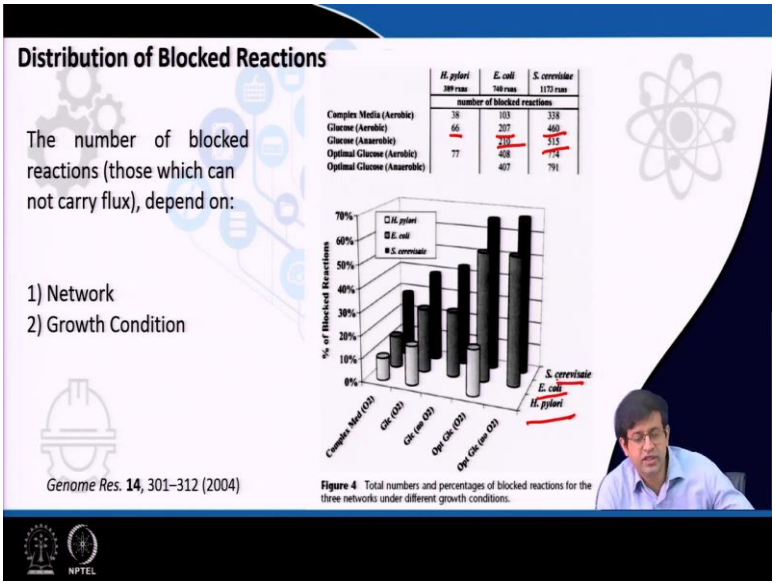

So, there are distributions of the block reaction where you can see the number of block reaction depends on the condition. So, the block reactions are the reaction which actually does not carry any flux. So, because the cell grows as long as the biomass equations are satisfied but there are many reactions which are not actually coupled with biomass reaction. And for those reactions it may not be necessary that they carry fluxes.

For example the aerobic condition you see for H. pylori there are 38 block reaction but if you go to aerobic from minimal media to glucose then the number of block reaction increases. So, from complex media to glucose, from aerobic to if you go for E. coli if you see that the aerobic, in aerobic glucose you have 207 block reaction. But if you go to anaerobic then the number increase the number of block reaction increase that means if you increase the constraint then your block reaction increases.

So, if you are applying more constraint it means that many reactions are actually blocked. So, you may not see any flux in those reactions, so, because the network is much more constrained. Similarly for Saccharomyces cerevisiae also you see you the moment you go from aerobic to anaerobic that is 460 to 515 reactions; the 515 reactions are actually blocked. So, as you know in saccharomyces cerevisiae many reactions are actually the substrate is oxygen the moment you actually become anaerobic.

And then what happened the most of the reaction they do not carry any flux because the substrate is missing. So that is why when you put more constraint there the chances are that you have a much more reactions are actually blocked. So, here I have added the blocked reactions are plotted for different organism. So, for H. pylori you can see that the number of block reactions are less. So, as you go to E. coli more complex organism, the E. Coli, saccharomyces cerevisiae has many more block reaction. And an E. coli has in between H. pylori and saccharomyces cerevisiae.

So, more blocked reaction means the network is much more the H. pylori network is much more compact. So, there are less flexibility so, more the block reaction the network has more

flexibility and if there are less block reaction for example H. pylori we have a more compact network.

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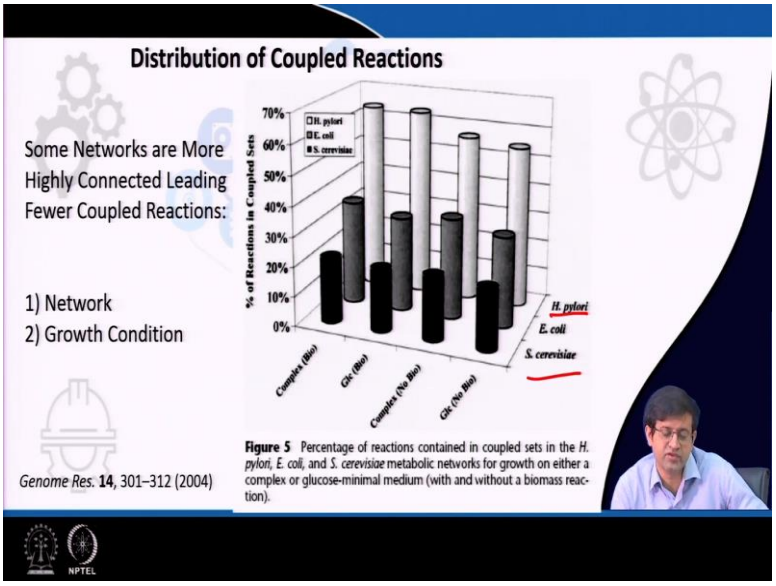

Similarly, for distribution of coupled reactions also you can plot organism like saccharomyces cerevisiae, E. Coli, H. Pylori. So, H. pylori, has more coupled reaction. So, some networks are more highly connected leading to fewer coupled reaction. So, some reactions are actually very well connected for example saccharomyces cerevisiae we have more connected network. So that is why the percentage of coupled sets are also very less for example, H. pylori is less connected network that is why the coupled reactions are more.

So, this way you can characterize different network based on the number of coupled reaction it has and it also says how much they are connected with each other. So, this property you can evaluate from organism to organism and see how it is affecting the network.

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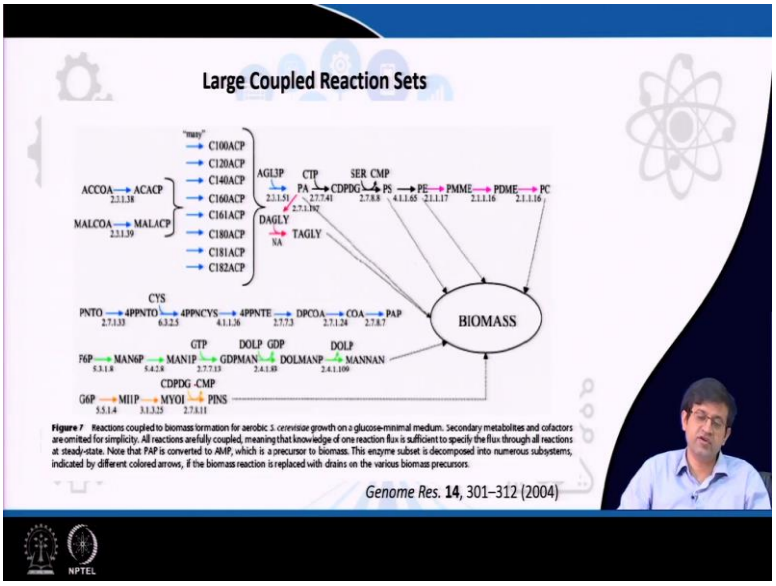

So, this is the large set of coupled reaction that has been evaluated for saccharomyces cerevisiae and glucose minimum condition, the secondary metabolite and cofactors are omitted for simplicity. All reactions are actually here are coupled and what do you can see that if I know the flux for one reaction then all other fluxes are estimated when the reactions are coupled so the biomass component is shown over here.

So, the biomass component how it is actually contributing to the biomass. And those reactions which are coupled are shown over here and that reaction which participate in the biomass equation and all these reaction you do not have to evaluate flux for all the reactions because they are actually coupled. So, if I know the flux for one reaction the other flux is only way determined, so that because they are all coupled set of reaction.

But for a directionally coupled reaction if I remove some reaction then definitely the coupled reaction, the directionally coupled reaction that is following reaction you will not carry any flux. So, this kind of method can be applied to know which the reactions if you remove or you knock off certain genes then that will lead to some other reaction not getting any flux.

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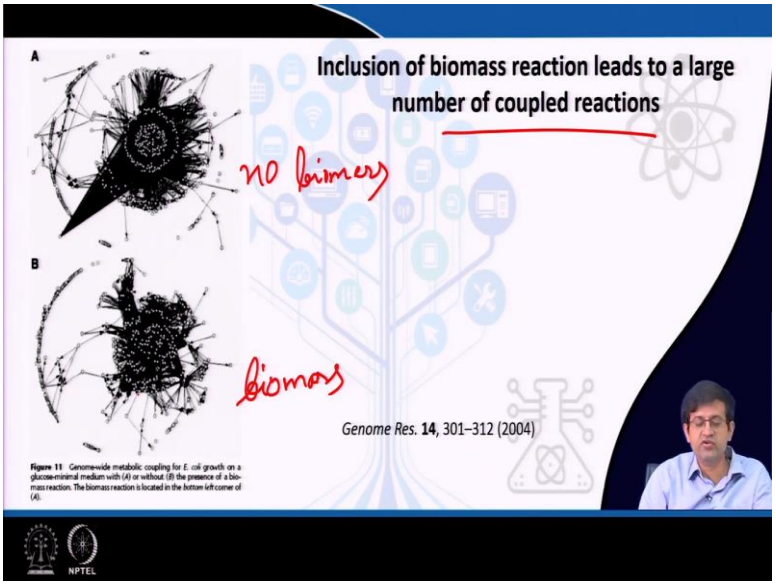

So, this is another representation, where you can see that when you include the biomass equation in the network then you to see the number of coupled reaction increases. So, there is a large number of coupled reactions when you include the biomass equation in the network or the biomass reaction. So, this has been calculated for E. coli where you can see that where in the condition A where we do not have the biomass in this case no biomass and as soon as add biomass you can see that biomass reaction then your network size increases.

Network size in the sense the coupled reaction increases there are many coupled reaction the moment you increase the biomass equations.

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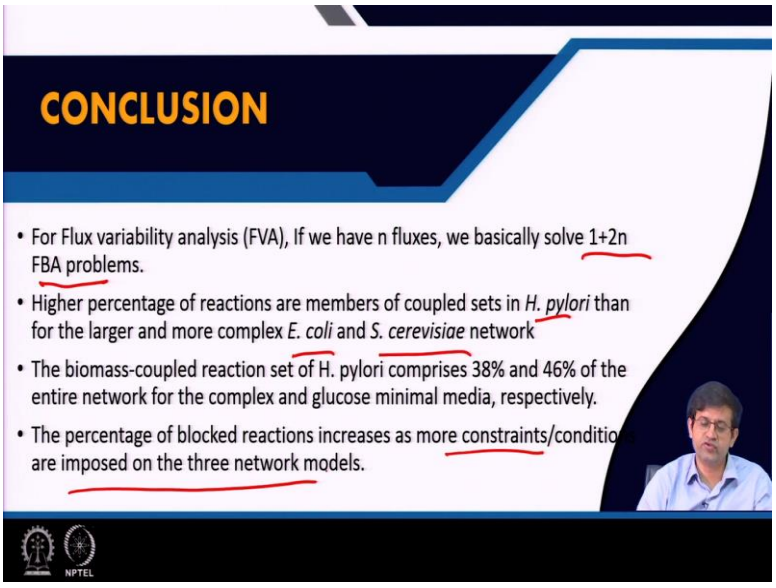

So, in conclusion what we learned that for flux variability analysis if there are n fluxes in the network we basically solve 1 + 2n FBA problem. So, sometimes these calculations are very lengthy for suppose we have 2000, 3000 reaction in the network then the number of FBA problem we have to solve is basically for 1000 reaction it will be 2000 + 1 reaction, 2001 optimization problem. Higher percentages of reactions are member of coupled in H. pylori than larger and more complex E. coli.

So, H. pylori has a relatively in smaller network but the number of coupled reactions are more compared to E. coli which are more complex then you can see that H. pylori has a more compact network so, and the connectivity is also very less in H. pylori. Where as in saccharomyces cerevisiae and E. coli there are more connected network and the biomass coupled reaction for H. pylori comprises the 38% and 46% of the entire network for the complex and the glucose minimal media.

So, in this way you can see how many biomass coupled reactions are there that is an estimate and see that how it is changing with actually glucose media and the percent of the blocked reactions increases more as you add more constraints. The blocked reactions increases as more constraints are imposed on the 3 network model that is E. coli, h pylori and saccharomyces cerevisiae. So, this we you can know that the blocked reaction increases where if you apply more constrains in the network.

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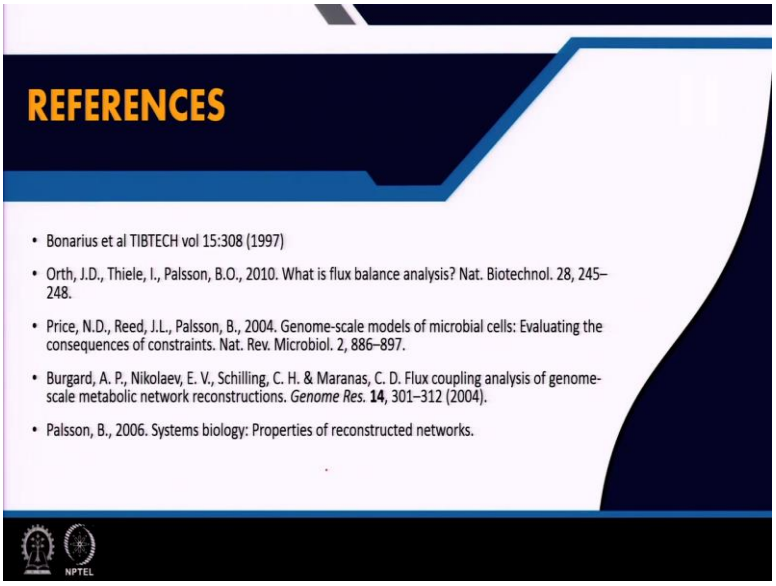

So, these are the reference you can follow for your study for hopefully we will start reading about flux variability and flux coupling and thank you for listening. We are closing thank you.