Bioreactors
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Lecture – 20
Culture status, metabolic flux analysis

Welcome to lecture 20, this is the NPTEL online certification course on bioreactors. In the previous 2 lectures, we had looked at bioreaction stoichiometry as one of the means of getting some insights into what is happening at the cell level in the bioreactors. The cells in the bioreactor are the actual factories. We worked out a problem in the previous lecture. Now, let us go forward with the module itself, the module 5.

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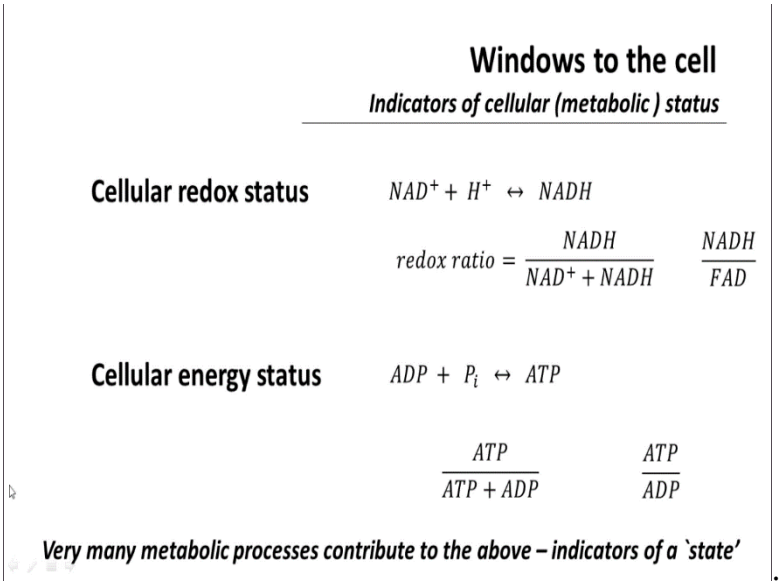

We are going to begin talking about windows to the cell. Now, we said, we will first consider it as a black box, the cell, and then, we will open up windows and look through the windows to see what is happening inside the cell. So, the windows to the cell are the indicators of cellular status of the cell, or indicators of metabolic status of the cell. These terms, cellular status, metabolic status, energy status, and so on and so forth, redox status, and so on, these are kind of soft terms. You know, you need to, you should not take them too strictly. These are kind of indications of what could be happening and these are kind of common terms that are used. They are not, of course fully understood, but they are useful. The cellular redox status is a well-known indicator and cellular

energy status is another well known accepted indicator of the cellular, or the metabolic status of the cells. The cellular redox status is typically derived from NADH, Nicotinamide Adenine Dinucleotide, and the hydrogenated form of that. This conversion takes place in various metabolic reactions, or accompanied with various metabolic reactions in the cell

$$NAD^+ + H^+ \leftrightarrow NADH$$

So, this has a reducing power which is taken forward for so many different important functions in the cell. So, the redox ratio, one of the ways of looking at it, can be given as:

$$redox\,ratio = \frac{NADH}{NAD^+ + NADH}\ or\ \frac{NADH}{FAD}$$

We have already seen that, N A D H fluorescence can be used to monitor the cell concentration, because the intensity of N A D H fluorescence can be directly linked to cell concentration. Here, it is redox ratio, the N A D H divided by the total N A D H pool, which consists of N A D plus and N A D H. This is the redox ratio. Similarly, or in, in some situations, the ratio of N A D H to Flavin Adenine Dinucleotide, another important such molecule, is also taken to be the redox ratio. For energy status, it is somewhat, a little more straight-forward.

We all know the energy is made, or energy currency of the cell A T P, Adenosine Triphosphate is made by the phosphorylation of Adenosine Diphosphate. So, the ratio of A T P to the pool A T P plus A D P, sometimes A M P is also added here, but, let us just look at these two; A T P divided by A T P plus A D P is taken to indicate the cellular energy status.

$$ADP + P_i \leftrightarrow ATP$$

$$\frac{ATP}{ATP + ADP}\ or\ \frac{ATP}{ADP}$$

So, lot of measurements are made on these, correlated to the happenings inside the cells; correlations with respect to what is important from a bioreactor context, and efforts are made, so that, these indicators are varied in a particular fashion, or kept constant at a particular known value, to achieve a desired performance by the cells in the bioreactor.

So, that is one more step towards understanding what is happening at a cellular status, cellular level. Sometimes, even A T P by A D P is considered the cellular energy status. There are various ways of defining this, and I have just given you a couple of them. What one needs to understand is that, very many metabolic processes contribute to the above. So, it is not a one to one kind of a relationships. So, many things contribute to redox status. So, many things contribute to energy status. So, one needs to have some level of, you know, interpretation; one needs to interpret this in that kind of fuzzy fashion; it is more of an indicator. You may want to look at this paper which has also been given in your notes :

Scheper ,Th., Gebauer, A., Schugerl, K. 1987. Monitoring NADH-dependent culture fluorescence during the cultivation on Escherichia coli. Chem. Eng. J. 34: B7 – B12.

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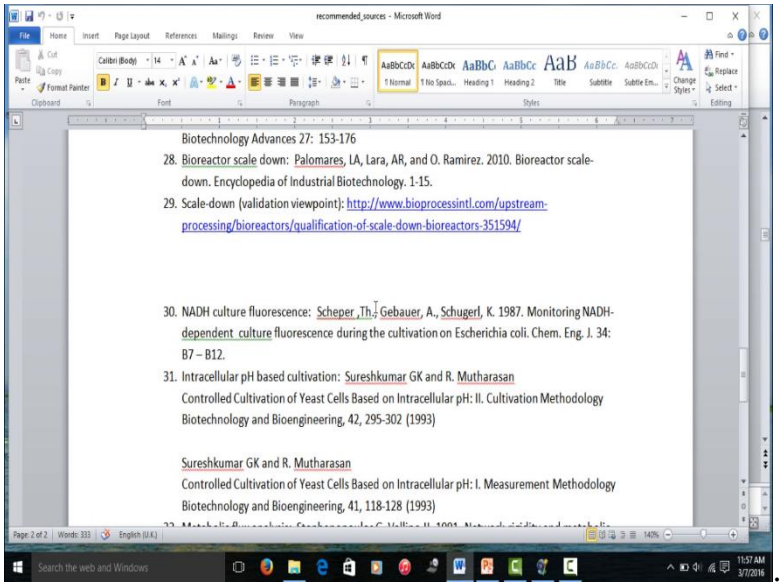

This is number 30, N A D H culture fluorescence, the authors are Scheper, Gebauer and Schugerl. This is the paper for N A D H culture fluorescence. The title of the paper itself is monitoring N A D H dependent culture fluorescence during cultivation on *E. coli*. It was published in a Chemical Engineering Journal in 1987. This is something that gives you some relationship to the culture energy status. Let me, I do have a paper here, yes, let me briefly tell you how this is done, with energy, with N A D H status.

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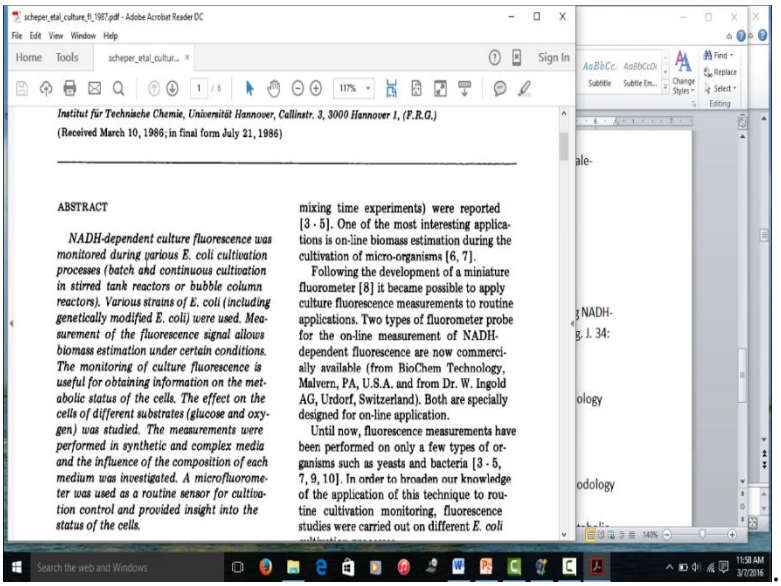

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ABSTRACT

NADH-dependent culture fluorescence was monitored during various E. coli cultivation processes (batch and continuous cultivation in stirred tank reactors or bubble column reactors). Various strains of E. coli (including genetically modified E. coli) were used. Measurement of the fluorescence signal allows biomass estimation under certain conditions. The monitoring of culture fluorescence is useful for obtaining information on the metabolic status of the cells. The effect on the cells of different substrates (glucose and oxygen) was studied. The measurements were performed in synthetic and complex media and the influence of the composition of each medium was investigated. A microfluorometer was used as a routine sensor for cultivation control and provided insight into the status of the cells.

mixing time experiments) were reported [3 - 5]. One of the most interesting applications is on-line biomass estimation during the cultivation of micro-organisms [6, 7].

Following the development of a miniature fluorometer [8] it became possible to apply culture fluorescence measurements to routine applications. Two types of fluorometer probe for the on-line measurement of NADH-dependent fluorescence are now commercially available (from BioChem Technology, Malvern, PA, U.S.A. and from Dr. W. Ingold AG, Urdorf, Switzerland). Both are specially designed for on-line application.

Until now, fluorescence measurements have been performed on only a few types of organisms such as yeasts and bacteria [3 - 5, 7, 9, 10]. In order to broaden our knowledge of the application of this technique to routine cultivation monitoring, fluorescence studies were carried out on different *E. coli*

To very briefly read the abstract, N A D H dependent culture fluorescence was monitored during various *E. coli* cultivation processes, batch and continuous cultivation, in stirred tank reactors, or bubble column reactors. Various strains of *E. coli*, including genetically modified e coli, were used. Measurement of fluorescence signal allows biomass estimation under certain conditions; this we have already seen. The monitoring of culture fluorescence is useful for obtaining information on the metabolic status of cells. The effect of cells of different, on different substrates, glucose and oxygen was studied. The measurements were performed in synthetic and complex media, and the influence of the composition of each medium was investigated. A microfluorimeter was used as a routine sensor for cultivation control, and provided insight into the status of cells

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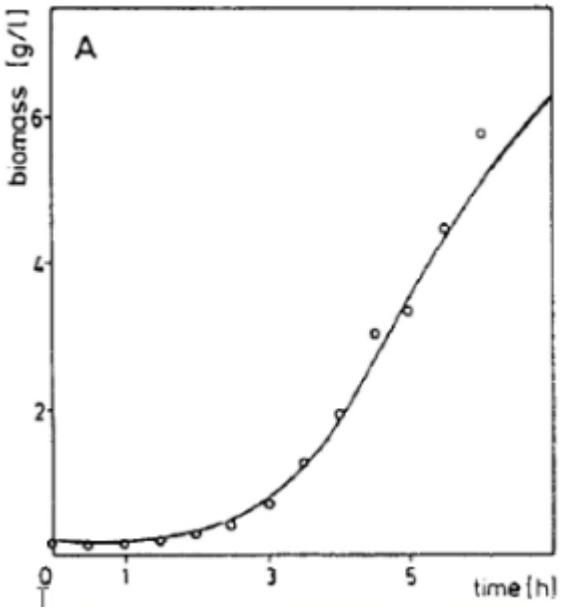

What I would like to point out is, this figure, biomass versus time. The points are experimental points and the line is given by culture fluorescence. You see a very nice relationship. This is what we talked about as a measure of the cell concentration itself. This is biomass, or cell concentration in grams per liter versus time by 2 methods; one is by the traditional gram per, the O D method, and the other one is by the culture fluorescence method. It agrees in many situations; it does not agree in one such situation; this is, this is a bubble column conditions.

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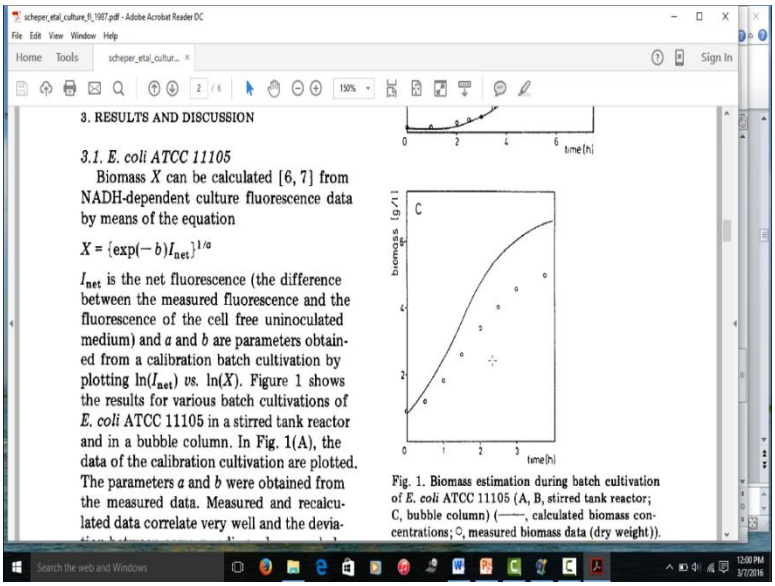

If it is a stirred reactor, there is absolutely no problem, whereas, in the bubble column, there seems to be interferences, which leads to a difference between these two. So, one should be careful while using culture fluorescence in bubble column. It makes sense, because bubbles will interfere. Then, one more thing I will point out on this paper. Here, you see this figure.

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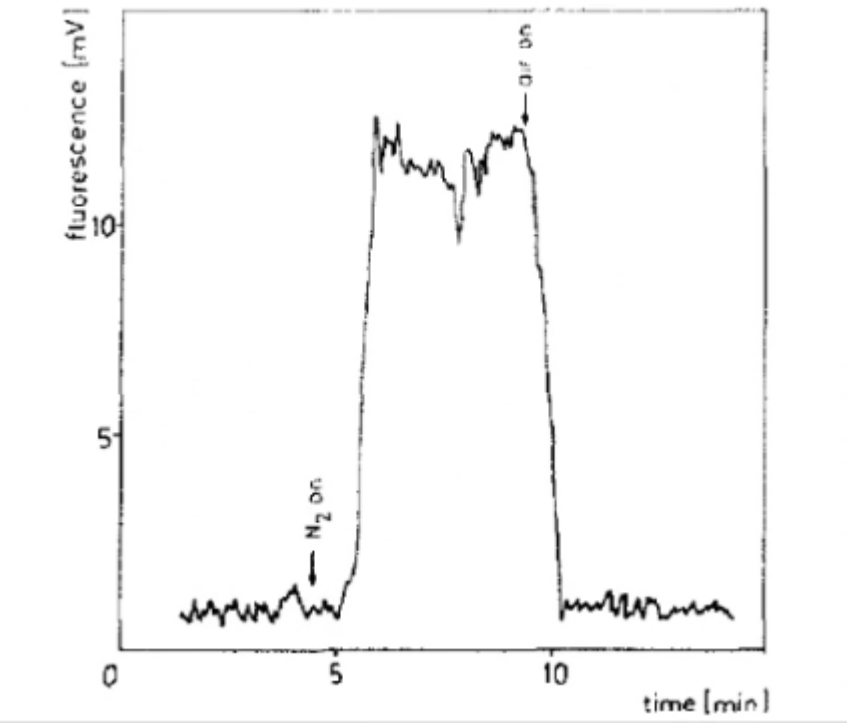

This figure is culture fluorescence versus time. The fluorescence was as a certain level, when oxygen was used. When oxygen and air was employed, let us read the, what this says; that does not matter. You have culture fluorescence at a certain level, when nitrogen is introduced, within few seconds, the culture fluorescence goes up; the anaerobic status goes up; you, when nitrogen is employed, the cells become anaerobic, because the oxygen is no longer available as final electron acceptors, right. So, the culture fluorescence at an anaerobic state is much higher than the culture fluorescence with an aerobic state because you will find N A DH forming more. So, this very clearly indicates the aerobic state of cells; when oxygen is turned back on, or air is turned back on, it comes back to its original level.

So, this is a nice indication of what is happening inside the cell, just by measuring the culture fluorescence.. You can read this paper if you are interested. I, for the purposes of this lecture, I think this is good enough. So, that is using culture fluorescence.

Now, let me talk about some of our work which was done quite a while ago, but it also on the same lines as this. This is measurement of intracellular pH, you know, this is pH inside the cells. This is distinct from the pH of the medium; pH of the medium, we know, goes up and down, because of acid base additions, and we said that, the pH needs to be controlled at an optimum value for best bioreactor performance and so on and so forth.

We are talking of intracellular pH, pH inside the cells. Now, the pH inside the cells can somewhat be related to the energy status of the cells, because there is something called a Mitchell's hypothesis. In fact, Peter Mitchell won the Nobel Prize for this hypothesis. This essentially says that, a hydrogen ion gradient across a membrane, intact membrane, is what provides the energy for A T P formation from A D P.

So, in some sense, we can link the energy status of the cell to the intracellular pH level. There are some fluorescent dyes that indicate the pH through their fluorescence, by getting inside the cell and so on, but we could not really use them, because those dyes leaked out; BCECF - AM is one such dye; it is, of course, some detail; it might be of interest to some, so, that is why I am mentioning it briefly. These dyes could leak out. So, we used a dye called 9-Aminoacridine, which is a weak base whose distribution depends on the difference between the extracellular and the intracellular pH. There are also these processes which take place across the plasma membrane in lower organisms such as bacteria and so on; so, that is what we were using.

So, what we did was, we developed this method of intracellular pH measurement online continuously in a bioreactor and then, we used intracellular pH to grow cells at a particular intracellular pH which is an indicator of energy status, after studying that different intracellular pH levels indicate different metabolic conditions in the cell. So, what we did was, we measured intracellular pH through fluorescence, and we found that, glucose addition, the addition of the substrate, can modify intracellular p H. So, the glucose additions were done so that, the intracellular pH was maintained at a certain higher level compared to the base level, sorry, the higher energy level; and when we did that, we could reduce the anaerobic product formation by about 4-fold. So, if it is

anaerobic growth, then the outcomes are much less in the aerobic growth, in terms of cell yield and so on. So, we were able to do that much better and we could reduce the anaerobic product yield. So, these things could also be done. There are a couple of papers on intracellular pH which are given here; I would not discuss them further, because of the introductory nature of this course, but if you are interested, you can look at these 2 papers given under 31, and know more about the intracellular pH. Let us move forward from here.

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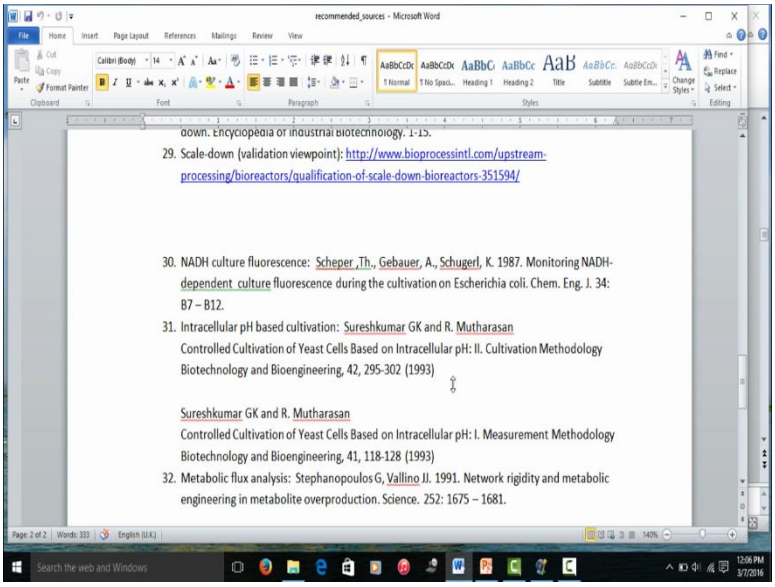

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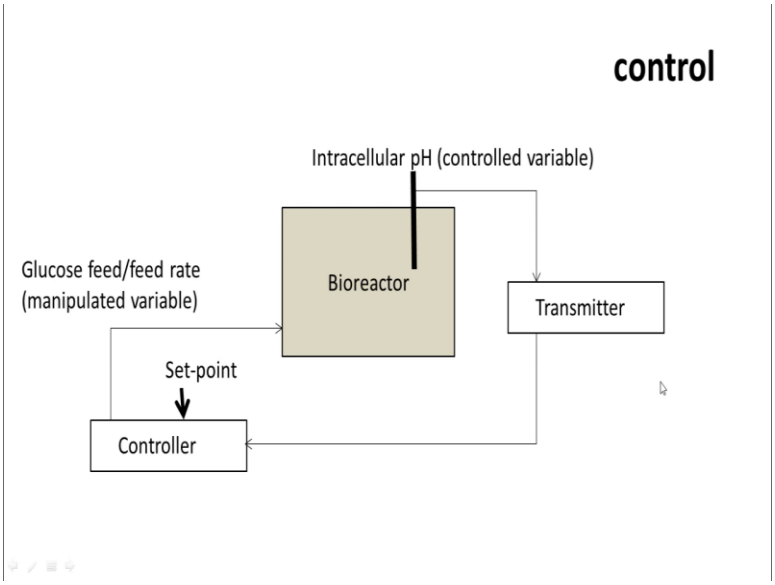

So, the control, can be explained with the above diagram. Intracellular pH was controlled using fluorescence; it is not a pH probe; pH probe will measure only the pH of the medium; then, transmit it and the controller was the one that determined the glucose feed, whether it needs to be fed, or the feed rate of glucose. So, that became the manipulated variable, to maintain the intracellular pH at a certain set point, to reduce the anaerobic product formation. So, this is the control schematic of the whole thing, ok.

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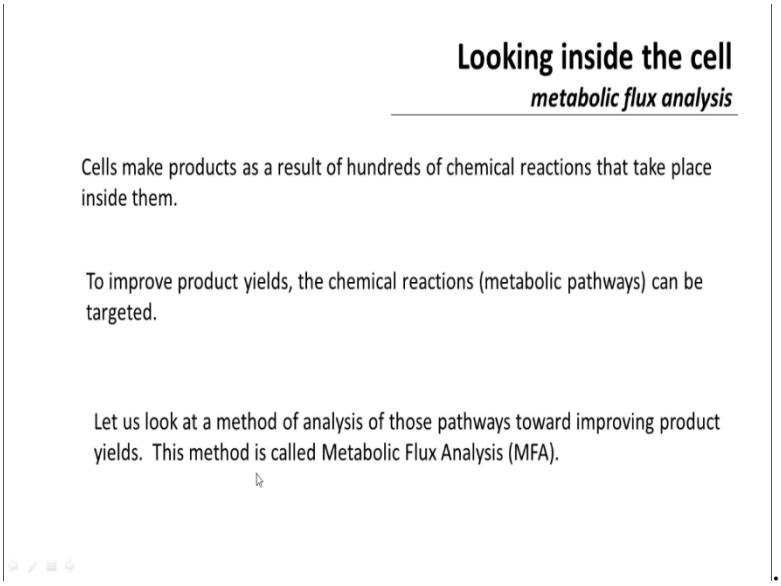

So now, let us starts looking inside the cell, through various, you know, windows, doors, and so on and so forth. I will tell you one example of such things, of looking inside the cell, and that is called metabolic flux analysis. We will spend some time on this, metabolic flux analysis. It is interesting. It is, it became popular in the early 1990s. The method was known from the 70s onwards, 1970s onwards. It became popular when Vallino and Stephanopoulos published their paper on using metabolic flux analysis to provide them with directions to improve lysine production; lysine is an amino acid that animals cells cannot make, and therefore, and that amino acid is required for a protein. So, we need to supplement it through diet, or other means and therefore, lysine is a huge product.

You know, large amounts of lysine are produced annually, and therefore, it is a, it is commercially a very important product. So, if you improve the lysine production a little bit, then the profits could be high, or the price could further be brought down, and all these made the use of metabolic flux analysis a popular one in the early 90s. And, a lot of work went on for the next 20 years or about 15 years on metabolic flux analysis. We will look at the principles of metabolic flux analysis next.

So, as an, as an introduction, cells make products as a result of hundreds of reactions that take place inside them; you already know this. To improve product yields, the chemical reactions, or the metabolic pathways can be targeted. And, let us look at a method of analysis of those pathways, toward improving product yields. This is our main aim. We are going to look at a method of analysis toward improving product yields and that method is called metabolic flux analysis, or MFA, for short.

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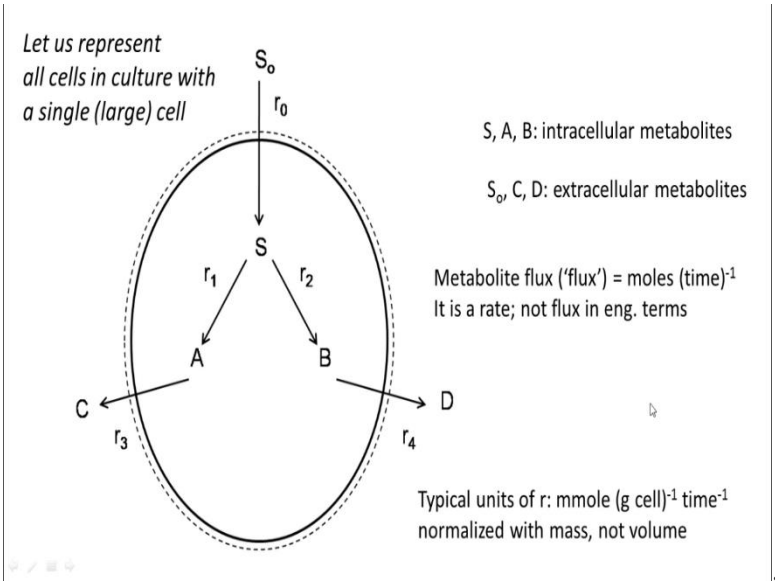

To know what metabolic flux analysis is all about, let us start with this.

Let us represent all the cells in culture by a single large cell, to begin with, as shown in the slide above. So, the volume of the cell equals the volume of all the cells in the bioreactor; the remaining volume is the broth volume. This could probably be less than 10 percent of the, even in the concentrated situations, it would be about 10 to 20 percent, right, of the total volume. And, let us consider these set of reactions that are occurring in the cell, hypothetical set of reactions, but these are good to understand the principles of metabolic flux analysis.

S_o is the substrate that is outside, that goes inside and gets converted to S; through probably the reaction r_0; then, S could get converted to A or B. It gets converted to A at a rate of r_1; this r 1 is moles per time. And, it gets converted to B at the rate of r_2 moles per time. B gets converted to D at the rate of r_4 which comes outside. A gets converted to C at the rate of r_3, which also comes outside. So, this is the situation here. Let us say that, this is some important metabolic pathway that is happening in the cell. This is the case, just put a note that S, A, B are inside the cell and therefore, are intracellular metabolites, whereas, S_o, C and D are extracellular metabolites, and metabolite flux is a common term that is used. The term flux is very different from the engineering term flux. Engineering term flux is amount transferred per unit area, perpendicular to the direction of transfer per unit time; that is our flux. In the case of metabolic flux, historically moles per time,

or, in other words, the rate has been used; an amount rate has been used, to indicate flux. We will continue using that, because it is widely accepted in the literature. It is a rate, and not flux, as in engineering terms. This we need to keep in mind.

So, the typical units of r i.e. r_0, r_1, r_2 are milli mole per gram cell per time; note that it is normalized with respect to mass and not volume, right; or rather, expressed in terms of mass and it has not been normalized with volume.

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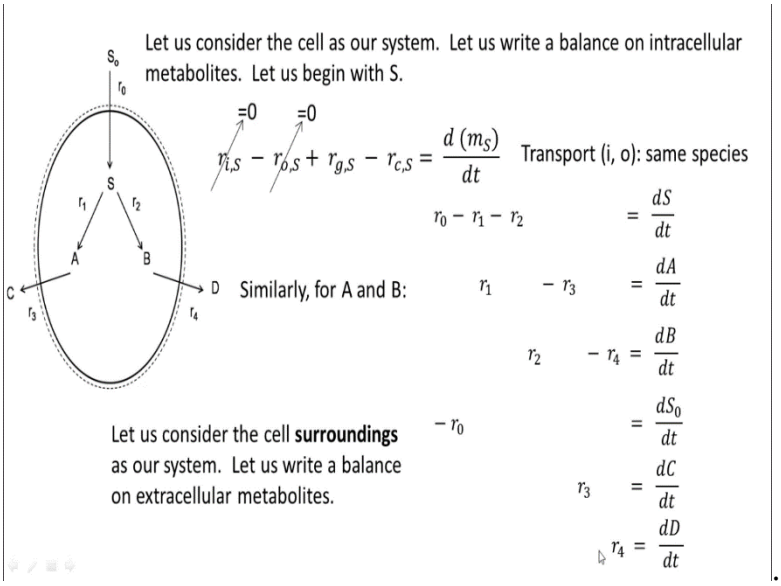

Now, let us consider this large cell as our system. Let us write a balance on intercellular metabolites. First, I would like you to note that, if we are writing balances on intercellular metabolites, we consider the cell as our system; this is our system; and we write the balances on the metabolites inside the cell. Let us begin with this S here.

$$r_{i,S} - r_{o,S} + r_{g,S} - r_{c,S} = \frac{d(m_S)}{dt}$$

So, rate of input of S minus rate of output of S; plus the rate of generation of S minus the rate of consumption of S equals the rate of accumulation of S. If S_0 has come in as S_0, then we consider it as transport across the system boundary. These two terms, input and output have relevance as you know, only when there is transport across the boundary of the system. So, if S_0 has become S_0 here, then, we can consider it as the input term; otherwise, we consider it as a reaction term, which could be accounted for in generation or consumption. But, we need to make sure that we count it only once; we cannot count

it as input, as well as generation, even if you want to follow a different strategy for doing this. If you follow this strategy that, if S_0 becomes S_0, then we consider it as transport; if S_0 becomes something else inside, then, it becomes a reaction. Similarly, A going to C, this, we are going to consider it as a reaction, because A has become C; A has not become A outside. Then, it can be looked at as transport. The main thing is that, there should not be any double counting.

So, there is, if you look at that, there is no input of S into the cell. There is no output of S into the cell; that is not going anywhere; whereas, there is definitely a rate of generation and a rate of consumption. The rate of generation is r_0; the rate of consumption, is through 2 reactions. Therefore,

$$r_0 - r_1 - r_2 = \frac{dS}{dt}$$

$$r_1 - r_3 = \frac{dA}{dt}$$

$$r_2 - r_4 = \frac{dB}{dt}$$

$$-r_0 = \frac{dS_0}{dt}$$

$$r_3 = \frac{dC}{dt}$$

$$r_4 = \frac{dD}{dt}$$

Similarly, for A and B, A going, A is formed at the rate of r_1; it gets converted at the rate of r 2. So, generation is r_1, consumption is r 2; r_1 minus r 2 is d A d t. Similarly, r 2 for B, formed at r 2 and getting consumed at r 4. Therefore, r 2 minus r 4 is d B d t. Now, there is a trick here; I want you to pay some attention.

We are going to consider the surrounding of the cell as a system. When we focus on extracellular metabolites, we will consider the surroundings of our cell as a system, and write our balances. That is what turns out to be consistent with our approach here. So, if you write a balance on the extracellular metabolites, on, first one S naught, S naught is getting out of the system here. This is our system now; it is getting out of the system. Therefore, there is no input, but there is an output. And therefore, minus r zero, which comes from here. It is not being generated; it is not being consumed; so, minus r zero is d S zero d t. Similarly, for D, it is being produced here; you know, the generation term alone exists. There is no input; there is no output; there is no, I am sorry; this is , there is no… In the case of the earlier one, there is no input; there is no output; there is no generation of S zero; there is consumption of S zero through the reaction r zero; it takes it to the other side and therefore, r zero is the consumption term here. I have just lined up r zero, r 1, r 2; that is the reason why r zero is here, and it is actually the consumption term. Therefore, minus r zero is d S naught d t; you need to be a little careful with these things.

In our system here; C is an extracellular metabolite, inside its system now. This is being generated at the rate of r 3 and therefore, this is the generation term. So, that is what is r 3 here r I, r o, and r c are zero. And therefore, it is plus r g equals d C d t.

Similarly, for D, this is, here, it is being generated at the rate of r 4 by this reaction; input output and consumption terms are zero. Therefore, plus r 4 equals d D d t. So, this is the way you go about it. Once you get used to it, it is simpler, but you need to be a little careful; you cannot make errors like I inadvertently made by switching the systems in my mind; be a little careful.

So, now the reason for me writing it this way is that, can you see a matrix evolving here? Now, I have just lined up r zeros, r 1s, r 2s, r 3s and so on. So, the set of these equations can be written in a compact form as this.

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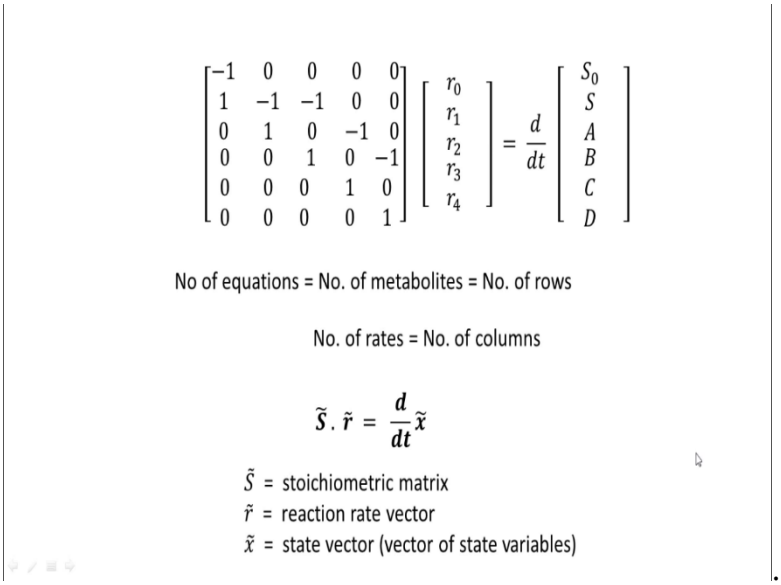

In our first equation, we will write it in this order, S zero, S, A, B, C, D. Our equation, you know, the way we do matrix multiplication, right; this term into this term, plus this term into this term, plus this term into this term, plus this term into this term, plus this term into this term, equals this term. So, minus r zero is, you know, the other terms are all zero for the first term, minus r zero is d d t of S zero, which is what this one says; minus r zero is d dt of S zero; I will show you one more thing.

r zero minus r 1 minus r 2, the rest of the terms are zero, equals d d t of S. If you remember, that was the first equation; r zero minus r 1 minus r 2 is d S d t. One more equation, this is for completeness, and then, we will go forward. This first term is zero; r 1, r 2 is zero; r 1 minus r 3 is d d t of A.

r 1 minus r 3 is d d t of A, right. That is the way the representation happens in a matrix form. Therefore, this matrix represents the set of equations that we got by doing material balances on the various metabolites in the cell, extracellular and intracellular.

The number of equations would equal the number of metabolites, as we have seen and that would equal the number of rows that we have here, right; here, we have 1, 2, 3, 4, 5, 6, and we had 6 metabolites, S naught, S, A, B, C, D. So, 6 and therefore, that equals the number of rows. And, the number of rates would equal the number of columns. Here, there are 5 rates and 5 columns; r zero, r 1, r 2, r 3, r 4 and there are 5 columns here, in this matrix.

So, this is what is called a stoichiometric matrix, $\tilde{\boldsymbol{S}}$, stoichiometric matrix multiplied with the rate vector, or the rate matrix r is d/ d t of the state variable as it is called, state variable matrix $\tilde{\boldsymbol{x}}$.

$$\tilde{\boldsymbol{S}} . \tilde{\boldsymbol{r}} = \frac{\boldsymbol{d}}{\boldsymbol{dt}} \tilde{\boldsymbol{x}}$$

$\tilde{S}$ = stoichiometric matrix

$\tilde{r}$ = reaction rate vector

$\tilde{x}$ = state vector (vector of state variables)

So, this is a compact representation of the entire set of equations, and this kind of a representation becomes much easier even if you have 100s or 1000s of equations. So, of course, cumbersome to first to convert them into a computer program, but once you have converted them into the computer program, you can do all sorts of modifications, if you look at it in this form. So, that is the reason why we got it into this form; reaction rate vector and x is a state vector, vector of state variables; in other words, the metabolites are state variables.

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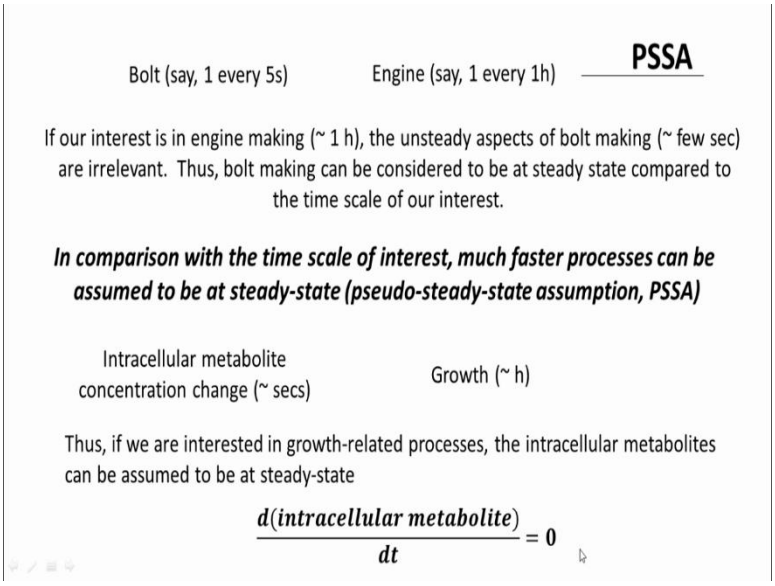

This is an important concept now. We had looked at something called a pseudo steady state approximation earlier. Let us revisit that. We talked about the rate at which a bolt is made; a bolt made every, one every 5 seconds; and an engine, a car engine made 1 every hour. We said, since this is made every 5 seconds and this is made every hour, even if it takes 7 seconds, or 3 seconds, or 4 seconds to make a bolt, that variation in time, the unsteady state, unsteady nature of this process will not affect the nature of engine making. Because, this process is very fast; this process is very slow. If you focus on the slower process, then, the much faster process can be assumed to be at steady state, whether it is actually at steady state or not; that is the pseudo steady state approximation. That is what is given here. If our interest is in engine making, about an hour characteristic time, the unsteady aspects of bolt making, a few seconds, are irrelevant. Thus, bolt making can be considered to be at steady state compared to the time scale of our interest, irrespective of whether this has been at steady state or not. So, you need comparison. So, in comparison with the time scale of interest, much faster processes can be assumed to be at steady state, and this is called the Pseudo Steady State Approximation. We are revisiting this this is an important concept; a little difficult to appreciate in the beginning, but once you get a hang of it, this becomes very powerful. Similarly, if you look at the intracellular metabolite concentration change, it happens over seconds, whereas, growth typically happens over, let us say, a few, let us say, over characteristic times of hours. So, this is a slower process, growth compared to the, the process of intracellular metabolite concentration changes. So, in comparison to the growth processes, if our interest is in growth related processes, the intracellular metabolites can be assumed to be at steady state. So, this is the pseudo steady state of approximation. Now, see how it simplifies our approach. All the rate derivatives, the time derivatives of intracellular metabolites can be set to zero by the pseudo steady state approximation. If we are interested in growth related processes, which we are, growth and product formation, in the case of a bioreactor, definitely we are related only in, definitely we are interested only in such processes, and in such a case, we can directly take the intracellular metabolite variations with time to be zero, or the, this process to be at steady state.

$$\frac{d(\textit{intracellular metabolite})}{dt} = 0$$

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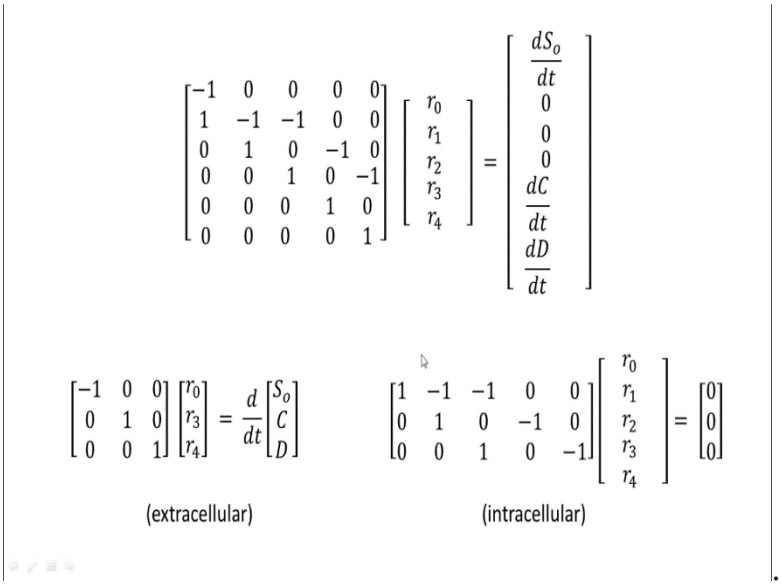

If you do that, then, the intracellular metabolites are A, B, C, they can be directly put to zero. The other rates are extracellular metabolites. Let them be there. If this is the case, then we could split up this matrix, as related to extracellular and intracellular matrices, because intracellular matrices, intracellular metabolite related matrices will vanish. I have just taken r zero, r 3 and r 4 and written the stoichiometric matrix from this part, which is relevant to that, to give us d d t of S naught, C and D. And, this is for the extracellular part; the intracellular part; the extracellular part is this; minus 1, zero, zero, zero, 1, zero, and zero, zero, 1, corresponding to r naught, r 3 and r 4, and they correspond, correspond on this side to d d t of S naught, C and D; whereas intercellular which corresponds to A, B and C which are actually zero here, can be taken from this particular, from the A, B, C part of the matrix here, this particular part of the stoichiometric matrix. So, I have represented this as a matrix that represents extracellular metabolites, and a matrix that represents intracellular metabolites. You can take a look at the details, and convince yourself with time. Let us move forward now.

So, the metabolite flux analysis. This is something like this. What all can be done with metabolite flux analysis, right. This stoichiometric matrix gives us the various rates; the stoichiometric matrix, sorry, this representation gives us stoichiometric matrix, the various rates and the rates of accumulation. So, let us see what all can be done, or what are the common things that are done with such a representation, which would be useful for us from a bioreactor point of view.

So, estimation of intracellular metabolite flux from extracellular metabolite rates. Extracellular metabolites can be measured by some of the methods that we talked about in an earlier module, module 3. So, by measuring those we can get an estimate of the intracellular metabolite flux, right, the rates at which things are going on inside the cell. Identification of branch point control, nodal rigidity, in cell pathways, this can be done. Identification of alternate pathways can be done; analysis of fluxome, metabolome, and many omes can be done. Ome is nothing, but a complete collection of everything that happens; a genome, for example, is a complete representation of collection of all the genes in the cell. Fluxome is the complete representation of all the fluxes in the cell and so on and so forth. You can analyze that using this approach. We are going to look at only the first two, as examples of what can be done with metabolite flux analysis.

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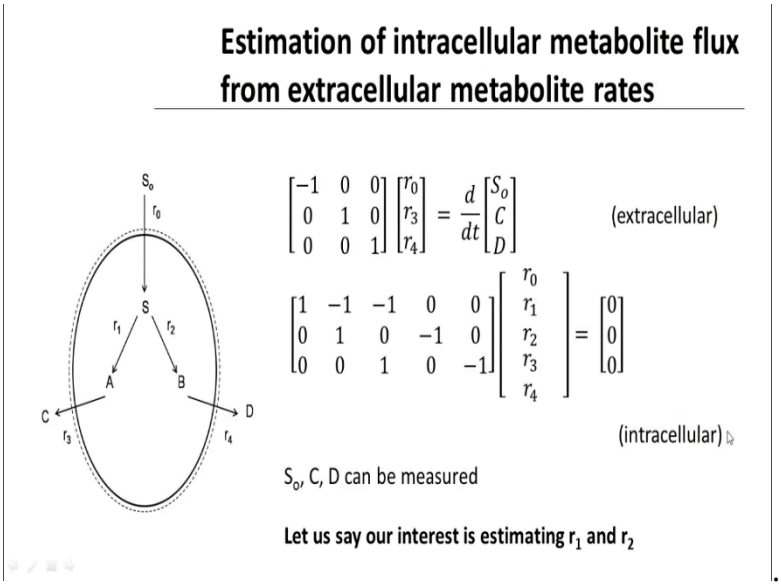

So, let us look at the first thing, estimation of intracellular metabolite flux from extracellular metabolite rates. This is the same figure that we saw earlier; S naught going to S, which further goes to A and B; A goes C and B goes to D; S naught, C and D are extracellular metabolites; S, A and B intracellular metabolites. So, we are going to measure S naught, C and D and then, get the rates that are involved here. How do we do that? We know that, the extracellular, the formulation corresponding to extracellular metabolites is this; we had already seen that earlier; note the rates that correspond to extracellular metabolites. This is d/ d t of S_0, C and D. The equations corresponding to extracellular metabolites have been written in this matrix form and, this is the

intracellular one. If S naught, C and D can be measured, you can calculate this, right. You take points at reasonably closely spaced time intervals. Then, you can actually get the rates of variation of S naught, C and D in the extracellular space. Knowing this, knowing the rates of S naught, C and D, let us say, our interest is in estimating r 1 and r 2; that is our interest; that is what we said here, estimation of intracellular metabolite flux or rates from extracellular metabolite rates.

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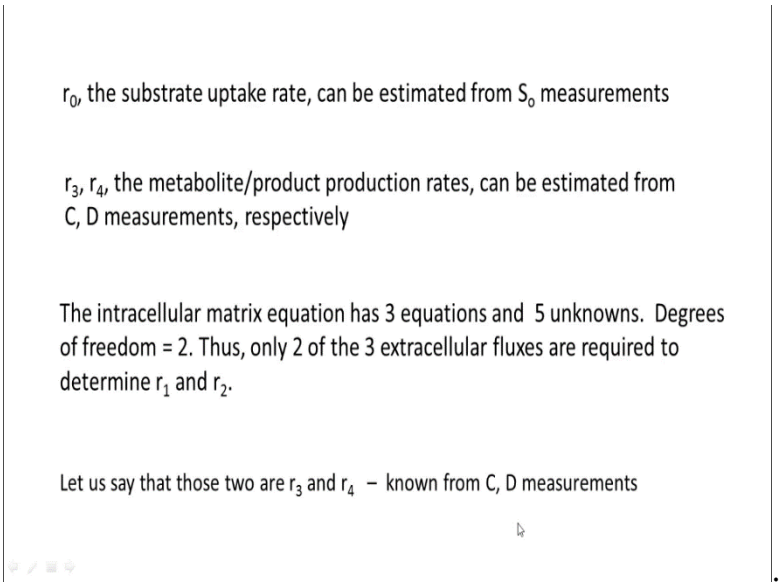

r 0, the substrate uptake rate can be estimated from S naught measurements; that is ok; directly gives you r naught; r 3, r 4, the metabolite, or the product formation rates can be estimated from C and D measurements respectively. The intracellular matrix equation has 3 equations and 5 unknowns, right. Number of equations, number of unknowns; you know the degree of freedom concept; the degree of freedom, degrees of freedom is the number of independent variables that are needed to be known to solve this set of equations completely. We have 3 equations, 5 unknowns. So, 5 minus 3 is 2. So, we need 2 of the 3 extracellular fluxes to determine r 1 and r 2. Let us say that, those two are r 3 and r 4 which are known, which can be known from C and D measurements. We take measurements of C and D concentrations at reasonably closely spaced times, and we have these concentrations. We have these rates, as derivatives C 2 minus C 1 divided by t 2 minus t 1 and so on so forth.

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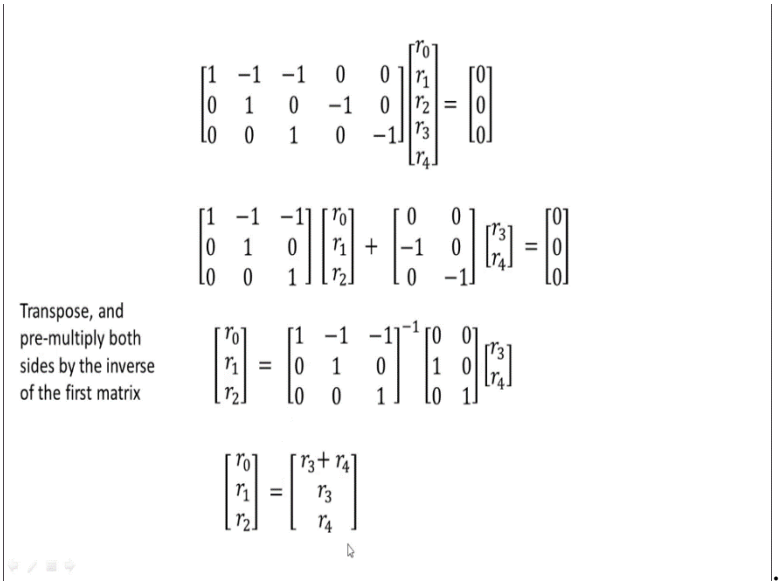

So, this is the matrix here. The intracellular matrix which we have taken to be zero, which we had represented as, which can be represented as this part alone corresponding to r naught, r 1, r 2, and this part alone corresponding to r 3, r 4, right; that will essentially be the same as this, you can test it out. So, minus 1, minus 1, minus 1, zero, 1, zero, zero, zero, 1, this corresponds to r zero, r 1, r 2. So, we have written that separately; and, zero, zero, minus one, zero, zero, one, this corresponds to r 3 and r 4, and so, we have written them separately as zero, zero, zero.

Now, if we transpose this matrix equation, this is an equation consisting of matrixes; we transpose these, and pre-multiply both sides by the inverse of the first matrix. You should be a little comfortable with linear algebra to do it. So, a little too difficult to get into those first principles, in this lecture. So, if you follow it, it is fine, otherwise, you need to take it on belief, and then, go and check the matrix algebra, and then, understand these things better. So, if we premultiply this matrix with its inverse, this will drop out; and, pre-multiply this, with this same inverse matrix, this term will remain and anyway, this is zero. So, this is going to drop out. So, we have transposed this equation and therefore, all the minus 1s have become 1s.

These zeros will remain the same. So, this equals this; that is what is the first step; and then, we have pre-multiplied it with this matrix, sorry, with the matrix that is the inverse of this matrix, which is this. So, I I inverse is a unity matrix; we are not going to

represent that. This matrix times, this, into r 3, r 4 will directly give us r naught, r 1 and r 2; or, r 3, r 4, we are interested in by measuring r naught, r 1, and r 2; no, r 3, r 4 are the ones that are being measured here, calculated here, from the measurements of C and D. With that, we can get r naught, r 1 and r 2. So, r naught, r 1 and r 2, if you work things out, it will turn out in this pre-multiplication of this, this matrix would result in, if you multiply, and so on so forth, r 3 plus r 4, r 3 and r 4. Therefore, r naught equals r 3 plus r 4; r 1 equals r3; r 2 equals r 4.

So, just by doing this kind of a manipulation, we directly get the intracellular rates based on the extracellular rates that can be measured; that is powerful.

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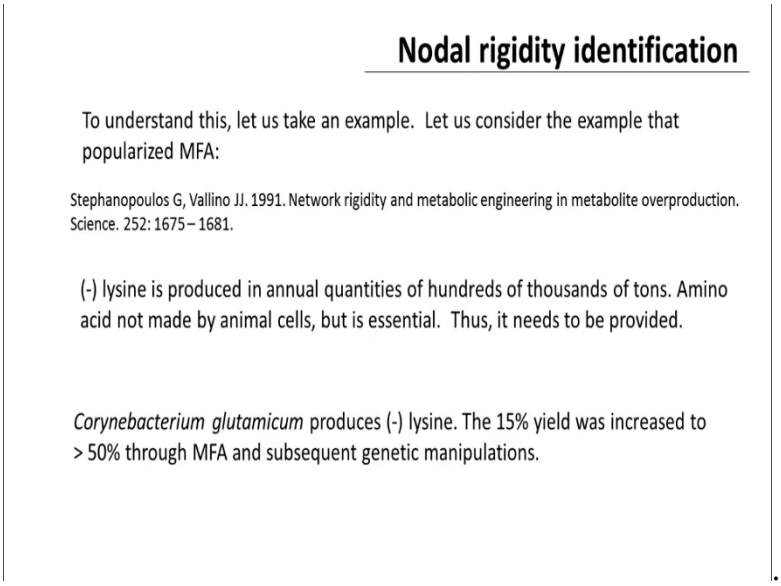

Now, let us look at nodal rigidity identification. This is what went into the lysine improvement that we talked about, Vallino and Stephanopoulos's work, early 90s. To understand this, let us take an example. Let us consider the example that is, that popularized the metabolic flux analysis, the lysine production. This is the reference here. It has been also given in your list of references. You can go and take a look at it; Stephanopoulos and Vallino, 1991, Network rigidity and metabolic engineering in metabolite over production. This was published in Science. And then, there is one other paper that was published in Biotechnology and Bioengineering in 1993, which gives some more details. As mentioned earlier, l-lysine, or minus lysine is produced in annual quantities of hundreds of thousands of tons, because this amino acid is not made by

animal cells, but is required for proteins and so on. Therefore, it needs to be provided. That itself causes, or gives you the reason for the large production of minus lysine. *Corynebacterium glutamicum*, which is an organism, this produces l-lysine. The yield, when this was being produced, was only about 15 percent. And, this 15 percent was increased more than 3-fold to greater than 50 percent through metabolic flux analysis. You see the power here. 15 percent has gone to 50 percent through metabolic flux analysis and subsequent genetic manipulations.

See, metabolic flux analysis gives you directions; based on that, you need to modify the organisms genetically, and then, in this case, it produced more than 3-fold yield.

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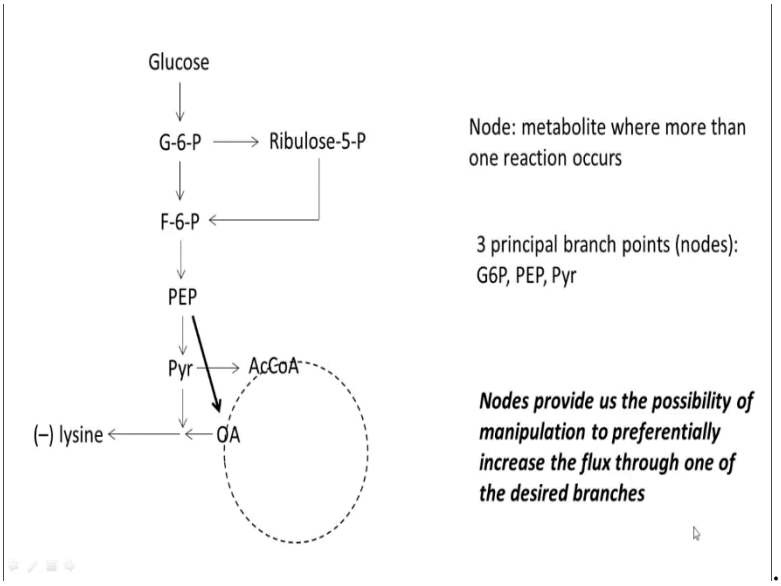
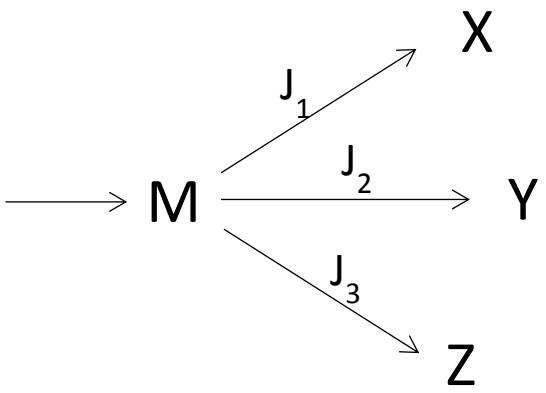


So, some details; this is the primary pathway that we should be interested in for lysine production. This is a part of glycolysis, glucose, glucose going to glucose 6 phosphate, to fructose 6 phosphate, phosphoenol pyruvate to pyruvate. And then, there is this branch point, which is important. Here, glucose 6 phosphate through ribulose 5 phosphate comes back to fructose 6 phosphate; phosphoenol pyruvate here goes to oxaloacetic acid and then to lysine; pyruvate can also go to lysine; and there is another branch point here; from pyruvate to acetyl Co a. People would recognize that, this is the T C A cycle, which is indicated by the dotted circle here; dashed circle.

Let us now define a node which is the metabolite, where more than one reaction occurs. So, there are three principle nodes, or branch points in this network, G 6 P, where there is

branch, branching, more than one reaction occurs; one reaction; second reaction; and then, P E P; this goes here into oxaloacetic acid; and pyruvate here, it is going to oxaloacetic acid and l-lysine. So, these are the 3 branch points here. These are the ones that one focuses on to do nodal rigidity analysis. Nodes provide us the possibility of manipulation to preferentially increase the flux through one of the desired branches. Suppose, we want the flux to go here to l-lysine and this is one of, one of the places where a possibility exists, to channel the flux more here, by some means, compared to the flux here; that is what is going to increase the l-lysine production, right. So, that is why we look at these nodes, the branch points.



So, in formal terms, if M metabolite undergoes 3 reactions with the rates J1, J 2 and J 3, or fluxes J 1, J 2 and J 3, in this terminology, to give you X, Y and Z, and let us say, that Y is the desired product here, J 2 needs to be increased selectively over J 1 and J 3, right. So, the split ratio as it is called, this is the flux through the desired branch divided by the sum of fluxes through all branches; that is the split ratio. It can be written as J 2 divided by the sum of all Js, J 1, J 2, J 3, in this case. So, J 2 by J 1 plus J 2 plus J 3; this is the split ratio.

$$split\ ratio = \frac{flux\ through\ the\ desired\ branch}{sum\ of\ fluxes\ through\ all\ branches} = \frac{J_2}{\sum_i J_i} = \frac{J_2}{J_1 + J_2 + J_3}$$

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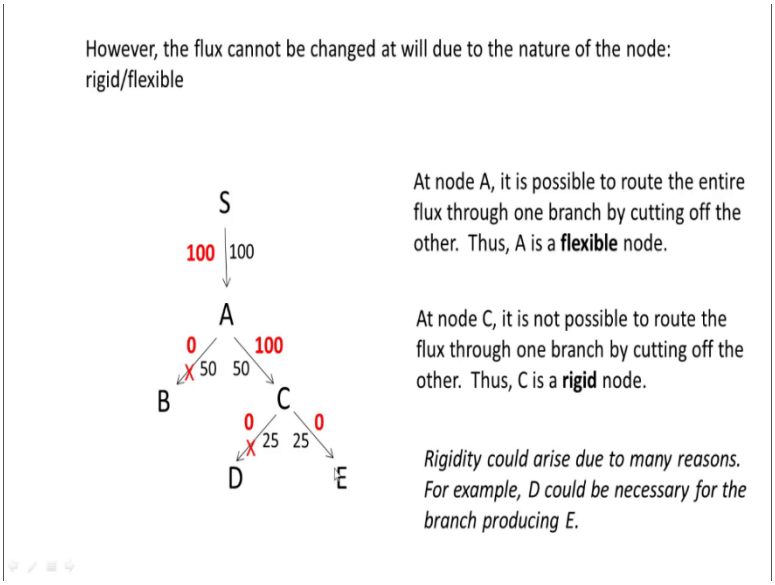

This is fine, but the main reason why we need to, why this is not so easy, is it the flux cannot be changed at will, due to the nature of the node; in all nodes, you just cannot say we will take more carbon through this branch, and stop by probably cutting off the enzyme that is responsible for this branch and so on and so forth. It will not happen. Let me very briefly tell you what it is. There are two kinds of nodes that are possible. It could be a rigid node or a flexible node. To do that, let us consider this example. S going to A; A goes to B and C, and C goes to D and E. These are normalized fluxes. S to A is 100; A to B is 50, and A to C is 50 and C to D is 25; C to E is 25 in some units, millimole per time, or whatever. So, this is the branching, when nothing has been done to this particular network. By some genetic, if we can cut off the enzymes maybe, for this branch, then, this branch can be cut off, and this branch also is cut off, let us say, then, this C to D conversion cannot take place. If it is, if the observed fluxes, not really observed it, let us say that, the fluxes on doing these 2 cuts, turns out to be something like this. On cutting off these, the 100 becomes zero here, but it is 100 here; whereas, by cutting off this, both these fluxes have become zero. Let us say that this has happened. It is not easy to observe this, but let us say, this has happened. If this has happened at the node A,

it is possible to reroute the entire flux through one branch; let us say, this is the desired branch. By cutting off this branch, we are able to reroute all the flux through this branch. And therefore, this is a flexible node, whereas, at node C, because we cut this off, this

also got cut off; we did not intend to cut this off, but just because we cut off this path, this also got cut off; such a node, C is called a rigid node. We cannot expect to increase the flux preferentially through one of the branch points, if the other branch point is cut off; that is a rigid node. So, we need to look for flexible nodes to manipulate things. Rigid nodes will not help us. So, rigidity could arise due to many reasons. For example, D could be necessary for the branch that is producing E in some way, some metabolic way, or some genetic way, it could be responsible; maybe through a feedback loop, or something like that. Therefore, if you cut this off, this also gets cut off.

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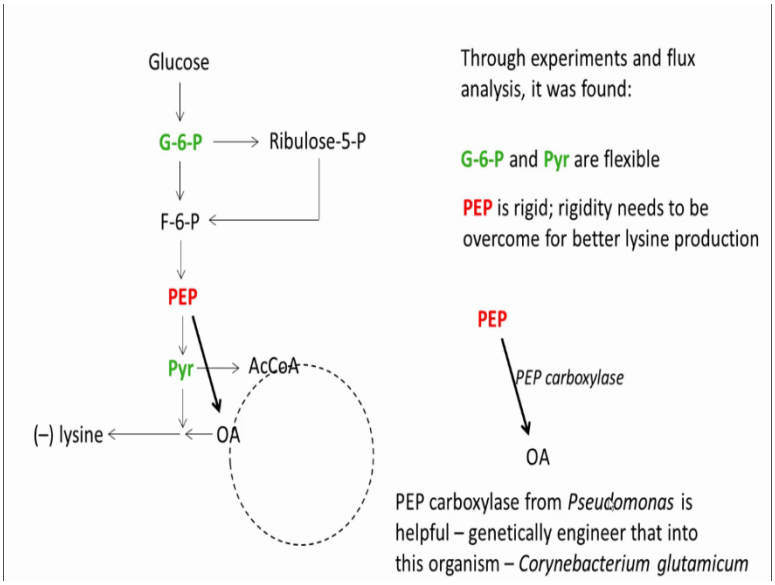

So, these 3 branch points are shown, G 6 P, P E P, Pyruvate, these 3 nodes. Through experiments and flux analysis it was found that, these 2 nodes, G 6 P and pyruvate, these are flexible. It is possible to reroute the flux through a desired branch by cutting off the other branch, whereas, P E P is rigid and rigidity needs to be overcome for better lysine production. You can, or in other words, P E P is going to OA. This is the desired branch. This is the P E P carboxylase enzyme that is doing this. P E P carboxylase from the regular organism leads to a rigid node, whereas, P E P carboxylase enzyme from pseudomonas is helpful, is helpful to break the rigidity.

Therefore, the approach was take *Pseudomonas* P E P carboxylase, express it in *Corynebacterium glutamicum*, and that has helped to decrease the rigidity of this node, and thereby, increase the production of lysine by about 3-fold, ok. So, in the last part, we

had actually taken the gene from some other organism, expressed it in the host organism, this is the recombinant DNA technology that I was talking about, and this is a way of modifying the cell to produce more. So, I have shown you an example, where the two major aspects, one is looking at the cell in detail, and then, modifying the cell are, have both been shown to improve the yield of a product, in this case l-lysine.

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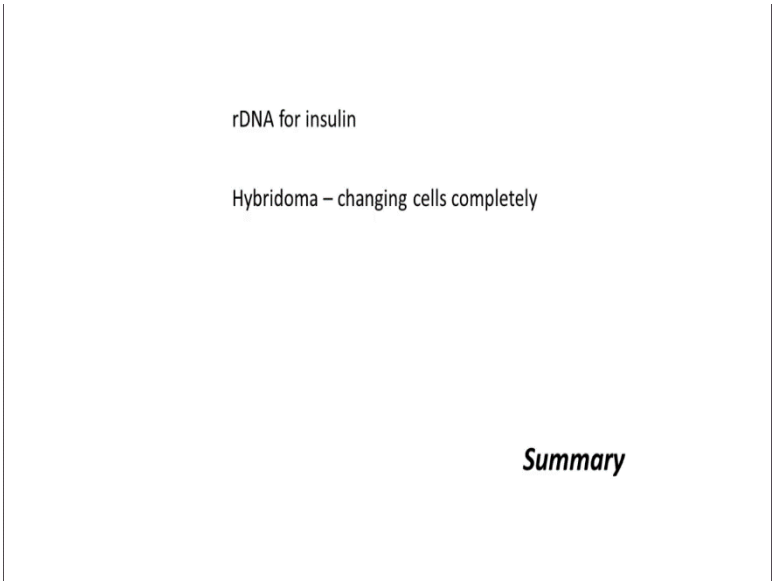

And, rDNA has been used heavily for insulin production.

Insulin is made by using a bacterium. They have taken the insulin gene, they took the insulin gene and expressed it in bacterium, and produced insulin; that was the way by which they could produce large amounts of insulin, and bring down the cost and availability and morbidity by significant, significantly. And, one more step further, this is changing, putting rDNA, rDNA technology is taking DNA and putting it into another organism. One step further is changing the entire cell. An example of that is hybridoma. You take a cell that is cancerous, that can grow forever; and you take a cell that can produce a particular type of antibody, put them together, you get a cell that produces only that kind of a antibody, and can live forever; that is changing the cell completely; that can also be done to make, bioreactor, and if you use them in bioreactors you can get production of the required product, the monoclonal antibody from bioreactors. So, in summary, if you look at the, look at module 5, we will summarize the entire course in the next lecture. In module 5, we began by considering the cells themselves, the actual

factories that produce the product. We said that, we could get some input into what is happening by considering the cell as a black box; and one of the methods of doing that is by stoichiometry, bioreactions stoichiometry, and the degree of reductance concept is an important concept there. Then, we said that, we will open a window and look at it. Some windows are the cell status indicators, the metabolic status indicators, energy status indicators, such as redox ratio, the energy ratio, and the intracellular pH. These three are examples. There could be others. And then, we said, we will go and change, we will go and look inside the cell; that is what we did by metabolic flux analysis, through the methods that we have described in detail here; some detail here, not all detail. And then, based on the input provided by the metabolic flux analysis, we could do directed change through rDNA technology, of the genetic machinery and improve the productivity, for example, l-lysine by more than 3-fold. And then, we said that, you could actually change the cell itself by bringing two types of cells together to produce monoclonal antibodies, or any other product of interest.

With this, we finish the 5 modules. In the next lecture, I will summarize, in brief what all we did in the course. See you in the next lecture.