Metabolic Engineering
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Lecture-26
Advancement in 13 C Metabolic Flux Analysis

Welcome to metabolic engineering course, today we are going to learn about advancement in 13 C metabolic flux analysis. So, far we learned how 13 C metabolic flux analyses have been done.

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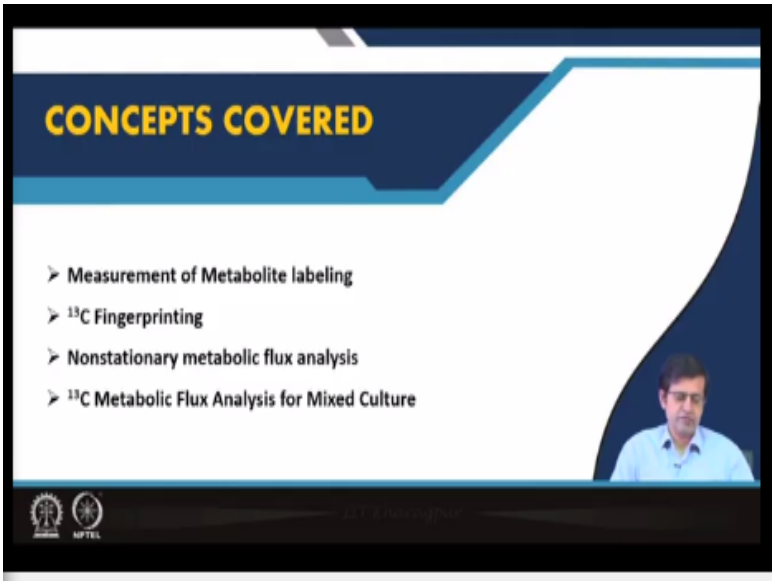

And then today we will cover some of the topic related to metabolic labeling, how you can measure the metabolite labeling. And another new topic that is 13 C fingerprinting which is helpful for metabolic engineering for identifying new pathways and then nonstationary metabolic flux analysis and 13 C metabolic flux analysis for mixed culture.

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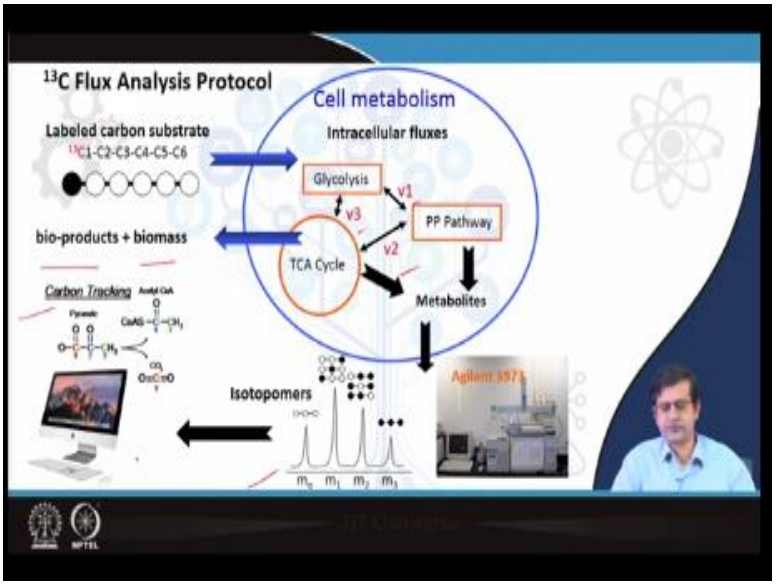

So, to start with the 13 C metabolic flux analysis protocols involve label carbon substrate. Label first carbon on the glucose and now the cell to grow on that label glucose. And the cellular metabolism which is happening inside the cell, if you want to know the intercellular fluxes, then you have to measure the extracellular byproduct. So, because you cannot measure an intercellular flux, that is why it is very important you measure all extracellular fluxes.

The extracellular fluxes which are easy to measure through HPLC or any other technique. So, you can measure the biomass and as well as the byproduct, these 2 things you can measure and how much carbon going in that you already know. And then how much coming out also you measure, intercellular fluxes like v1, v2, v3 if you want to measure for that you have to do the isotope measurement.

That is the metabolite measurement through GC Mass. Now using the GCMS you would be able to measure the mass distribution vector like m0, m1, m2, m3 and using those isotopomer you would be able to measure the fluxes provided. You have software to do that. So, 13 C metabolic flux analysis softwares are available, where you can track the carbon the 13 C MFA is nothing but carbon tracking experiment.

That you have to perform in the computer, you have the experimental values for the isotopomer and also the metabolic network and you try to feed the data that I have already explained in the previous class.

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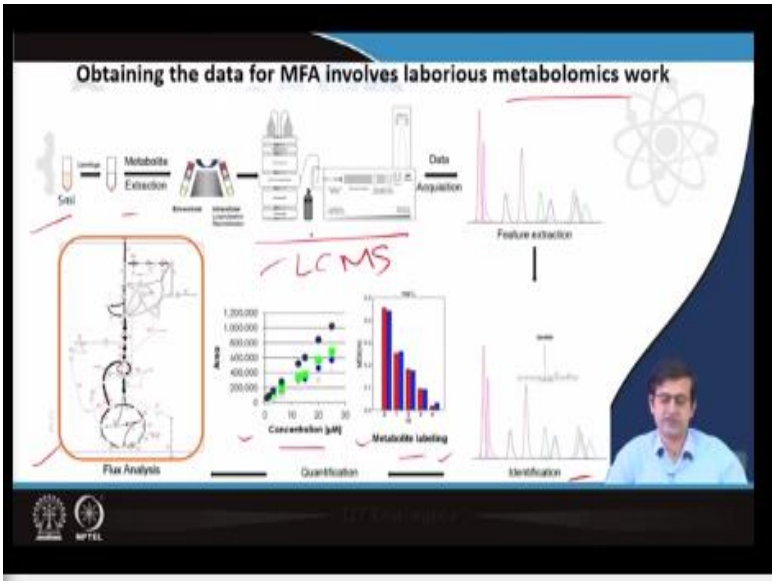

So, obtaining the data for 13 C MFA involves laborious metabolomics work. So, then you have to go through the steps required for metabolomics. The metabolomics is very important. Here you can see you extract some amount of culture from the cell culture what you are doing to it can be a micro bioreactor or a bioreactor and try to extract the metabolite. So, the extraction of the metabolites and another thing remain the same as metabolomics, you pellet the cell and then extract the metabolite and try to lyophilize the cell pellet in a mobile phase.

And try to extract the metabolite using the extraction procedures specified for particular organism and try to measure in a GCMS. So, this is the LCMS setup has been shown here not GCMS. So, assume the LCMS you can measure the intercellular amino acid. And then you do the data acquisition and then try to identify the metabolite then you get the labeling. So, when you get the labeling and also the concentration of the metabolites using HPLC. So, 2 things you need metabolite labeling and the concentration of the extracellular metabolite not the intercellular metabolite.

You try to get the concentration of extracellular metabolite which is coming outside the cell as a secretory product. And using these 2 data you will be able to calculate the fluxes using a software. So, this metabolomics work is very laborious, it takes many days to actually get the labeling done. Labeling experiment done in a HPLC or GC. The metabolite labeling for the intercellular amino acids are done in LCMS, whereas if you want to do proteinogenic amino acid then you have to do GCMS.

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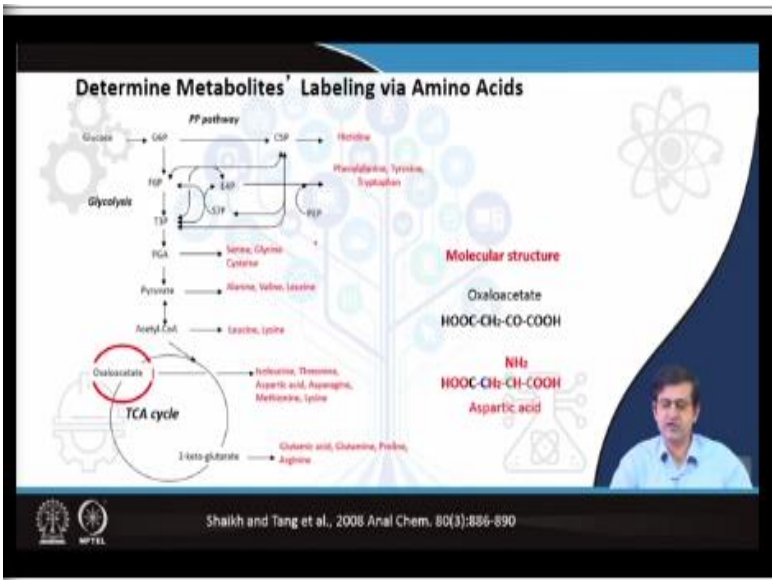

So, how do you derive the metabolite labeling? If you are considering using GCMS then you have to derivatize the amino acid.

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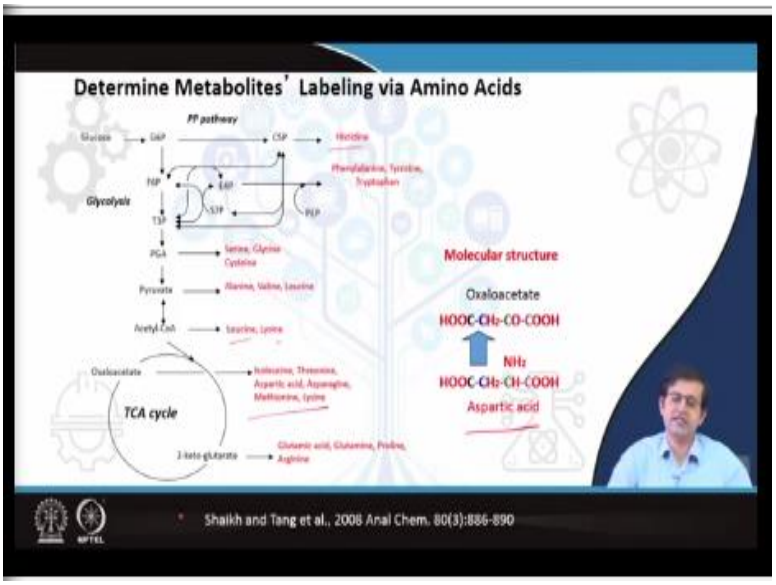

So, how the amino acids are label? You can see that aspartic acid is label and the labeling you are getting from oxaloacetate. So, this one to one reaction mapping is also there, but if you know the labeling of amino acid like aspartic acid then you would be able to know the labeling of oxaloacetate. So, various amino acids are shown over here, you can see that from oxaloacetate isolation, threonine, aspartic acid, asparagine, methionine, lysines are made.

And whereas acetyl coenzyme A, leucine or lysines are made and histidine is made from PPP pathway and then phenylalanine, tyrosines are also more made from PPP pathway. And then to 2-ketoglutarate metabolite is used for synthesis of glutamic acid, glutamine, proline and arginine. So, the proteinogenic amino acids are actually measured in a GC. So, if you want to measure the intercellular amino acid then you have to go for LCMS because intercellular amino acids are not measured in GCMS.

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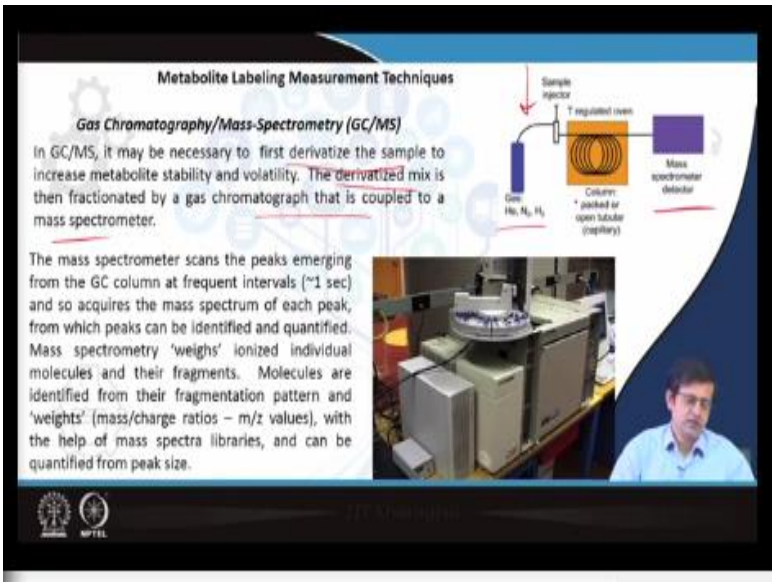

So, because the intercellular amino acid concentrations are very less, whereas proteinogenic amino acid, you can make more protein to get a more proteinogenic amino acid. For that you have to run GCMS for the proteinogenic amino acid, the GCMS apparatus is shown over here, for GCMS you have to derivatize the sample faster. So, what is derivatization and derivatized mix is then fractionated by GC gas chromatography and then it is coupled with mass spectrometer.

Here you can see the GC is coupled with mass spectrometer, where the mass is detected. In GC the fragmented metabolites are separated and mass spectrometer scans the peak emerging from the GC column at frequency interval of 1 second. So, acquires the mass spectrum of each peak for which peak can be identified, you can identify the peak based on the peak size. And the mass spectrometer actually weighs ionized individual molecule and their fragment.

Molecules are identified from the fragmentation pattern and weigh based on m by z ratio with the help of mass spectral library. Because we have a libraries of molecule, whose m by z values are given and based on that you identify that m by z value is actually falling in that category, then you call that this is that metabolite. This scheme is using gas chromatography, mass chromatography in GCMS you have to get the metabolite labeling. And here you can see that in a GCMS we have to supply gas like helium, nitrogen, hydrogen and here is a sample injector.

We inject the sample and then it goes to a column open tubular column or a capillary column. The capillary column allows the sample to go in gaseous phase and then it entered the mass spectrometer.

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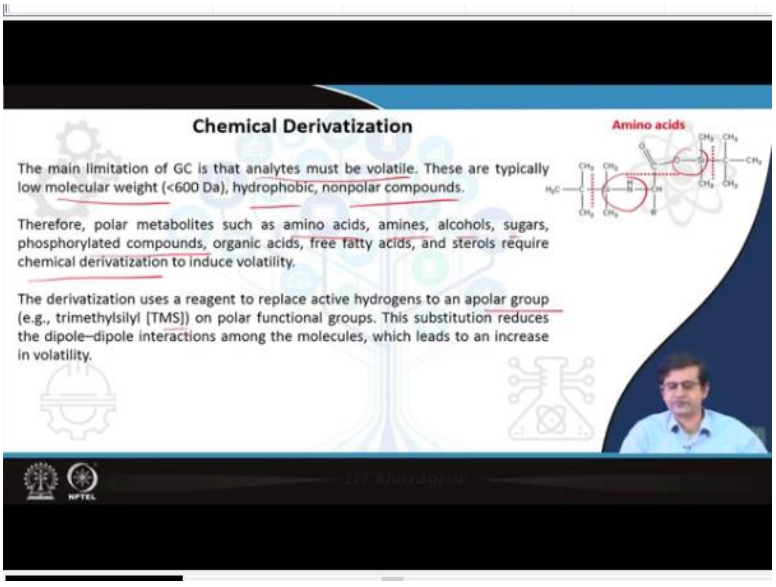

So, if you are using GCMS for especially, generally in flux measurement, you use mostly proteinogenic amino acid that is universally accepted. And the main limitation is that the GC and the analyze must be volatile. So, as I told the gas chromatography only allow the samples to be

measured which are volatile that is they are not polar. So, it should be volatile molecule, these are typically low molecular weight.

So, the volatile molecules are very low molecular hydrophobic non polar compounds. So, but the metabolite are mostly polar. As you know the metabolites are polar and they are hydrophobic, they have higher molecular weight, so they are typically not suitable for GC. So, what do you do then? So, therefore polar metabolite like amino acid, amines, alcohols, sugars, phosphorylated compound, organic acid, free fatty acid, sterols require chemical derivatization to induce volatility. So, what we will do?

We do chemical derivatization; the chemical derivatization uses a reagent to replace the active hydrogen to a apolar group. So, you replace the hydrogen with apolar group like trimethylsilyl TMS on polar functional group. This substitution reduces the dipole-dipole interactions among the molecule, which leads to an increase in volatility. So, this is the amino acid you can see. So, and then amino acid we have the polar group OH and the amine group.

These 2 groups can be replaced; the hydrogen present in this 2 group can be replaced with trimethylsilyl group. So, that it become derivatized. So, the polar groups are removed and more apolar groups are added. So, it becomes more volatile.

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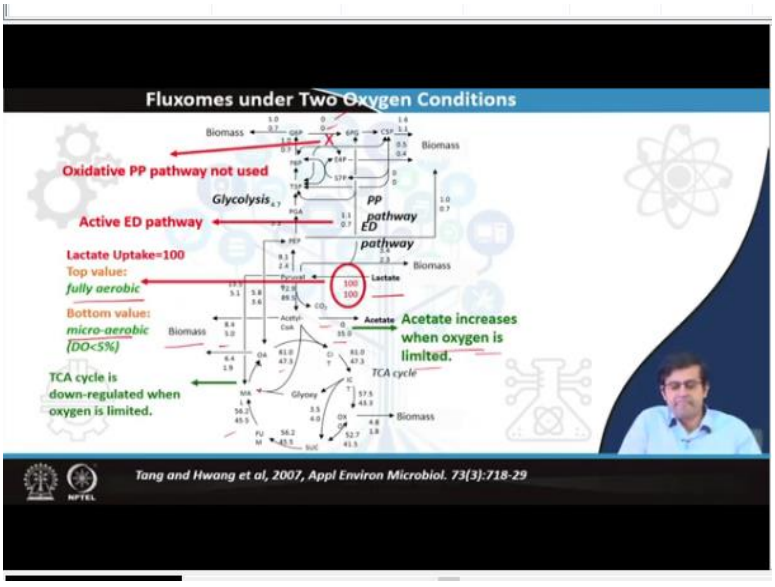

So, this way you can measure metabolic fluxes at 2 different conditions. So, where you can see the metabolic flux has been measured at 2 different conditions, what are the conditions? That is at 2 oxygen condition. So, 2 oxygen conditions is either fully aerobic or micro-aerobic. These are the 2 conditions they have used for measuring the flux and you can see how the flux profiles are changing in presence of different oxygen level.

So, the oxygen level was fully aerobic, where you can see the uptake of lactate, the cell is consuming lactate. So, this organism is consuming lactate which is like hundreds. So, the amount of lactate is going assuming that it is 100 and then what is the flux distribution? So, you can see that the top value is basically for example, the acetate the acetate increases when oxygen is limited.

So, when oxygen was there fully aerobic there is no acetate. So, acetate production is 0 and the moment we have micro-aerobic that is less oxygen, then you can see that acetate has increased to 35 and the organism is salmonella which produces acetate in absence of oxygen. And you can see that the TCA cycle is down-regulated when oxygen is limited. So, the fluxes in-side the TCA cycle see before it was a micro-aerobic it was 61 and then it reduced to 47 and then 56 reduces to 45.

So, all the fluxes in the TCA cycle has come down because the moment you have reduced the oxygen level and this allow for the cell to produce the carbon. The carbon that is less carbon in the TCA cycle that is why this carbon is secreted as acetate from a acetyl coenzyme A, because acetyl coenzyme A carbon flows into TCA cycle and TCA cycle is down-regulated because absence of oxygen.

And that is why acetate production has increased. And also the biomass has reduced, you can see the biomass which was earlier 8.4 it has reduced to 5 unit and then the fluxes are also in the oxidative we can see that flux are not going in PPP pathway. It was 00 right, 00. Now no flux is flowing in the PPP pathway, most of the fluxes are going into in glycolysis and then TCA cycle and then in the biomass formation.

So, this is a 2 condition they have used under 1 oxygen limited condition and another is fully aerobic, but they found that the acetate production has increased when oxygen is limited and also simultaneously the fluxes in the TCS cycle is down-regulated when oxygen has reduced. So, this way you can calculate the fluxes at 2 different conditions where the oxygen is limited and the oxygen is fully aerobic. So, using these 2 conditions you can calculate their 2 different conditions to know how these carbons are flowing in different metabolic pathway.

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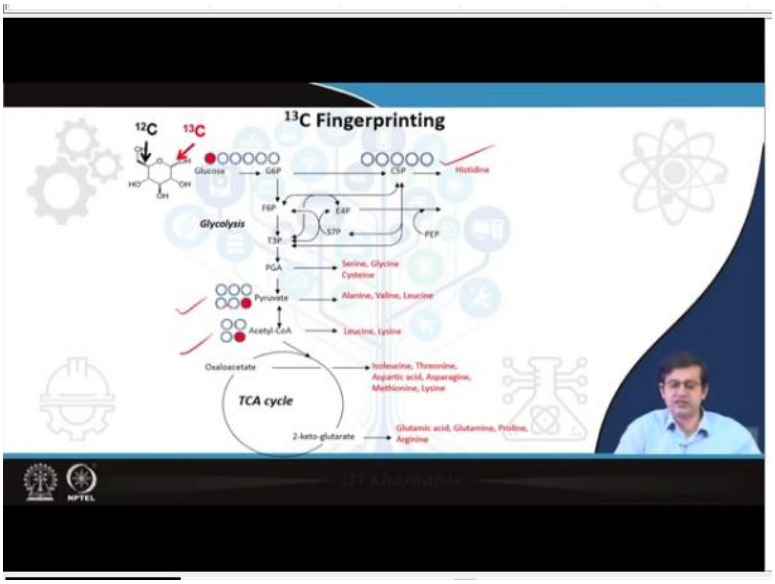

Now we learn about the 13 C fingerprinting. 13 C fingerprinting is a way you can actually identify why the carbon is flowing or you can identify a new metabolic pathway, any reaction which is not identify you are not sure about the metabolic pathway for a new organism then you can do 13 C fingerprinting. So, in 30 C fingerprinting you actually allow the cell to grow with 1 carbon glucose and you can see that when it goes to C 5 pathway that PPP we see none of the carbon cell label because 1 carbon is chopped off.

And you only have 5 carbons, and 5 carbons there is none of the carbon cell label. So, the only label carbon will flowing in the glycolysis and so we see the pyruvate and acetates are label and there is no labeling incorporation in PPP pathway and the labeling incorporation is only there in glycolysis. This way you can characterize different pathway and also know how the carbons are moving.

So, this you can estimate for different amino acid and see whether there is any labeling incorporation or not, if there is no labeling in corporation then you know that the carbon is label, the first carbon is not there is not flowing in that pathway, because only the first carbon is label.

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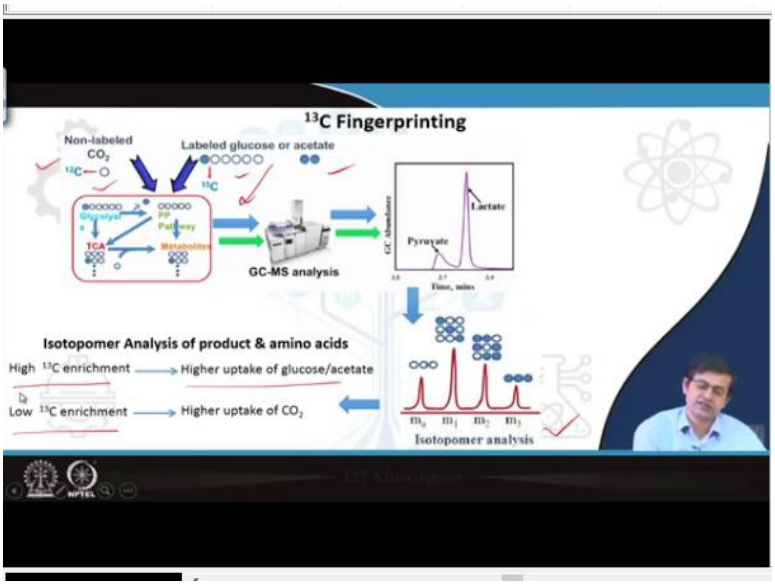

Then this is one more example of 13 C fingerprinting where you can see that we have non labeled glucose, carbon dioxide and label glucose or label acetate. So, in acetate both the carbons are labelled and in glucose is 1 carbon label and it will have a carbon dioxide which is not label at all. So, this allows to cell to grow on the substrate and we measure the labeling pattern in GC.

So, GC absorbance is shown over here and then you can measure the isotopomer m 0, m 1, m 2, m 3 and based on this labeling pattern you can say that the high 13 C enrichment, if the labeling pattern shows that is high 13 C enrichment then higher uptake of glucose and acetate. So, the cell is consuming more glucose or acetate and if there is a low enrichment 13 C enrichment then you can see that there is a lower uptake, there will be lower uptake rate of carbon dioxide.

So, based on the 13 C enrichment we can say that how much carbon is flowing. If you see that there is a higher uptake of glucose and acetate then you can say that the enrichment is more than this pathway is more active and if the enrichment is low than low uptake of CO 2. This way you can actually identify that how different pathways are consuming different substrate?

Here, the substrate is glucose and as well as carbon dioxide, both has carbon. But carbon if it is taking a lot of carbon dioxide then it is a higher uptake of CO 2. So, when the enrichment is very less that means, because carbon dioxide is not label. So, if you are finding that the metabolites have low enrichment of 13 C that means, what carbon dioxide is consumed more.

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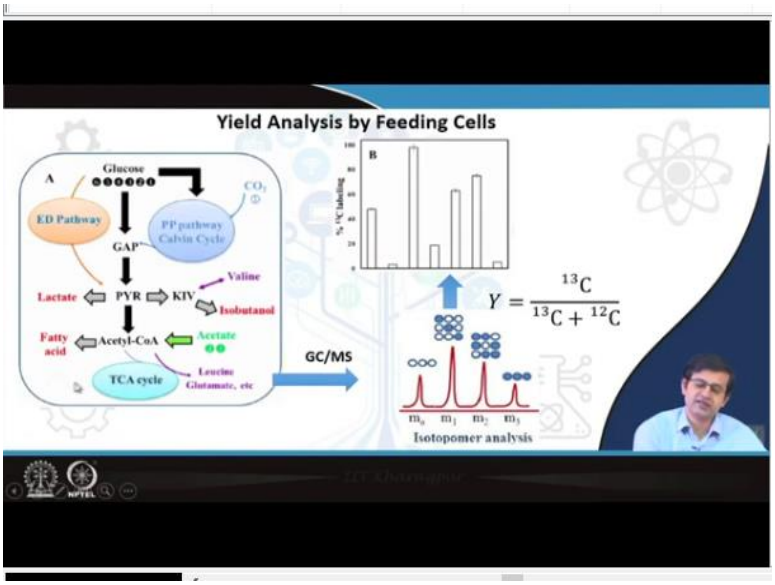

But if the enrichment is more then you can say that the 13C enrichment is more in the metabolites and you can see there the glucose and acetates are consumed more because they are the only metabolite label, the others are not. So, based on the labeling pattern, the high enrichment and low enrichment we can say that which of the subsidies taken more, if the 13 C enrichment is more than the glucose acetate were consume.

If the enrichment is low, then carbon dioxide is consumed more, because carbon dioxide is not label. And then we can also calculate the yield by feeding the cell with label glucose. So, they can actually calculate the isotopomer analysis, step by step calculate the metabolite and try to calculate the yield. Yield is nothing but how much 13 C enrichment the metabolites are making divided by the sum of both the sugar 13C labeled glucose and 12C.

Because, you know how much total carbon is going and how much you are actually getting through labeling experiment. So, these ratios can give you a yield analysis that how much of the

metabolites are forming in terms of label enrichment. These are all you can do by fingerprinting 13C fingerprinting can estimate the yield of different metabolites.

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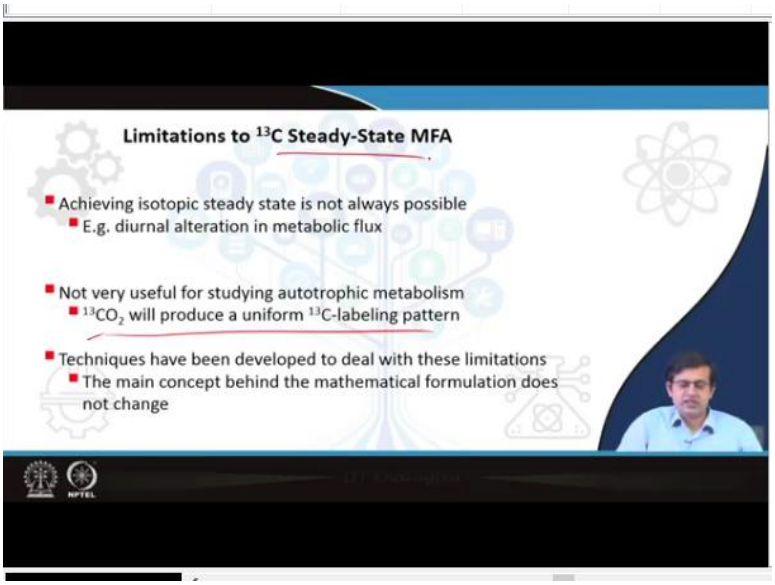

So, far we discuss about the steady state 13 C MFA. For the steady state 13 C MFA, what are the limitations? So, one of the problem is the isotopic steady state is not always possible. So, the steady state 13 C MFA is always the problem is that you have to reach to the steady state. That is, there is no metabolite accumulation, but not all cells will reach that state and that is diurnal alternation in metabolic flux.

And like photosynthetic organism, cyanobacteria is very difficult to reach the steady state not very useful for studying autotrophic metabolism. Autotrophic metabolism for example, is only consuming carbon dioxide. So, it has only one carbon and it will produce uniform labeling. You have seen that you always use 1 carbon label. So, there is a fraction of labeling. So, if you use carbon dioxide, since carbon dioxide is only 1 carbon.

So, if you label them, then all metabolites will be uniformly label. So, then you would not be able to measure the flux. So, because the autotrophic metabolism organism only consuming carbon dioxide, does not consume glucose. So, how do you measure flux for them? For that is the limitation in 13C steady state MFA, that we have to see how we can overcome this problem. Techniques have been developed to deal with these limitations. The main concept behind the

mathematical formulation. So, there are techniques which can deal this situation. So, in this lecture, we are going to deal this problem and how we can overcome this problem.

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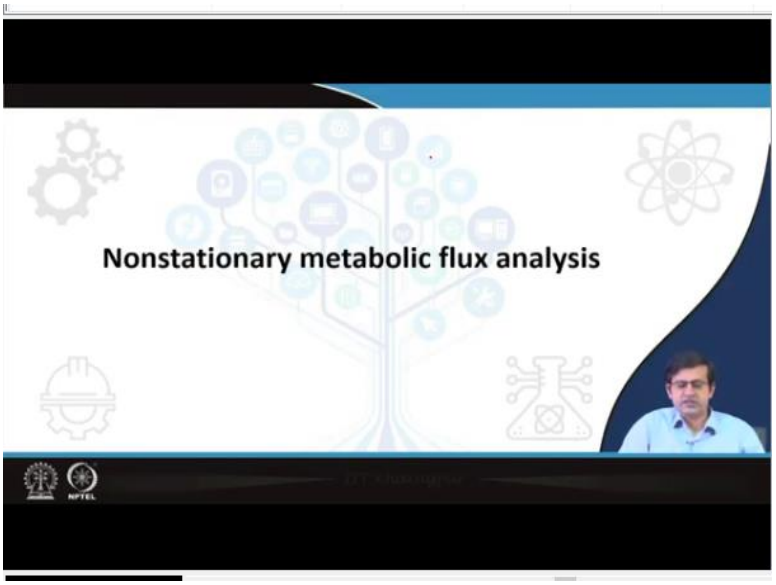

So, this new technique we are going to learn is about the nonstationary metabolic flux analysis. So, all this problem I have discussed can be overcome by using this technique. So, let us discuss about this new technique the nonstationary metabolic flux analysis.

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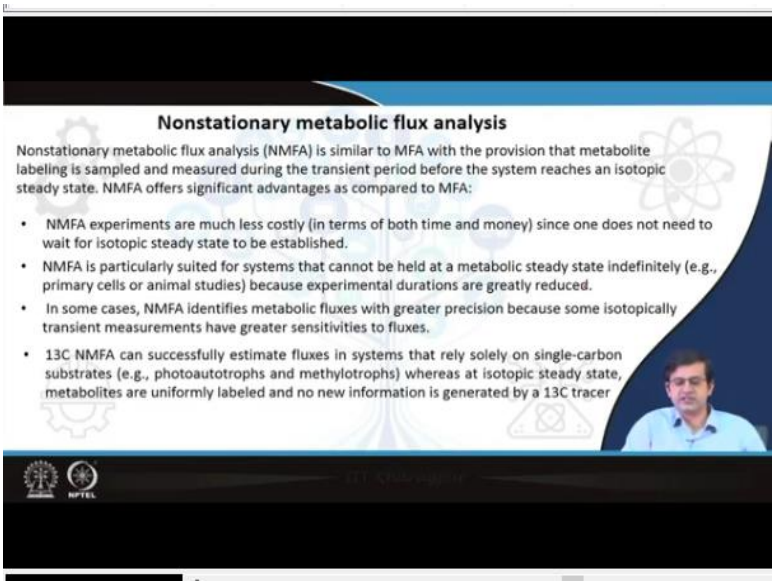

The nonstationary metabolic flux analysis similar to MFA with the prohibition that metabolite labeling is sampled and measured during the transient period before the system reaches a isotopic steady state. So, you do not have to go to isotopic steady state. In the transient period you can

measure the metabolite labeling. So, you sample the metabolite from the culture and NMFA experiments are much less costly, since it does not need to wait for isotopic steady state.

So, you do not have to wait for isotopic steady that is why the experiments are less costly. Nonstationary metabolic flux analysis is particularly suited for system that cannot be held at a steady state indefinitely that is primary cell or animal cells, animal cell studies. So, if you are not able to reach the metabolic steady state for example, the animal cell culture because experimental durations are greatly reduced.

So, in some cases nonstationary MFA identifies metabolic fluxes with greater precision because some isotropically transient measurements have a greater sensitivity to fluxes. So, you are measuring the transient period, in a transient period you are measuring the isotope labeling which has a greater sensitivity to fluxes, this is a good benefit about an NMFA. The 13 C NMFA can successfully estimate fluxes in a system that rely on a single carbon subsidy as I told that the organism only causing carbon dioxide.

The substrate has only one carbon, so if you label that carbon dioxide what will happen? All metabolites will be labelled in a steady state condition. So, it is of no use, that is why nonstationary MFA are very powerful where you can measure the metabolite in a transition period where it has not reached the steady state, if it has reach the steady state and then all metabolites will be label, fully label, because it is a single carbon substrate. So, those problems can be overcome by nonstationary 13 C metabolic flux analysis.

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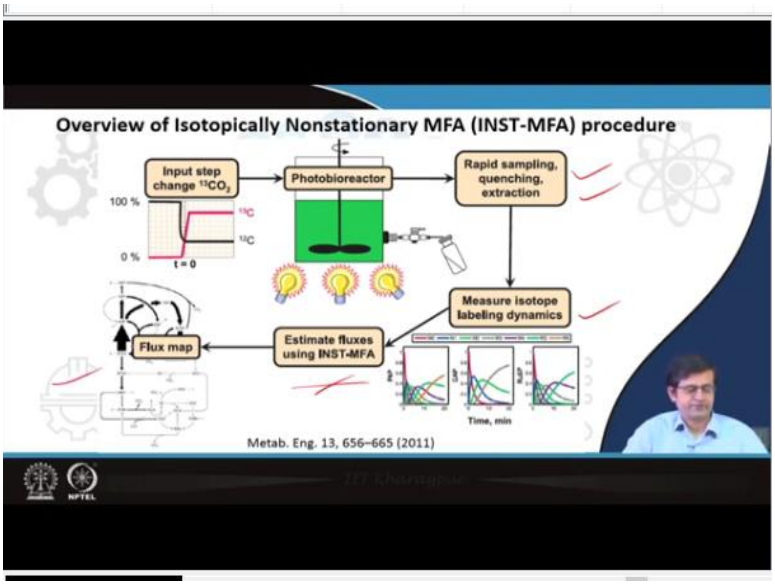

And using this people have study the nonstationary MFA that is INST MFA for photosynthetic organism like cyanobacteria where you can see that the carbon dioxide, they started giving carbon dioxide, and sodium bicarbonate in the form the sodium bicarbonate where carbon dioxide is label using 13 C and then they allow the photobioreactor to go on light and carbon dioxide and then have to do rapid sampling and quenching and extraction.

In rapid sampling, we collect the sample at a frequent time interval before it reaches the steady state and immediately quench the cell so that no more activity remain in the cell and measure the metabolite immediately and after quenching you try to measure the metabolite labeling and estimate the fluxes using isotropically nonstationary MFA, using these methods you can calculate the flux for all the reaction in the central metabolic network.

So, this is the overall procedure where you do not wait for steady state, before steady state you do rapid sampling and quench the cell immediately. So, that no further activity happen inside the cell and measure the isotope labeling using the software. For nonstationary 13 C MFA we have separate software INCA that can be used to measure the fluxes.

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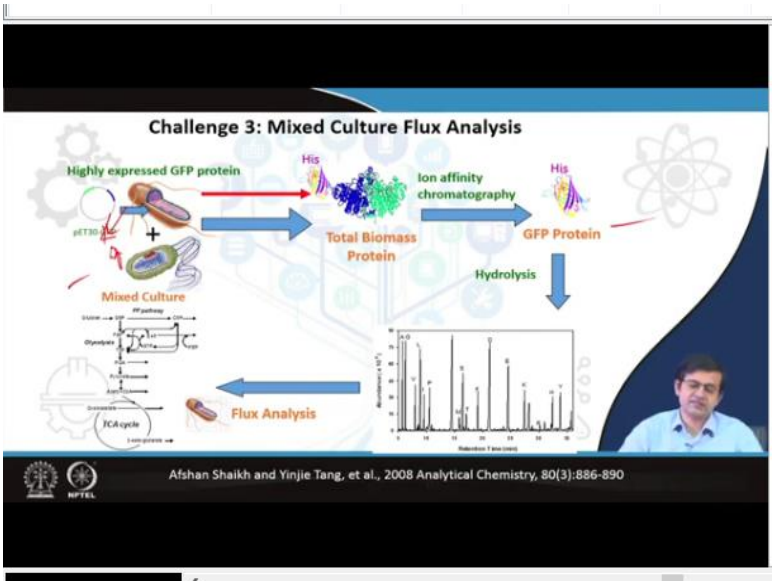

And then we have the other challenge is that they how we can apply 13 C metabolite flux analyses for mixed culture. How do you actually apply for a mixed culture? Because, if you measure the metabolites for entire culture then you do not know the metabolite is coming from this organism or that organism, you are in a confusion if you measure the metabolite labeling.

So, the challenges the metabolite flux analysis for a mixed culture is a problem, just for 2 organism and if you culture then you try to measure the metabolites, for these you lyse the cell, because you cannot separate the cell from the culture, that is the problem. And if you measure the metabolites for the combined culture, then you do not know whether the metabolite has come from this organism or that organism, because it is a common effect, combined effect is observed.

So, this is the major challenge in mixed culture. So, how do you overcome these challenges is also, we will discuss here. So, this problem can be addressed by measuring the protein label. How we can actually measure the protein inside the cell that can be very useful for getting the fluxes. So, for that instead of metabolite if you can measure the protein then you will be able to calculate the fluxes because proteins are made up of amino acid.

So, you can get the amino acid from the protein directly by using a software. So, if you have those amino acids then you can actually put into the same algorithm and calculate the fluxes. The metabolic fluxes can be calculated using the amino acid and those amino acid you can collect it

from the protein because protein has a signature of the organism. So, we know this protein has come from this organism. So, we can separate it out, we can separate the labeling profile.

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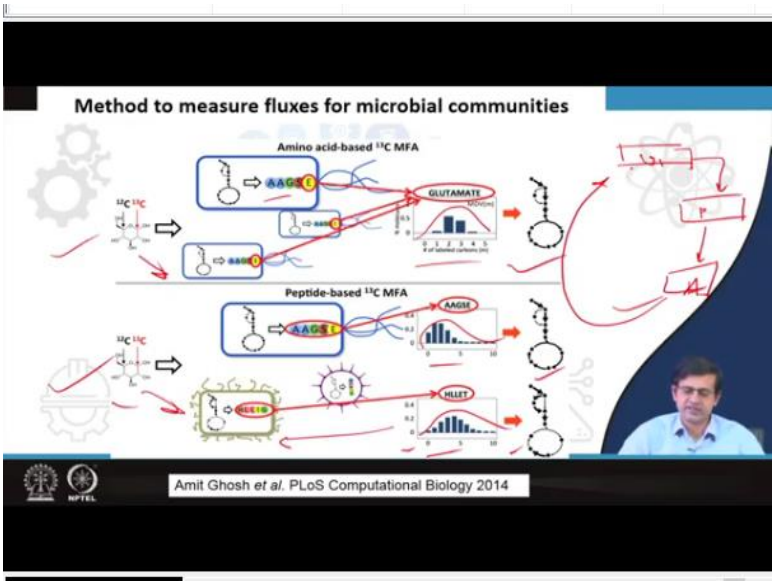

So, these challenges can be overcome by using a small peptide. So, in this algorithm, this is a new algorithm for measuring metabolic fluxes for microbial communities. So, in this culture you have 2 organisms, you can see that there are 2 organism, organism 1 and organism 2 and these 2 organisms consume label glucose. So, both of them consuming label glucose and what you see is, the metabolites are also label.

But if you can extract a peptide or a protein from the cell and then measure the labeling and then you use these peptide labeling for calculating the metabolic fluxes, then it will be organism specific. So, organisms specific metabolic fluxes can be drawn or calculated provided you measure the peptide labeling that is coming from a protein not the metabolic labeling. So, for microbial communities you are not going to measure the metabolite labeling rather than we may measure the peptide labeling.

So, small peptide that is coming from a protein can be measured and you can get a labeling profile like this. So, we get a labeling profile like this, like this. So, using this labeling profile there is a method which can be used to calculate the fluxes. So, we can see that fluxes are

estimated from the peptide labeling, because peptide is a signature of the organism. So, we can actually connect to the organism provided you have unique peptides.

The peptide you are choosing here should not be overlapping with the other organism, it's a unique. So, you have to choose a unique peptide because the measurements are done by you only. So, you have to measure those peptides which are unique to a particular organism. So, that there is no overlap. So, in this way you can measure using a mass spectrometer, proteomics experiment, for this you have to do the proteomics.

So, the proteomics experiment need to be done where you can measure the peptide labeling and using the peptide labeling you will be able to calculate the metabolic flux. Because, the algorithm you know that if you have a flux profile where you start with the flux profile and then you have a peptide labeling and from the peptide labeling, you calculate the amino acid labeling. So, you can deconvolute from the peptide you can decompose the peptide into an acid individual amino acid.

The labeling of peptide can be converted into amino acid labeling and then the remaining algorithm is same. So, from initial flux value you use the peptide labeling and from peptide labeling you get the amino acid labeling and then you compare with the experimental amino acid labeling or the peptide labeling and to try to fit the flux. So, this algorithm is also available where you can calculate the metabolic fluxes for microbial communities where the peptide labeling can be used, because peptide has a signature of the organism.

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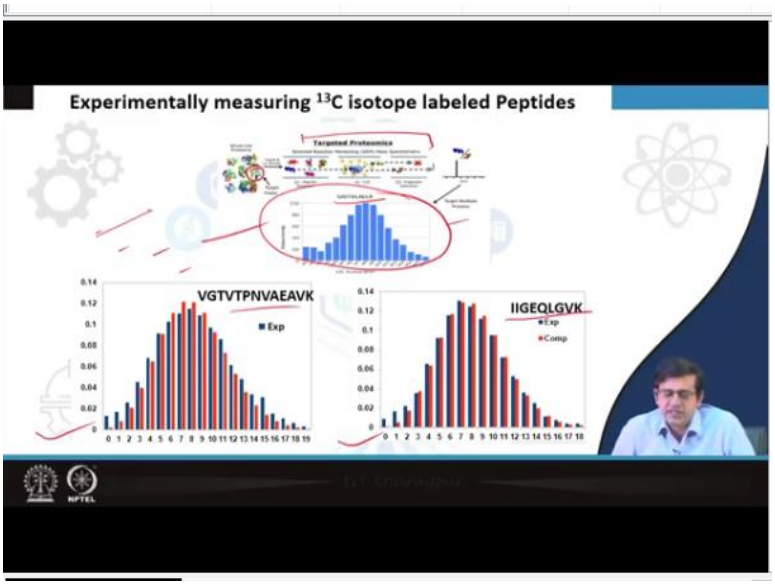

So, experimentally you can measure the 13 C label peptide. So, you can see that in targeted proteomics we have technology right now to measure the protein which are actually you can do trypsin digestion, the protein can be digested with enzyme and then in small, small peptide will be generated. Small, small peptide will be generated and those peptide you can put into mass spectrometer.

So, that you would be able to measure the protein labeling. So, this is the example where the small peptide was labeling has been measured, this is the sequence which is targeted. So, more number of labeling have been measured experimentally and then it is fitted with this is the labeling which was obtained for this peptide and then you can see that this is a fitting.

So, 13 C MFA which is used to fit the experimental labeling and the computational labeling. So, the computationally the algorithm is that we generally compared the amino acid labeling with the experimental amino acid labeling, but here we are comparing the peptide labeling. So, theoretically also you can make the peptide labeling using amino acid labeling. So, using a small mathematical algorithm, you would be able to calculate the peptide labeling and then in compared with the experimental labeling.

So, if the fitting is good and then that is where you stop the calculation and you get the experimentally derived fluxes. The algorithm remains the same just that you are comparing the

peptide labeling instead of amino acid labeling. So, experimentally you can measure the 13 C isotope label peptide because of targeted proteomics where you can measure the small peptide labeling as well and using the labeling profile, you will be able to calculate the fluxes specific to particular peptide.

So, you can use 1 or 2 peptide to calculate the fluxes i.e., 10 peptide, 12 peptide like that you can use to calculate the fluxes.

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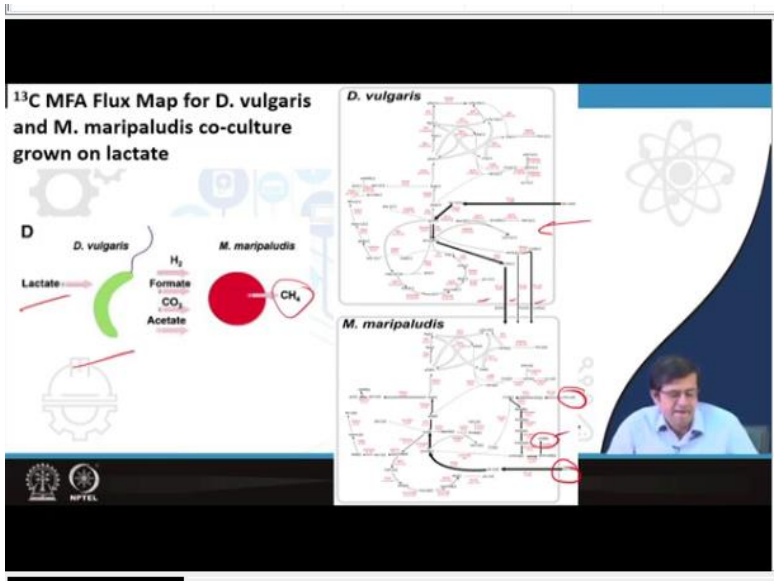

So, the metabolic fluxes have been calculated for a co-culture where you can see D. vulgaris and maripaludis, these 2 organisms co-culture has been the studied where this co-culture is producing methane from lactate. So, here I would like to know the metabolic flux profile for these 2 organism and how the carbons are flowing from. Initially the D. vulgaris is actually growing on lactate and you can see the lactate is going in and it is forming acetate, formate and carbon dioxide.

So, these are the metabolites it is forming by the first organism and these products are input for the second organism. The second organism the Methanococcus, which is methanogenic organism which convert the formate carbon dioxide into methane. So, methane is produced, it consume formate and also it will consume carbon dioxide and how methane is produced by methanogenic

bacteria and you can see how the carbons are flowing in different organisms are shown over here.

So, this is the example where the flux analysis have been done on the peptide labeling. So, here you have measured the peptide for these 2 organisms and using this peptide labeling you can calculate the flux profile for these 2 organisms individually because the peptide labeling you use instead of amino acid labeling. That is the difference that instead of peptide, the amino acids have been used for normal 13 C MFA.

But for co-culture you have to use the peptide labeling, the labeling incorporation which you get it from culture because it is a mixed culture, you would not be able to separate the metabolite that is why the peptide labeling are used, because peptides are basically signature of the organism. Here you can see the methane is produced and formate which is consumed here, the formate and then we have acetate.

So, we have acetate, formate and carbon dioxide, these are the carbon sources, which are consumed by the methanogenic organism which consume this carbon and produce methane gas.

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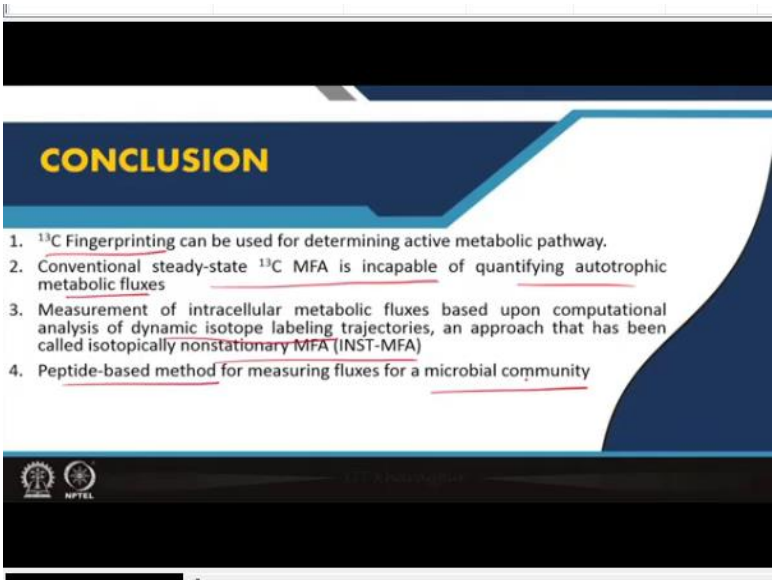

So, in conclusion, we learned about 13 C fingerprinting is a new technique where you can identify which of the metabolic pathway is active. We have given the example of how the cell is

going on carbon dioxide and glucose acetate. If the carbon enrichment is more then you say that the cell is growing more on acetate. So, this kind of study like the organism which have multiple substrate, suppose the organism consuming 2, 3 substrate.

Now, we do not know which substrate it is consuming more, in that case 13 C fingerprinting is very powerful, because we do not know what kind of substrate the organism is picking up and which one is more. So, those kind of experiment you can do why 13 C fingerprinting for a unknown organism, that can be efficiently be characterized by 13 C fingerprinting.

And also we saw the conventional 13 C MFA is incapable of quantifying autotrophic metabolic fluxes, the autotrophic metabolic fluxes because the autotrophs they consume this carbon dioxide which has only one carbon. So, those organisms, the conventional steady state MFA is not useful, they will not be able to calculate the fluxes, that is why measurement of intercellular metabolic fluxes based on dynamic isotope labeling.

So, using dynamic isotope labeling the nonstationary 13 C MFA are more powerful, where you can measure the fluxes by measuring the metabolized in transient period, that you can measure the metabolite labeling and you can calculate the fluxes before it reaches the steady state. So, you are not allowing the cell to go to the steady state before the cell reaching this steady state you will be able to measure the metabolite the isotope labeling trajectory can be measured and you will be able to calculate the fluxes.

And also we learned about peptide based method for microbial community, the flux analysis for measuring fluxes. Microbial community you can measure the flux by identifying the peptide labeling and using the peptide labeling you will be able to calculate the fluxes.

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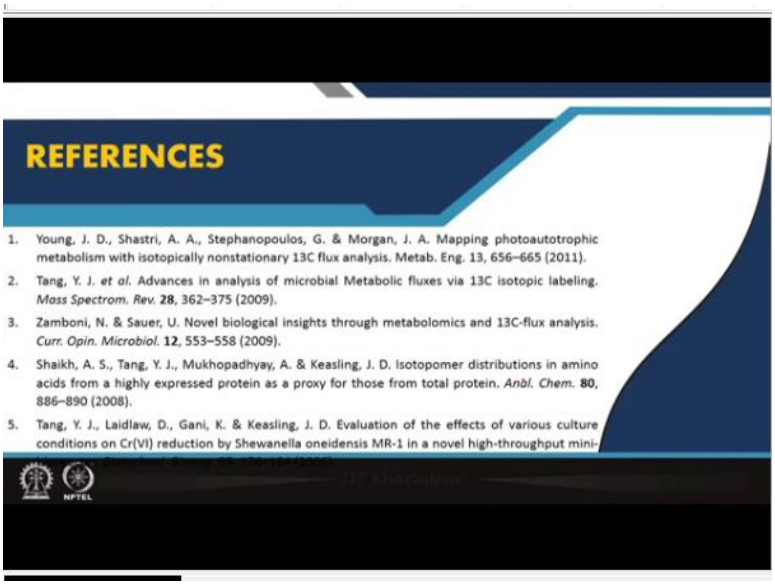

So, these are the reference mainly the nonstationary 13 C MFA, if you want to read more you can read this paper. And more information about steady state 13 C metabolic flux analysis are also given over here. You can read more about this. Thank you. Thank you for listening. So, we will meet on next lecture. Thank you.