**Bioreactors**
**Prof G. K. Suraishkumar**
**Department of Biotechnology**
**Indian Institute of Technology, Madras**

**Lecture – 09**
**Measurement principles and methods**

Welcome to lecture-9 on the course on Bioreactors under the NPTEL online certification program. Let us, in lecture-8, we looked at the solution to the practice problem 2.2. We are in module-2, which looks at the two major outcomes from bioreactors, the biomass and the products and some enzyme related aspects, the products from enzyme reactions. Let us move forward in that module, most likely we would complete the module in this lecture.

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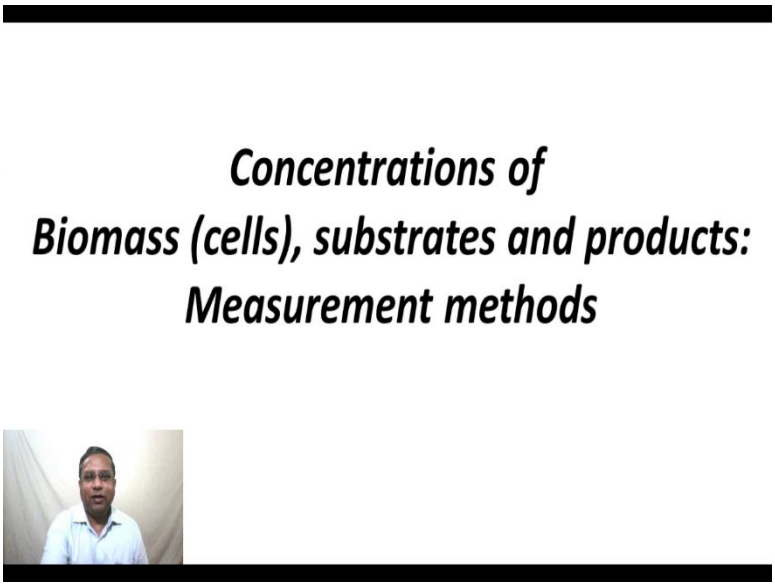


The last aspect that we are going to see in this lecture is the concentrations of biomass or cells, substrates and products, the measurement methods. We saw how crucial the information on biomass, concentration-substrate concentration and product concentrations were in terms of kinetics, in turn the design and operation of relevant reactors. Therefore, we need to have good measurement methods to find out the

concentrations of biomass, cells, the cells or biomass, substrates and products. We would look at some of the common measurement methods that are used for these.

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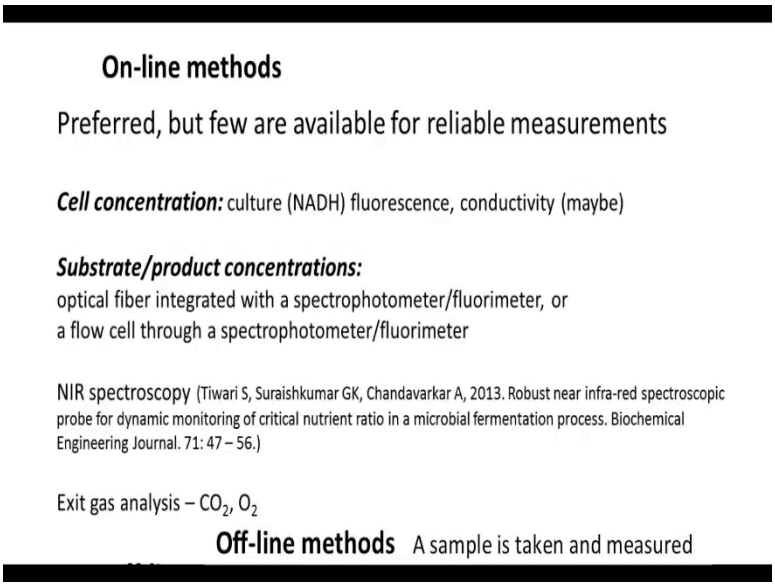


There are two kinds of methods, one on-line and the other off-line. On-line means that the bioreactor is not disturbed; it is measured on-line as the process goes on without any disturbance. Off-line, we take a sample from bioreactors and then measure it away from the line or away from the bioreactor, and that is why it is called off-line method. We will look at some on-line methods; they are of course, preferred because they are measured at the point of interest without disturbing the bioreactor. So, the best view or the best information comes from on-line methods, but few are available for reliable measurements and that becomes the difficulty with on-line methods. For example, if we look at measuring cell concentration, there are a few on-line methods; there is fluorescence method that measures the fluorescence of a molecule inside the cell that is called NADH. You all know NADH–nicotinamide adenine dinucleotide, the hydrogenated form of it. NADH is fluorescent, N A D is not and the level of NADH is a direct indication of the cell concentration, higher the NADH fluorescence higher the cell concentration. The relationship is known, has been established.

So, you could follow the increase in cell concentration through the NADH fluorescence or it is also called the culture fluorescence, the NADH fluorescence change. What is typically done is, the fluorescence measurement probe is placed in special well in the bioreactor, this is an optical probe. Before that I need to tell you that NADH, the optimal wave length pair for NADH is let say 340-460 nanometres. So, the excitation wavelength needs to commit at 340 nanometres, the emission wavelength needs to be measured at 460 nanometres. In a spectrofluorimeter, you have a nice cuvette or a some other well kind of a measurement. You have a controlled way of sending in 340 and measuring it at 460. Whereas in the bioreactor, typically what is done is a fibre optic probe is interfaced with a spectra fluorimeter. The fibre optic bundle, the some bundles carry the excitation wavelength from the spectra fluorimeter, and projected in an open ended geometry into the bioreactor. And then the emission wavelength at 460 is collected through the same fibre optic probe taken back to the spectra fluorimeter and measured, this is called an open-ended geometry of measurement. Well, those are the details, the main point here is that the cell concentration can be measured by following the culture fluorescence or the NADH fluorescence.

The conductivity of cells can also be measured; there are some probes for it; the reliability not so sure may be, that can also be used it is an on-line method. For the substrate and product concentrations, there are a few on-line methods. As I mentioned earlier, the optical fibre is integrated with a spectrophotometer or a fluorimeter or you could take a sample from the bioreactor as it is operating, but bring the sample back into the bioreactor after it flows through what is called a flow cell which is placed in the fluorimeter for measurement. This is another way of doing that. You could use absorbance fluorescence for some substrate, some products.

What is recently of interest, it is what is called near infra-red spectroscopic. In fact, we have paper on this, which was the work was a done by Sanjay Tiwari; it was developed as a part of Ph.D thesis and was also applied in the industry. The advantage here, let me talk of this as specific case, so that you will understand the advantage. Here this industry was a looking at using low levels of substrate, because the substrate was toxic to the cells for their one product - commercial product. So, the substrate needs to be maintained at a certain low concentration, now the order of about millimolar and so on. I think it is about

2.5 to 5 millimolar. So, if substrate concentrations need to be measured, need to kept at a low level and also the product concentration needs to be maintained at a certain level otherwise, it would become toxic. So, let us concentrate on the product here.

The older method that was followed was something like this. They would take a sample from the bioreactor, this is the production reactor. The measurement such as hplc and so on are available in the lab which is at a distance from the production unit, the production unit is large. Therefore, they need to take a sample from here, come there, measure it, it takes time for measurement may be about 20 - 25 minutes, and then bring it back. By that time, the product concentration would have gone up and they would have lost the batch, it would have become toxic to the cells, and they would have lost the batch. So, to avoid that, they definitely needed an on-line measurement, which would immediately tell them what the concentration was so that they could take an appropriate action to maintain the product levels at acceptable levels. So, to do that Sanjay and I had worked on NIR spectroscopy and developed a method for doing that and that obviously was very beneficial to the industry. That is a paper there, it was published in Biochemical Engineering Journal, if you are interested you can take a look at that.

Also the gas that comes out of the bioreactor, now we provide air for most aerobic bioreactor systems, the exit gas which consists of CO2 and O2 and of course, nitrogen and so on, that can also be measured to provide some input into what is happening in the bioreactor. So, those are the available on-line methods, a few are available, but off-line are the most used, although there are difficulties with off-line system such as the one that we talked about just now.

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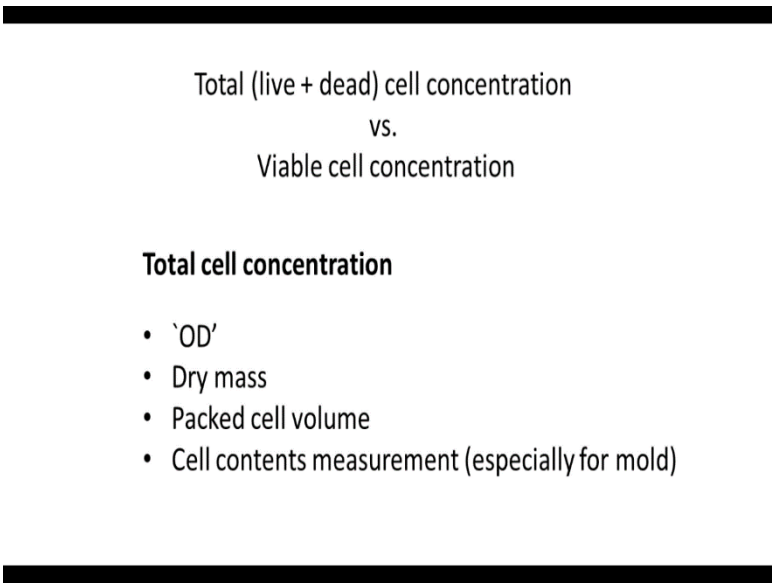


Let us look at off-line measurements. Let us look at measuring the total cell concentration. Take one at a time, I think that is okay. The total cell concentration, if you are considering a bunch of cells, consists of both live cells and dead cells. If we look at live cells alone, because the viable cells are the ones that are contributing to product formation and so on, we would want to follow the viable cell concentration. Sometimes the viability percentages are very high 97, 98 percent, that it does not really make a difference whether you follow viable cell concentration or total cell concentration. But there is a difference in principle and it can come in, it can turn out to be important, the difference can turn out to be important. So, you need to know that there is a total cell concentration measurement and a viable cell concentration measurement.

Let us first look at the methods to measure total cell concentration. The first one is what is called OD or optical density; OD stands for optical density. It is a historic term; it does not represent what it measures, but we will talk about it. You could also measure dry mass, take sample - large enough sample, dry it out completely and then measure the mass. That is the, you know the mass and that mass came from a certain volume, therefore mass of the cells by the volume gives you the total cell concentration. You could also use something called packed cell volume. This is the volume that is occupied by the cells in a rather concentrated solution of cells, typically in the industry. So, this is

the packed cell volume, is typically used in some industries as a measure of the total cell concentration. The fraction of volume that is occupied by the cells or you could also use cell contents measurement especially for molds, we said if you remember we said the molds were organisms that grow by extension of hyphae. So, the number of cells may not be an indicator, it's just one cell, but increasing in mass through extension of hyphae. For such kind of organisms we measured the contents of the cell. So, those are the methods for total cell concentration measurement.

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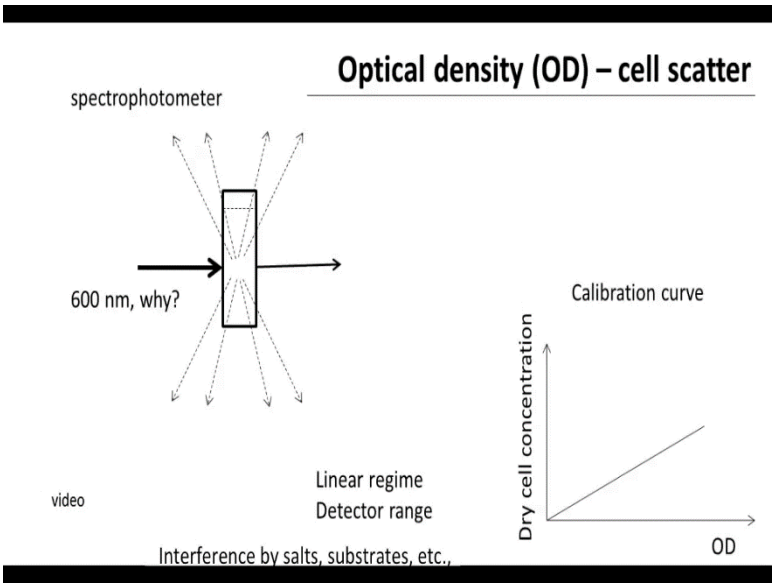


Let us see the principles one by one. The optical density, the so called optical density is measured by using a spectrophotometer, but it is actually a measure of cell scatter, it is not a measure of absorbance by the cell. This is something that you need to remember very clearly. We have cuvette here that is place let us say that the cuvette has a cell suspension here, fill till the measurement level, then there is a certain wave length that is shown on the cuvette. The cells, you know they are micron sized, so the mie scattering range comes into effect. They scatter and whatever light is not scattered is sent to the detector that is measured, so that is measured and related to the incoming light to get the extent of light that has been scattered. The light that has been scattered is proportional to the concentration of cells. Make this difference very clearly set in your minds; this is cell scatter and not cell absorbance.

Although the same instrument is used and something called optical density the term is used to which is a complete misnomer, but any way in terms of the principle is the misnomer it is a well-accepted term, but keep this in mind it is a measurement of scattering by the cells. And the cells scatter is, the amount scattered is proportional to the cell concentration. That is the reason why we used about 600 nanometres. The principle is scatter; the scattering occurs maximally when things are not, when the wavelengths are not absorbed by the cell. So, we would like to work with wavelengths that are not absorbed by the cell. So, anything in the range of 500 to 600 is actually fine. In fact, I have even used 450 in certain cases so that is perfectly fine, because you are measuring the extent of light scattered. Six hundred is a very typical number you could use that, anything between 500 and 600 is perfectly fine.

Then what is done is using a calibration curve between the dry cell concentrations. Remember we did talk about dry cell concentration measurement, you take a large enough samples, you dried out the cells completely using a vacuum oven over night and then you measure the mass of cells that was present in that particular volume, therefore you have a dry cell concentration. Before you are going to dry that you take a sample and measure the OD and thereby you can relate dry cell concentrations, various values of them to the OD, and you would get a straight line in a certain range and that is the calibration curve. You could measure OD and get the relevant dry cell concentration using this calibration curve, and that is what is typically done.

The linear regime is important while measuring, you need to pay attention to that. The linearity, there are two aspects there. If you plot cell dry cell concentration versus OD, it is going to be linear till a certain point, and above a certain dry cell concentration, the linearity is going to break down, that is a law of nature. So, it is going to be linear here and above a certain dry cell concentration, it is actually going to saturate. We should not operate in this region, of course we measure, but we limit our range of operation to this linear region. In other words, we need to measure only in this linear region, if it happens to be in a higher OD region, we should actually dilute the sample to get the OD reading in this range and then get the dry cell concentration corresponding to the measured OD and multiplied by the dilution factor to get the actual OD. If you follow all this, it is fine;

otherwise just keep this in mind whenever there is a need for measurement, you go through this in detail or look at some reference book. You can always ask me too.

There is another reason for the loss of linearity that is because of the detector range. If the amount of light reaching the detector goes below a certain level or if the amount of light that is scattered is very high, then the detector saturation happens, then also there is a problem. So, we do not work in the region of detector saturation. Depending on the spectrophotometer, I would feel very comfortable working only with OD values say between 0.1 and about one or so, that way I am pretty much in the linear regime and in the normal detector region. Of course, this depends on the spectrometer, the maximum value and so on and so forth.

You need to worry about, in during this measurement, interference by salts and substrates also, because we are taking a sample here, drying out the whole thing. When we dry out, whatever is soluble in the broth is going to contribute to the mass measurement. So, the mass would contain, the mass that is measured would contain dry cells plus the salts plus unutilised substrate plus the other things. So, we need to have an appropriate control by which we can account for the mass of salts and substrates and so on, so that we need to take into account while measuring OD. This is a finer point, I just thought I will mention this here during the measurement.

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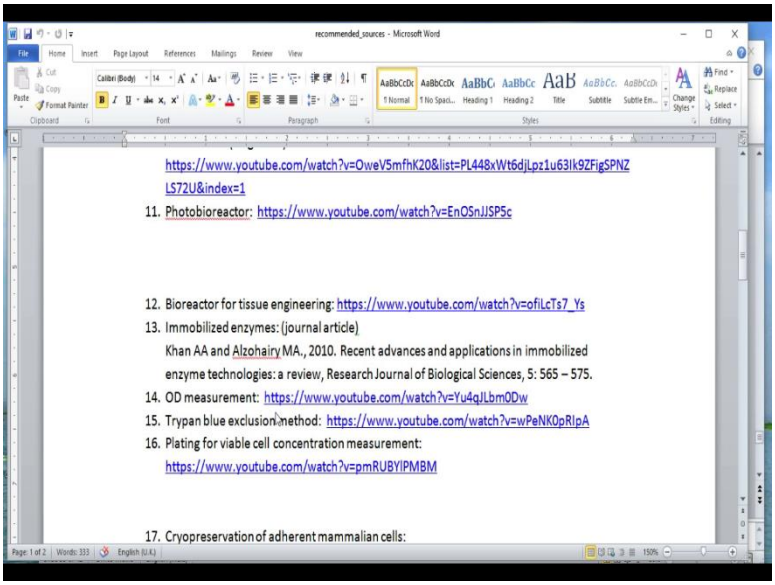


There is a video here let me show you the link, which can give you some more details. You may want to take a look at; it is what is given in number 14 OD measurement, you can go and take a look at this video.

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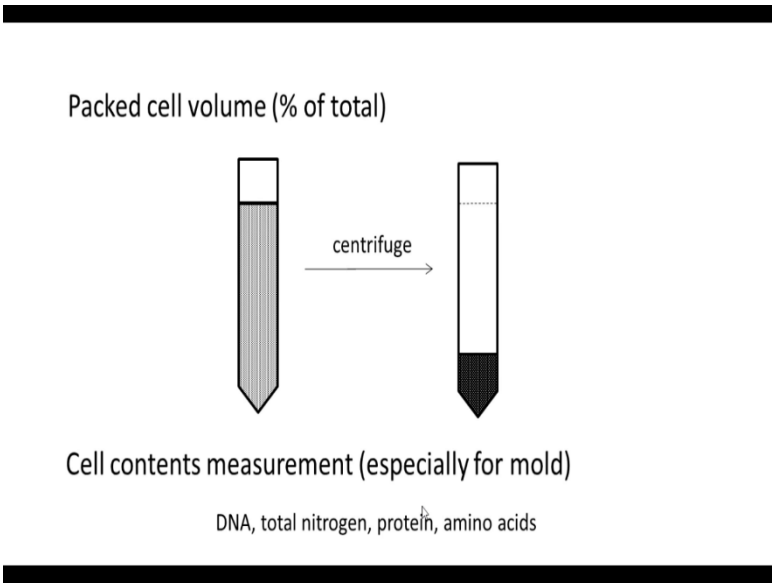

Let us move forward. The packed cell volume, the percent of total, this we mentioned earlier. You know if this is going to be a cell suspension, you spin it down using a centrifuge, and the cells are going to occupy this volume here, the total volume is this. So, this volume by the total volume is going to give you the percentage packed cell volume. This is used in the industry. The next is cell contents measurement, especially for mold which grows by extension of hyphae not by division of cells. The amount of DNA, the amount of total nitrogen, the amount of protein, the amount of amino acid in cells could be measured. And if we assume that the percentage DNA per cell does not vary too much, then this becomes a direct measure of the cell concentration itself. This is the basis for the measurement of cell concentration through the measurement of its contents. The typical contents that are measured are DNA, total nitrogen, protein and amino acids in that order of importance. DNA is usually preferred because it does not vary too much during the various parts of cell cycle and so on, before it splits up ofcourse. So, total nitrogen could be measured, protein could also be measured, but there would be variations; amino acids there are some variations, but these are all accepted methods for cell contents measurement. Those were the methods for measuring total cell concentration.

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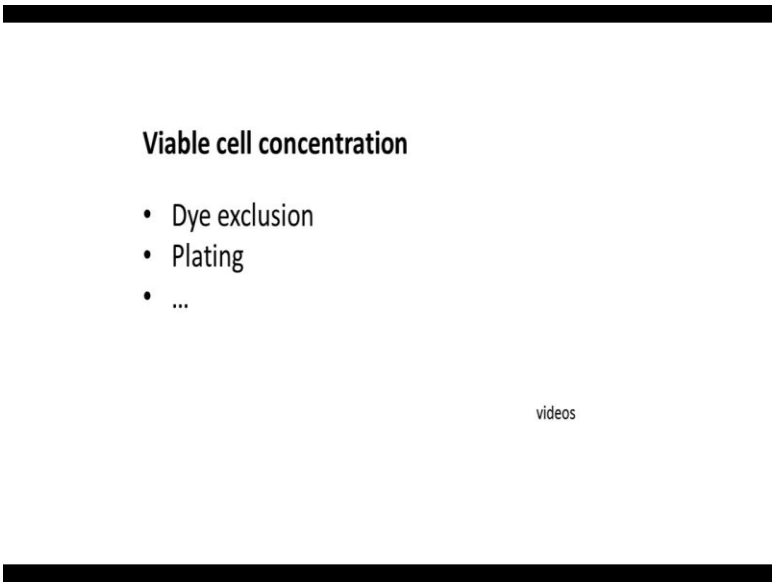

Now let us look at the methods for measuring the concentration of cells that are live, that are viable, if the viable cells are the once that are going to actually contribute to the functioning of the bioreactor. The methods are dye exclusion, plating and others; we will just look at these two methods. There are videos here, before I get into the videos, let me just tell you the principle of dye exclusion and plating. Dye exclusion means or is based on the principle that certain dyes which can be visualized are excluded by living cells. So, in the presence of dye, the cell is going to appear un- dyed, you know uncoloured. So, by counting the number of cells that are not coloured and by counting the total of cells, you can have percentage viability or the viable cell concentration itself from direct measurements. There are slides specialized slides that can be used for these measurements. They are called neaubauer chambers, haemocytometers as they are called and there are ways by which you could reliably measure the number of cells in a particular volume and therefore, the cell concentration. That was dye exclusion method principle.

The plating principle is that you have an agar plate - a solid medium agar, it contains agar. And then you take a certain low concentration of cells and plate them here, and assuming, when you plate them here, the cells are going to multiply and when they multiply to a certain extent, the colonies that arise from each cell become visible, you know one cell becomes two cells become four cells, eight cells and so on, as and as long as there are let’s say millions of cells that would form a colony. And at that stage, the colonies become visible to the naked eye and you can count the number of colonies. Assuming that each colony arises from a single cell, you could count the number of colonies and relate them to the number of cells that were there at the time when the plating was done. These methods take time, rather the plating method takes time. You will have to keep it overnight and so on even for bacteria which grow fast, but that is a method that can be used because colonies will arise only from live cells. So, as long as you make sure that the cell concentration is measurable by counting the number of colonies you could use this.

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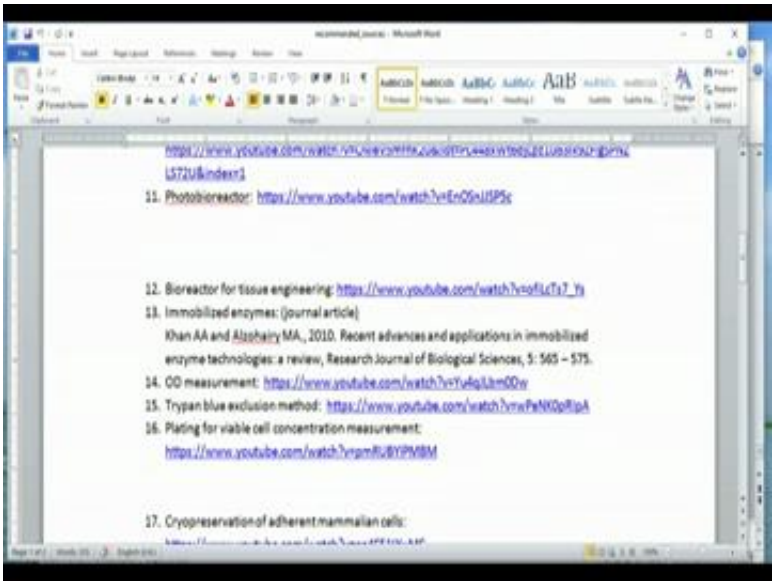


The videos here let me show you the details of the videos. You may want to look at them for the trypan, the number fifteen for the trypan blue exclusion method. And sixteen for plating for viable cell concentration measurement; these are nice videos that you can look at for the details of the measurement.

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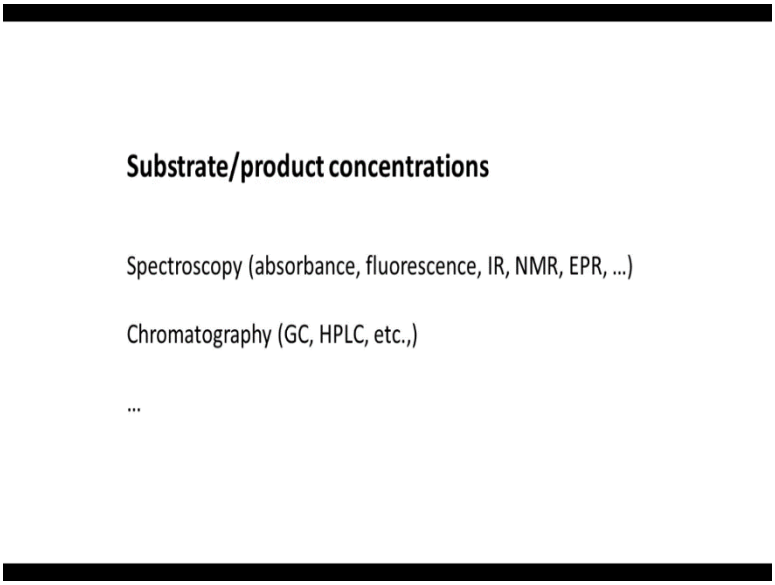

For the substrate and product concentrations, spectroscopy, many kinds of spectroscopies can be used; absorbance spectroscopy which we normally know, the absorbance at a particular wavelength which is particular for that molecule is used to measure the concentration. Fluorescence, we looked at NADH fluorescence and so on; they could be fluorescence substrates, fluorescence products that can utilise this method. IR spectroscopy – infra-red spectroscopy, we did talk of something related to that near IR. NMR - nuclear magnetic resonance spectroscopy, EPR - electron paramagnetic resonance spectroscopy can all be used. Spectroscopic methods can be used for off-line measurement of concentrations of substrates and products. Chromatography, chromatographic methods can be used - gas chromatography, high pressure liquid chromatography – HPLC and so on they can be used. And there are many such methods that can be effectively used.

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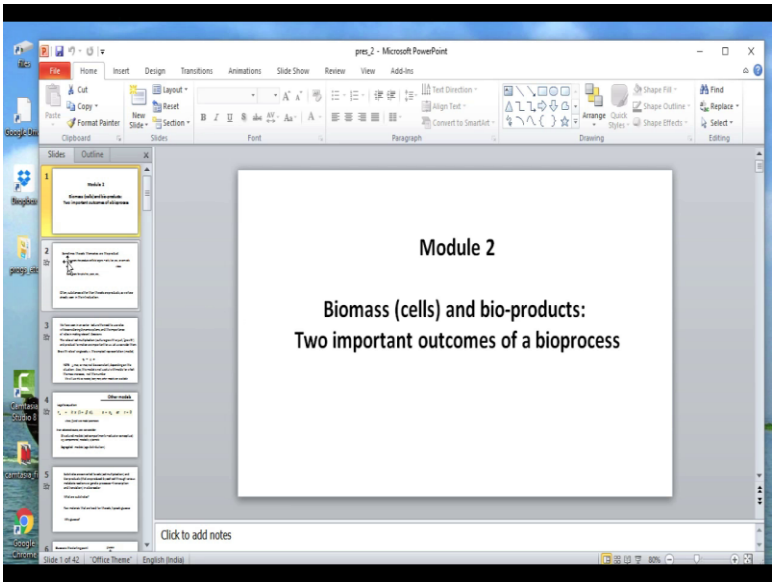


I think we have come to the end of a module 2. Very briefly summarizing what we did in module 2. We looked at biomass and bio-products as two important outcomes of a bio process, that is, that was the main theme of module 2. We looked at the details of these products and then the models for the rate of cell formation, simple models as well as some detail models.

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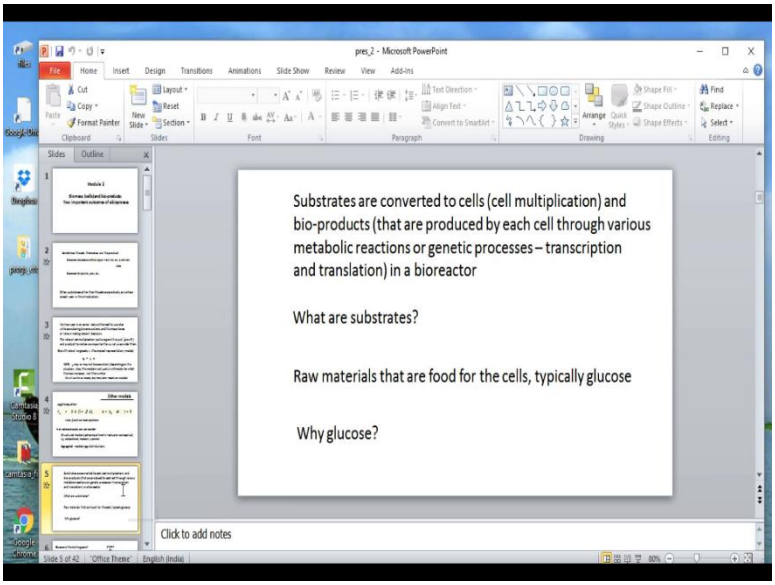


And then how, what are substrates, and how they are converted and how the substrate affects the growth rate, we had seen that, some models for that.

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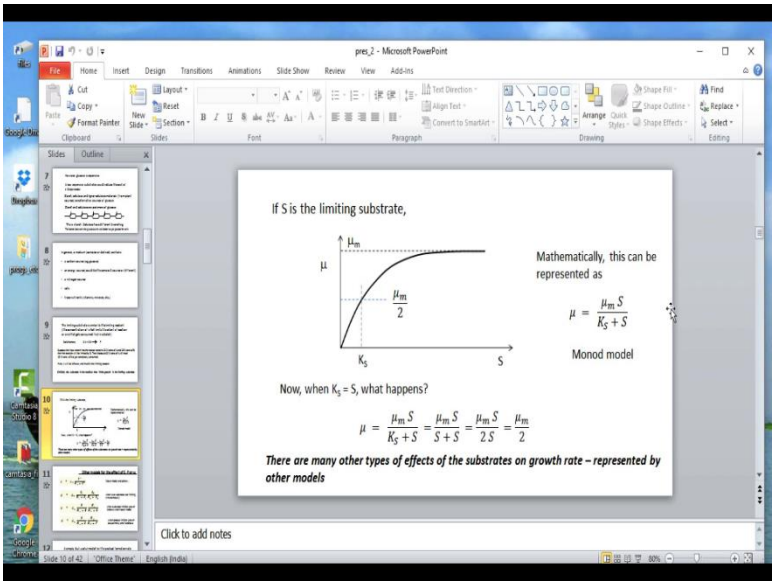


And also when we looked at that we showed that the Monod model gives us the effect of substrate on growth rate, that is the simplest representation.

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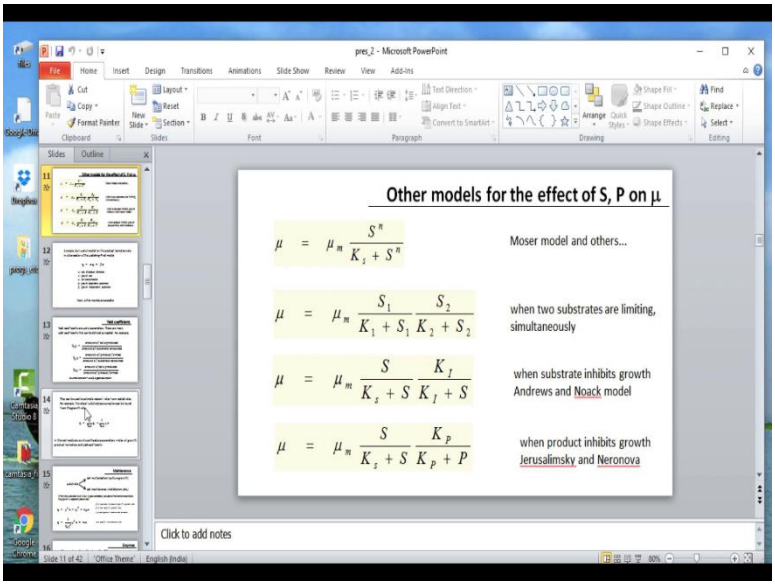


There are other representations that are given here.

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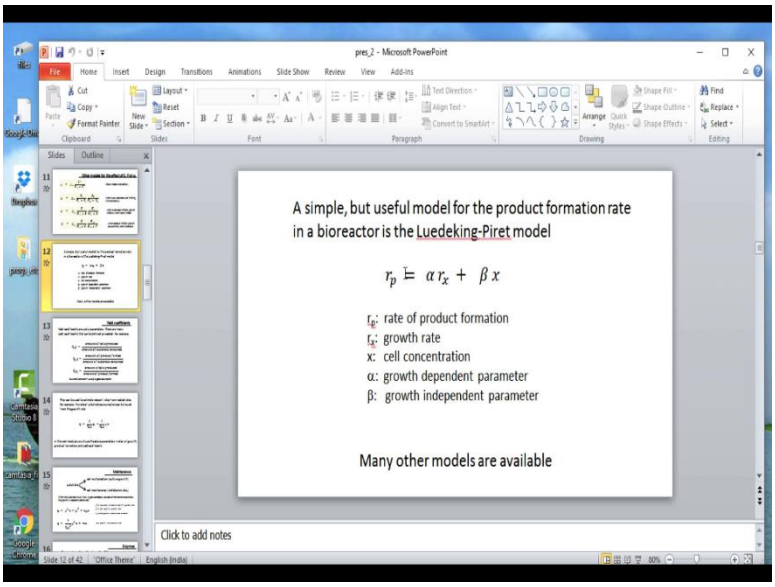


And then for the product formation, we have the Luedeking-Piret model,

$$r_p = \alpha r_x + \beta x$$

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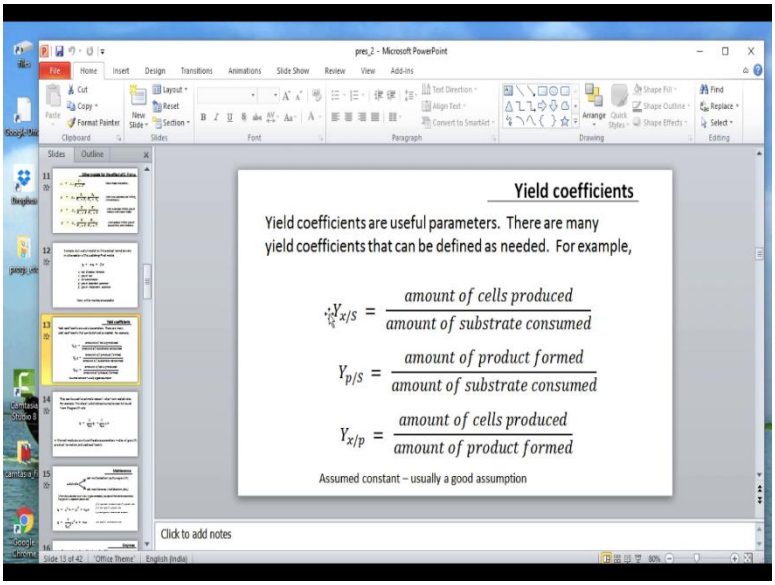


Then we talked about a few concepts such as yield coefficients and maintenance.

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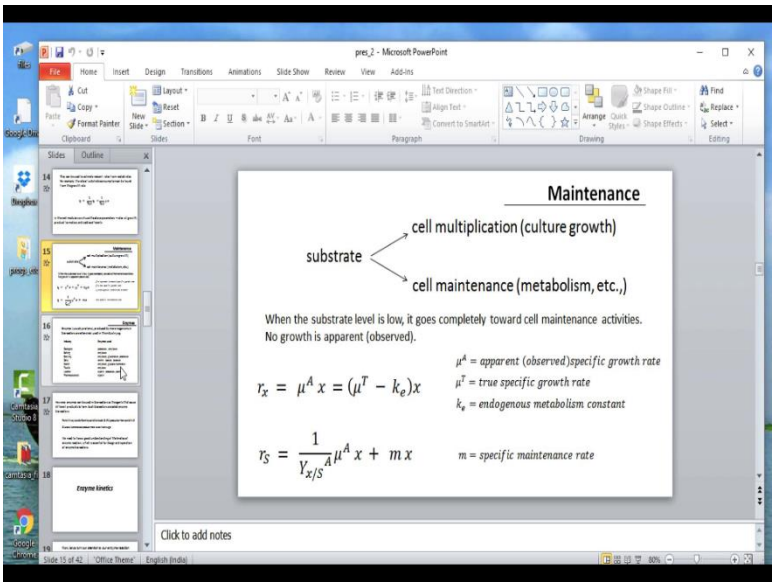

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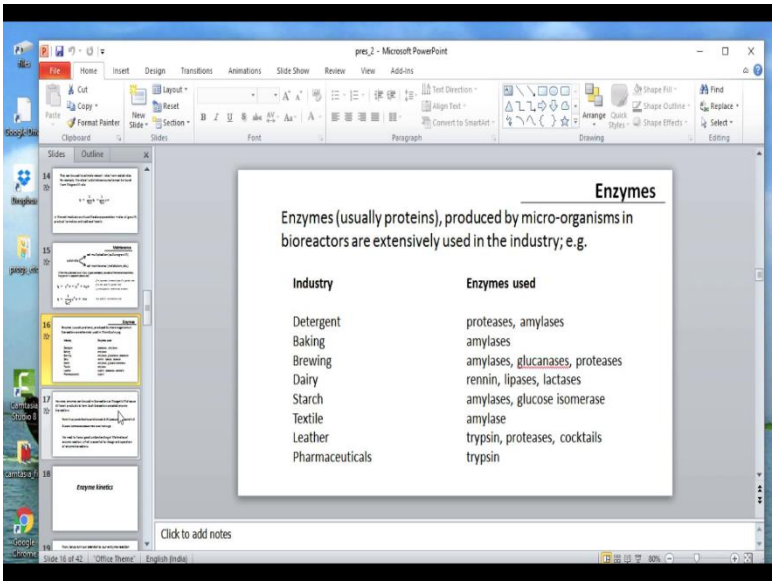


Then we looked at enzymes, some enzymes that are used in the industry; enzymes that can be used for bioconversions in a bio reactor. And we said that we need to know the kinetics of the enzymes. We looked at Michaelis-Menten kinetics in detail we went through the derivation. Then we also looked at inhibition kinetics, the competitive, non-competitive and the uncompetitive inhibition kinetics.

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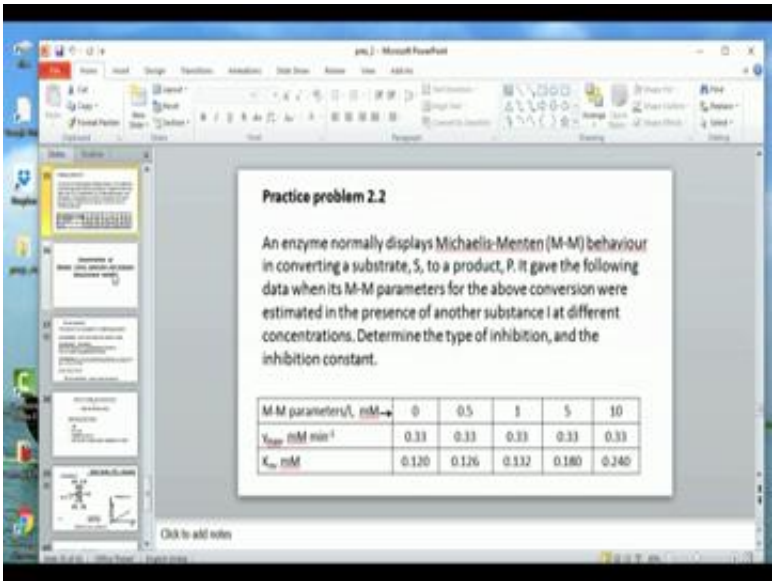

We worked out a couple of problems based on those.

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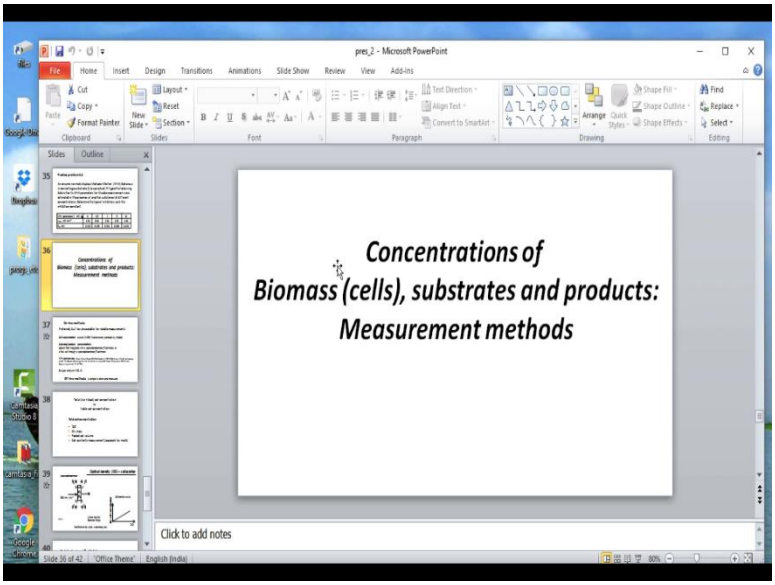


And in this lecture, we looked at methods to measure the concentrations of biomass or cells, substrates and products.

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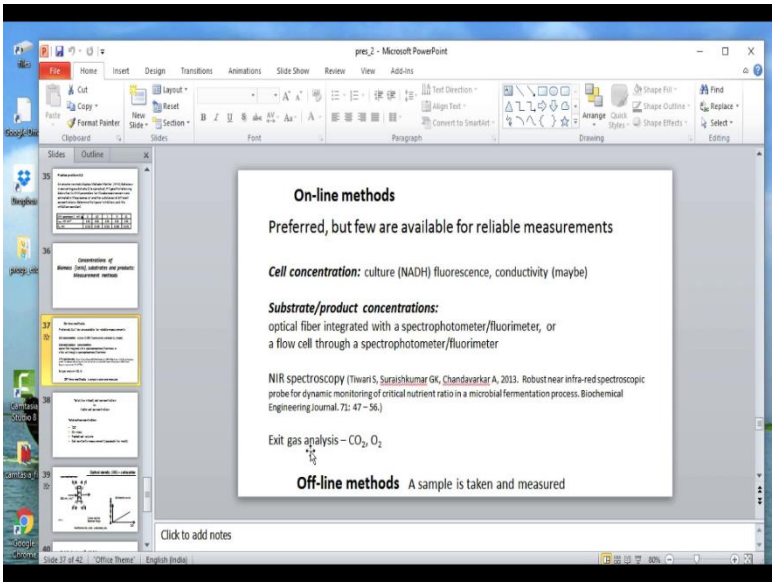

We said that they were two different kinds the on-line and the off-line methods. On-line a preferred, but the reliability of the methods available are to a certain extent, very few methods are highly reliable. Then we looked at the principles, we looked at methods for total cell concentration measurement such as OD measurement, the packed cell volume measurement, the cell contents measurement method. And methods for viable cell concentration such as dye exclusion and plating. Finally, some methods for substrate and product concentrations we also saw. This concludes module 2, and this is lecture 9. When we meet in lecture 10, we will take things forward with module number 3.

See you.