Plant Cell Bioprocessing
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Lecture - 25
Plant Cell Bioreactors -Part 1

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How is it different from microbial cultivation?

Characteristics	Microbial cells	Plant cell suspensions
Size	2-10μm	20-40 μm (variable cell size & shape during growth)
Homogeneous suspension	Often	Aggregates up to 2mm in dia. Leading to culture heterogeneity (w.r.t physical and chemical environment)
Growth Rate	Rapid, doubling times of 1-2 h	Slow, doubling time of 2-5 days
Shear stress tolerance	High	•Presence of cellulose based cell wall of high tensile strength makes them more sensitive to shear due to rigidity (less flexible) . •Large vacuoles make them more sensitive toward osmotic and physical stress.
Aeration requirements	High	Low
Cultivation time	2-10 d	2-4 weeks
Product accumulation	Often extracellular	Mostly intracellular

Most of the reactors in industry are working with configurations which have already been standardized for microbial fermentations. The same reactors are modified for plant cell cultivations, in general. But one has to be clear how plant cell cultivations would differ from microbial fermentations.

So, in terms of size, microbial cells are pretty small, at the range of 2 to 10 micron size while plant cell suspensions form aggregates, which can go upto 50 to 100 cells together with each plant cell being around 20 to 40 microns. So, you can imagine there is a big difference between the aggregated plant suspension and uniform microbial cell suspensions.

Hence microbial suspensions are homogeneous, while plant cell cultures forms aggregates eventually leading to high cell density which is the reason for non-homogeneity arising in the reactors.

Growth rates in microbial fermentations are quite rapid. The doubling time of microbes is in hours while in plant cell fermentations the doubling time is generally around 2 to 5 days. So, this means that the reactor cultivation time would be much longer than the microbial fermentation. So, keeping aseptic environment, also becomes one of the critical factors here.

Shear stress tolerance: Plant cells are shear sensitive, in comparison to microbial cells. This is because they have vacuoles and the cell contents are stretched onto the cell membrane. Majorly this would be the scenario when you are working with matured cells or if the production phase is happening in the matured cells.

So, there is one large vacuole. The cytoplasm and the cell contents are stretched on to the cell membrane. The presence of a cell wall accounts for a rigid structure, Due to the presence of cellulose based cell wall which is of high tensile strength, makes the cells more sensitive to shear and less flexible.

Aeration requirements: Specific oxygen demand of plant cells is much less than microbial cells. So, what will this imply? You would not like to have as much aeration as you would see in microbial fermentations. But as the cells would grow there will be high cell density, and aggregate formation will take place. This will lead to mass transfer limitations.

Now, in order for the oxygen transport rate to increase such that the oxygen reaches till the inner core of the aggregates, OTR has to be manipulated. The only controls which we can change are aeration rates and rpm, which is what is generally done. But then balancing between the shear forces which will get generated and the oxygen transport rate which is required is important.

It has to be noted that in spite of the fact that specific oxygen demand is less, the oxygen requirements for plants cell fermentations will keep on varying during the cultivation. So, cultivation time with continuous fermentation in microbes you will see it can go up to days, 2 to 10 days or so, but in plant cell fermentation if you go to continuous cultivation, it can reach up to a month or so.

Product accumulation with microbial fermentations is generally extracellular, but in plant cell fermentations you will find it, it is often intracellular. This means that the impact on

productivity is much higher because anything which impacts the cell can also impact the product forming inside which can lead to change in the yield of the product. If you want to make the process continuous, the downstream part is affected because there is an extra step involved in disrupting the cell wall and taking the product out.

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Implications on bioreactor design

The differences	Implications
•Lower oxygen demand than microbial cultures due to lower metabolic rate •Specific oxygen demand is low during exponential growth but high O_2 transfer rates required at high biomass concentration.	Variable oxygen transfer rates required during entire cultivation period
More shear sensitive than microbial cultures	Cultivation under low-shear conditions
Cells often grow as aggregates or clumps unlike microbial cultures	Mass transfer limitations can limit the availability of nutrients to cells within the aggregates
Cell to cell contact can influences secondary metabolism in plant cells	Optimal degree of aggregation/ self-immobilization required for product synthesis
Volatile compounds play significant role in plant cell metabolism (e.g., CO_2 or ethylene)	Optimum gas-phase composition required

Now, what are the implications on bioreactor design with these factors? Lower oxygen demand than microbial cultures due to low metabolic rate; that was one. Specific oxygen demand is low during exponential growth, but high oxygen transport rates are required at high biomass concentration. What does that mean? I just spoke about this.

Specific oxygen demand is low during exponential growth. What is specific oxygen demand? The amount of oxygen per unit time per unit biomass is low because the metabolism is slower in comparison to microbes. But what is high? High oxygen transport rates are required as the biomass concentration is increasing. What does that mean? The two are different specific oxygen demand, which determines what? The overall?

Oxygen uptake rate. So, with increasing biomass concentration the oxygen uptake rate is increasing, so oxygen transport rate has to be increased and moreover cell aggregation is happening. So, therefore, variable oxygen transfer rates are required during the entire cultivation period. Plant cells are shear sensitive than microbial culture. So, this means what? That cultivation has to be done under low shear conditions.

So, what can be done? You will generally observe that Rushton turbine is used for microbial fermentations, 6 plate, and it is the most commonly found configuration of the impellers. But, in plant cell fermentations, marine impellers, setric impellers or centrifugal impellers which can give better mass transport rates are used. This is because of better kLa which you will observe with less energy dissipated or with less shear forces generated and are preferred over these high energy dissipating impellers like in microbial fermentations.

Higher diameter to width ratio is preferred and large diameter impellers are preferred. Why? In microbial fermenters radial impellers such as rushton turbine are used. Now, in plant cell fermentations you will observe that axial impellers are preferred or steric impeller which can provide both axial and radial movements. I said aggregation will happen, if there is not much suspension created, the cells will settle down which will eventually lead to loss in productivity in the critical phase which is the log phase.

Now, cells often grow as aggregates or clumps unlike microbial fermentation. So, mass transfer limitations, can limit the availability of nutrients to cells within the aggregates, then cultivation has to be done under low shear conditions. Cell to cell contact - now we also have been hearing that, even though one would like free suspension in a reactor or well dispersed homogeneous suspension, but plant cells do not behave that way.

Now, while preparing the inoculum even when optimizing the shake flask, people who have worked that microbes do not know the requirements of plant cells, would try to keep the suspension as uniform as possible. So, but then eventually you will observe that there is a loss in yield of the product, because we now know that cell to cell contact leads to a differentiation signal which helps in secondary metabolite yield and productivity.

So, there has to be a balance between homogeneity and even cell to cell contact. Plant cells are self-immobilizing, sometimes when you are working with tissues you will find that the growth would not begin until unless all the tissues come together. I used to observe this during my research, when I was working with hairy roots. When we used to inoculate roots, the roots used to be very small in size, we used to cut sections, 2 cm long, and we used to then inoculate into the shake flask.

Initially, although we used to put the required amount of inoculum, but the culture growth was not happening. Why? What we used to observe is until unless all the roots

fragments which we had dispersed in the medium come together and they form a clump, and then growth begins and then we used to see that log phase used to begin.

So, this is an indication that self-immobilization is a prerequisite for the growth to begin although it depends on the species also. So, that is why sometimes you will observe that support structures are needed, immobilization of your cell mass is needed. Maybe you need any immobilization like polyurethane foam, which is what we used. We used polyurethane foam then designing reactors where we could inoculate the roots. So, that they could stick together and then the lag phase could be reduced. But, then all that has to be optimized.

Cell to cell contact can influence secondary metabolism in plant cells. So, optimal degree of aggregation and self-immobilization is required for product synthesis. Now, because you are trying to have high cell density in the reactor, what you would do?

You would try to increase the airflow rate, so that the oxygen transport rate can improve. But then you may observe that your secondary metabolite yield has gone down because the volatile components like carbon dioxide or ethylene which is crucial for secondary metabolism (it plays a role say as a signalling compound), may affect the secondary metabolite yield this is again what we observed with hairy roots.

We then eventually optimize the inlet gas composition with carbon dioxide also. So, plant cells, they are more sensitive to gaseous components like carbon dioxide or ethylene, which needs to be optimized unlike the microbial fermentations.

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CULTIVATION IN BIOREACTORS

Choice and design of most suitable reactor is determined by following factors:

- ✓ Shear environment
- ✓ Oxygen transfer capacity
- ✓ Mixing mechanism
- ✓ Foaming & Aggregation
- ✓ Maintenance of aseptic conditions for relatively longer cultivation periods
- ✓ Capital investment.

Modulation of factors like mixing (rpm) & aeration (air flow rate) to balance between the mass transfer requirements and shear sensitivity of plant cells.

So, what are the things which have to be kept in mind when you are designing reactors or reactor operating strategies for plant cell fermentations? The shear environment in the reactor, the oxygen transfer capacity of the reactor, mixing mechanism in the reactor, foaming and aggregation. It is observed that in plant cell fermentations there is much more frequent foaming than the others because of the large amount of sucrose which is used along with the proteinaceous medium which might alter the surface tension, and because of which the air bubbles are not allowed to escape, so they stabilize the foam. So, there is much more foaming in the plant cell fermenters. Then aggregation which can eventually lead to mass transfer limitation has to be taken into account.

Maintenance of aseptic conditions for relatively longer durations. So, as I said earlier the doubling time is in days. So, your batch time generally varies from 2 to 3 weeks and if you are planning to do continuous mode, it can go even beyond one month or one and half months, so that is the time period which we are looking for maintaining sterility.

Then capital investment; obviously, that is common, be it any fermentation that has to be looked into. You cannot end up in making such a complicated design which is not able to get scaled up or which might be incurring a lot of cost. So, modulation of factors like mixing and aeration is to balanced out, the mass transfer requirements and the shear sensitivity have to be taken care.

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Factors affecting Reactor Design

Rheological characteristics of plant cell culture

- Plant cell cultures are viscous at high concentrations (~80% PCV)
- Act as non-Newtonian fluids (shear stress and shear rate correlation dependence on aggregate size and cell conc.)
- Culture rheological properties get affected by medium viscosity and osmolarity
- Culture rheological properties can affect mixing and oxygen transport

So, what are the factors which will affect the reactor design? Let us talk about the rheology now rheological characteristics of plant cell cultures, what does it say? Plant cell cultures they are viscous. So, as the cell density increases, they tend to become non-Newtonian in nature. Non-Newtonian means?

Student: Viscosity is not constant

So, which means your viscosity is no more proportional to viscous force or your shear forces and shear rate, but it becomes a function of shear. Since it acts as a non-Newtonian fluid, shear stress and shear rate correlations depends on aggregate size and cell concentration and thereby fluid properties. Now, culture rheological properties will get affected by medium viscosity and osmolarity. What is osmolarity? Now, viscosity would keep on changing so we can understand. What about osmolarity? What is osmolarity?

It is also used as one of the indirect estimations ways to indirectly estimate your biomass. So, what is it related to?

Salt concentration inside the cell?

So, it is the solute concentration in the reactor, osmolality per unit gram or moles of solute per unit, amount of the solvent, osmolarity is the amount of solute per unit volume of the solution.

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Cell aggregation, foaming and wall growth

- Plant cell suspension cultures form aggregates [Diffusion limitation resulting in nutrient (especially oxygen) concentration gradient in the aggregates]
- Foaming is frequently encountered in plant cell bioreactors with aeration [Initial high sugar concentration and proteins released during cell lysis toward the end of cultivation]
- Wall growth due to cell floatation creates thick layers of necrotic cells [built up byproducts such as proteases or superannuated cell organelles secreted can inhibit the cell growth]

Now, cell aggregation foaming and wall growth in plant cell fermentations. Now, plant cell suspension cultures they form aggregates, so diffusion limitation can happen within the aggregate. So, there can be external mass transfer limitation. So, when we are talking about oxygen transport rate, we are only taking into account what? The external mass transfer limitation which is your k_L or k_G.

But and we neglect the k_S part the solid liquid mass transfer. We were neglecting it till the point. Why we were neglecting it? Your kL is what? It is the overall mass transfer coefficient which involves only the gas liquid boundary, but we are neglecting the solid liquid boundary. There will be a solid liquid limitation, boundary limitation on, isn't it. What solid? Because of the cell.

So, why we were neglecting?

Student: Earlier the cells were very small.

Very nice because it was made for?

Student: microbial cultivations.

So, you could assume that, but if you are working with plant cells or tissue cultivations it depends on the size and the size of the aggregates. If your cell suspension is uniform,

you are much closer to that assumption so that you can neglect, but if you are working with tissues much larger than the bubble size then can you neglect that boundary? No.

Moreover, when you are working with aggregates or when you are working with immobilized cultures, can you neglect the internal mass transfer limitation. Internal means from the surface solid surface till the inner core. People have even found that when they are working with root cultures there are intracellular gradients of oxygen diffusion limitation. So, oxygen gradients are observed till the inner because the size is appreciable.

Now, foaming is frequently encountered in plant cell bioreactors with aeration, initially. So, I have already told you because of the proteinaceous medium, or the proteins come out once the cells are lysed because of the shear forces or the high sugar concentration. Now, wall growth due to cell flotation creates thick layer of necrotic cells. So, plant cells as I said earlier during my discussions that they have a tendency to adhere to the glass walls.

So, you will observe in lab scale reactors on non-movable parts because of the large size. So, once they get stuck there or once they adhere to those walls then subsequent growth will cause a film or an aggregate size to increase, the innermost layer will be devoid of the nutrients, availability of nutrients, so then you will observe necrosis.

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Mixing

- To keep the cells in suspension and provide a homogeneous environment
- At high cell density, culture viscosity makes mixing difficult
- Impeller design- shear generation, power requirement, fluid circulation ability under high viscosity. Large diameter impeller can provide high homogenization but lead to less bubble dispersion
- Baffles enhance mixing

So, mixing is done to keep the cells in suspension and to provide homogeneous environment. Now, at high cell densities culture viscosity makes mixing difficult because they tend to aggregate. Now, there has to be a balance between homogeneity and suspension.

So, you would choose impellers which can create more amount of shear force or more energy dissipated onto the medium, so that the bubbles can be well dispersed inside the medium and there are more circulation velocities radial velocity is generated, so that it is homogeneous in nature. But then this will eventually lead to larger shear forces. So, you will observe that impeller design is a crucial thing in plant cell fermentations.

Shear there, what is to be considered? The amount of shear which is generated, power which is required, because that will involve cost. So power dissipated, power required to drive the impeller, fluid circulation ability of the impeller and high viscosity. So, what is preferred is you will observe in plant cell fermentations, large diameter impellers are preferred because of their high homogenization, but less bubble dispersion.

So, again although you can improve mixing, because you have larger diameter impeller, but what you are giving away is because then this will create lesser shear force on the sparger for the air bubbles to be dispersed. So, what will happen? Eventually, you will observe lesser dispersion of gas bubbles, lesser oxygen transport rate. So, things have to be manipulated during the fermentation as the plant cells would grow and baffles.

Now, baffles we know enhances mixing. But you will observe baffles in literature are found to be detrimental for plant cell cultivation. So, people say that it is better to run plant cell fermentation without baffles because mixing requirements or air dispersion or oxygen transfer requirements are much lesser than the microbial fermentations. So, you can afford to remove baffles.

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- In pneumatically agitated vessels, superficial gas velocity (hence liquid circulation velocity) affect the suspension efficiency.
- Particle suspension improves with a high reactor aspect (height-to-diameter) ratio
- For the same power input per unit volume, mixing time in airlift reactors is longer than in stirred tanks
- Mixing, a limiting factor for pneumatically agitated reactors at high plant cell concentrations

Now, in pneumatically agitated vessels, superficial gas velocity affects the suspension efficiency. Now, there is no moving part. So, in your airlift reactors and bubble column reactors what is causing the circulation currents? The air bubbles. So, particle suspension is what is going to keep the cells in suspension in suspended state, and not allow them to settle.

Now, particle suspension, improves with high reactor aspect ratio. This is what has been observed. Higher is the aspect ratio, (aspect ratio means height to diameter ratio, better is the suspension ability.

So, for the same power per unit volume, mixing time in airlift reactors has been found to be much longer than the stirred tank, isn't that obvious. Now, mixing is a limiting factor for pneumatically agitated reactors at high plant cell concentrations. So, even if you are choosing an airlift reactor for plant cell fermentation because there are no moving parts and the air can keep it suspended, but then mixing is a limitation.

So, that has to be taken care as the cell density would increase. So, don't you think I am giving you a pro and a con, a merit and a demerit. What does that mean? So, how will you choose? Why am I giving you a merit and a demerit? Everything has a merit and a demerit that is what I can understand from this. So, what does this mean? What is needed?

Student: Optimization

Right. So, which means that depending on the species don't think that without knowing your culture requirement you can just begin with, ok, now I have optimized everything in shake flask let me do a batch now in stirred tank reactor. No, it never works like that. Your batch once you have even optimized everything in shake flask, you need to take it to the reactor, choosing the right kind of a reactor configuration would be very crucial for reproducing the results which you have obtained in shake flask at the reactor level.

And then once you have chosen the right reactor configuration then the second stage of scale up begins which is what should be the right scale up criteria now to increase the volume of this reactor configuration.

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Oxygen demand and supply

- Dissolved oxygen level to be maintained above a certain critical concentration.
- High culture viscosity, cell shear sensitivity, and cell aggregation make oxygen transfer in plant cell cultures a challenging problem.
- Impeller design and sparger design affect aeration and in turn oxygen transfer.
- Factors affecting $k_L a$ are superificial gas velocity, culture viscosity, and reactor geometry.

So, oxygen demand and supply: Dissolved oxygen levels to be maintained above the critical concentration. So, that is same for any kind of fermentation. So, we need to ensure that the dissolved oxygen, the bulk oxygen concentration in the reactor is always maintained above the critical oxygen levels. High culture viscosity, cell shear sensitivity and cell aggregation would make your oxygen transfer in plant cell fermentation a challenging problem.

Now, give me a way just think logically, I know that cells would be shear sensitive, my assume cells will be aggregating and I cannot increase aeration and rpm because that will create shear.

What would you do then? We have now learned about so many things in optimization strategies, what would you do to improve your biomass or your yield with this limitation? Cell shear sensitivity, culture viscosity, cell aggregation is causing oxygen transfer limitation.

Student: So, we can add pure oxygen which will decrease the limitation.

So, we can optimize the inlet gas space concentration. What else? But that is an expensive strategy, you cannot afford to have pure oxygen in it. So, which means that oxygen transfer in plant cell fermentations are much more critical or the key limiting nutrient than the carbon. Generally, for microbial fermentations oxygen is not the limiting nutrient even in your Monod's model, that S is never O oxygen, that S is carbon or nitrogen.

At the maximum, when you work with products you will find some other critical nutrient which might be affecting the product. But in plants cell fermentations or with hairy root cultures I could find that because carbon was still available it was not growing which means that we need a model where now O_2 becomes the limiting factor and not Carbon. So, that is how it shows that the plant cell fermentation, you cannot directly mimic what you know about microbial fermentations.

So, you need to first understand the culture itself, its requirement and then work and use the, right configuration. So, impeller design or sparger design affects aeration and in turn oxygen transfer. So, this is also critically needed. I will not go in to this. I will just show you.

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Mixing

- To keep the cells in suspension and provide a homogeneous environment
- At high cell density, culture viscosity makes mixing difficult
- Impeller design- shear generation, power requirement, fluid circulation ability under high viscosity. Large diameter impeller can provide high homogenization but lead to less bubble dispersion
- Baffles enhance mixing

So, when we say that impeller design, high homogenization or power requirements shear generation, just saying does not help. Shear generation means what? How will you design an impeller taking into account shear generation, power requirement or circulation ability. How will you design?

So, generally what is done is you look in literature and get to the empirical correlations which from where you first we need to define how would we define shear generation, how can shear be quantified in terms of these reactor operating parameters. So, you can see literature you will find there are many empirical correlations where people have correlated the shear forces generated with power input with impeller speed, with impeller width.

So, one such empirical correlation depends on the amount of energy which would be dissipated on to the medium depending on the power input, time duration and the volume of the reactor or the volume of the liquid.

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Design equations for two-phase and three-phase mixing without shear damage in bioreactors

- Cumulative energy dissipation on cells related to power dissipation

$$E = \frac{1}{V_L}\int P\phi \, dt \text{ (Wongsamuth and Doran, 1997)}$$

where E is the cumulative energy dissipated on the cells per unit volume, V_L is the reactor working volume, P is the power input by agitation, ϕ is the volume fraction of cells in the culture, and t is time.

- Maximum allowable impeller speed is determined by impeller power number

$$N_{P_0} \text{ or } N_{P_g} = \frac{P}{\rho N_i^3 D_i^5} \qquad \text{(Doran, 1995)}$$

where N_{P_0} is the unaerated power number, N_{P_g} is the power number with gassing, ρ is broth density, N_i is stirrer speed, and D_i is impeller diameter.

So, eventually this power generation is also connected like in your power number. Given a power number this power generation would eventually be correlated with the impeller speed, the diameter of the impeller. So, now, you know how these operating parameters will be impacting the shear force.

Now, first thing is you need to see the threshold of your culture. How would you see the threshold of your culture? You see do a simple experiment. I am just giving you an example. Do a simple experiment, keep on increasing the rpm, check the viability of the cells. Once you know that this is the maximum rpm at which we can work, work backwards at this rpm, what will be the shear generated; so, which means this becomes your threshold shear force. So, then you can fix up your rpm and similarly your power input.

Suppose you want to quantify how much at this volume, will be the shear force generated or this may even help you to choose the right kind of impeller. Once you have fixed on that, this can be the maximum shear which can be tolerated. Then you choose that kind of an impeller or the power input such that you go do not go beyond this shear force generated.