Plant Cell Bioprocessing
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Lecture – 16
Optimization strategies- Part 1

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Disadvantages related to natural plant extraction of phytochemicals

1. Public awareness on plant based therapy due to low cost, less side effects, and environmentally sound
2. Low concentration of product
3. Limited supply to meet ever-increasing demand
4. Endangered species, may lead to extinction
5. Specific environmental condition
6. Area under plantation for particular species
7. Long time for growth of the whole plant
8. Non-uniform and inconsistent quality/quantity due to nature dependence

So, the disadvantages of natural plant extraction are as follows: low concentration of product in the plant. That may be because of the demand and supply difference and there can be limited supply. Further, it can be an endangered plant because of extensive uprooting by the industry. Then it can be an endemic plant and is able to grow only on a particular region. Thus, there can be limited supply for these reasons.

Now, being an endangered species, as I said can be a limitation which may lead to ultimate extinction of the plant. Natural plants, because it is seasonal, the desired compound will have temporal and spatial variations. So, a long time is required for the compound to get generated and maybe it only comes up when the plant matures. So non uniform and inconsistent quality and quantity is a problem.

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Alternative production method

Chemical synthesis-
- high cost involvement
- impact on environment
- synthesis not possible in case of large complex molecules

Biotechnological methods- Plant cell technology

So, what are the alternative production methods for such high value phytochemicals? Some as I said we had seen earlier have complex structures and biosynthetic pathways. So, these high value and low volume metabolites are complex structures with too many chiral centres, and it has been observed that chemical synthesis is very difficult. And even if people are able to do it, it becomes a multi step process. So, biotechnology-based methods are an alternative. One is plant cell based bioprocess, what else?

Student: Protein engineering.

Where? So, you are talking about heterologous expression, that is in some other host eukaryotic system. But, for some of the secondary metabolites, the most frequently used system is yeast. But the challenge is that some of the biosynthetic pathways for high value metabolites is not elucidated.

So, going on to how to get those intermediate steps, you need to know which proteins are involved. Also, it is such a wide and big spectrum like for example, 40 or 50 steps are involved and if it is a heterologous system, the pathway will not be present. So, all those steps have to be included, so it is very challenging which may cause low yields which can make the process non-economical and challenging.

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Advantages of producing compounds from Plant Cell Technology

- Control of supply of product independent of availability of plant itself and climatic, geographical and governmental restrictions etc.
- High growth and productivity as compared to natural plant.
- Reduction in time and space requirement for the production of desired chemicals.
- Strain improvement can be done for the enhanced production of desired compounds.
- Ease in the extraction and purification of the final product

So, the most feasible process which is proposed is plant cell based bioprocess where the machinery is present. But you need to work around the different other bottlenecks which is low yields and scale up of the process. So, using plant cell technology for producing these high value metabolites which are low volume in nature can be advantageous.

There can be control of supply why? Because the product is independent of the availability of the plant and it is away from climatic, geographical and governmental, restrictions. Then high growth and productivity, so how is it compared to natural plant?

Student: Productivity because we can culture them in less amount of time.

Right.

Student: We can optimize the production according to our needs.

Because these are amenable to optimizations. So, you can see that time is a big factor. So, reduction in time and space is logical. Now, strain improvement: the cell line itself is amenable to optimisation. You can manipulate the cellular yields to improve the $Y_{P/X}$ which is product yield per unit biomass. So, there is ease of extraction and purification.

Because you are doing process optimisation, you will be optimising the conditions so as to increase the productivity of your desired compounds. So, the relative abundance of

your desired compound becomes more than the other small molecules or other impurities, so thereby the downstream processing can become easier.

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Plant cell cultivation-critical requirements

Appropriate explant (any plant part)

A suitable growth medium containing energy sources, inorganic salts and growth regulators to supply cell growth needs. This can be liquid or semisolid

Aseptic conditions to prevent microbial contamination

Optimum culture conditions

External culture conditions: light, temperature and agitation

Internal culture conditions: medium components, pH, degree of mixing and aeration.

So, what do we need for plant cell cultivation, an appropriate explant is needed, a suitable growth medium Now what is the growth medium composed of? You will have major salts, minor salts, energy sources, growth regulators in it which will fulfil the plant cell growth and this medium can be in liquid or semi solid form.

Aseptic conditions are required, so as to prevent contamination specially from microbes. Optimum culture conditions: environmental conditions and other physical and chemical parameters.

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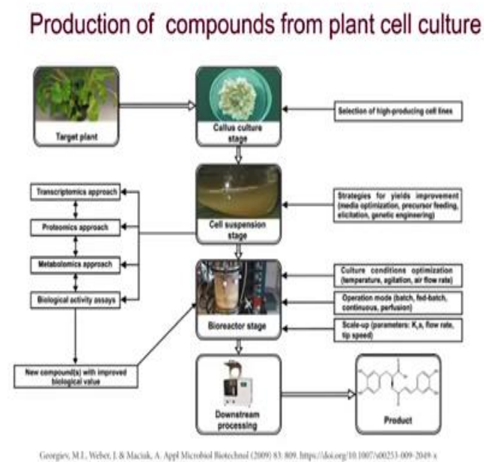

So, this is a schematic. We know we begin from the plant parts then we bring it in the form of a dedifferentiated form which is the callus. From that callus you can select the cell line, bring it in suspension and do the optimisation where yield enhancement strategies are implemented.

So as to improve the $Y_{P/X}$ what kind of strategies can be employed? Media optimisation, precursor feeding, elicitation, and genetic engineering which can be metabolic engineering can be done. Then coming to the bioreactor stage, you again optimise the bioreactor operating parameters which we also call as fermentation parameters. Then you can optimise the mode of cultivation which can be feeding strategies. Why do we have to have feeding strategies, what is the aim, why are we talking about modes of cultivation every time in the reactors, why cannot the reactors be run as batch?

To remove any nutrient limitation and nutrient inhibition, in order to improve the biomass or product productivity. And why in a batch is it getting limited? Because of the nutrient imitation. So nutrient feeding is done to get over this nutrient limitation.

And now with this logic can one dump in all the substrate, so that it is never limited? No, inhibition can play a role. So, there comes the concern of how you feed and when do you feed. So, then after you have grown the biomass it comes to downstream processing, downstream processing means separation and purification of the product.

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Approaches to enhance productivity

- Optimization of medium composition.
- Optimization of environmental factors like light, temp, pH and agitation.
- Addition of precursors.
- Cell permeabilization for product secretion.
- Addition of elicitors.
- Immobilization of plant cells.
- High density cultivation in suitable bioreactors
  - Mathematical modeling

So, what are the approaches which can lead to improvement in yield and productivity of the secondary metabolite? Optimisation of media composition, optimization of environmental factors, addition of precursors, cell permeabilization, addition of elicitors, immobilization of plant cells and high density cultivation in reactors. And in some of these, in order to reduce the hit and trial and minimise the number of experiments which you would like to conduct to reach to the optima we will see how mathematical modelling is used with case studies.

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Screening and Selection

- Plant cell cultures are genetically heterogeneous (change in chromosomal and intra chromosomal rearrangement).
- Epigenetic instability (changes in gene expression or cellular phenotype caused by mechanisms other than changes in the underlying DNA sequence) causes genetic instability leading to product accumulation only in some population of cells.
- Productivity in such mixed population of cells is liable to be unstable since change in any factor, environmental or nutritional which favors the growth of a particular sub population of cells thereby changing the composition of the culture.
- The variation in the culture offers the opportunity of isolating from the mixed population of variant cells the ones with high productive capacity and thus developing from them cell lines of high productivity.

Now, yesterday if you remember I was talking that in order to start a bioprocess development you would like to begin with the highest yielding cell line, so logically you would like to pick up first the plant variety which is highest yielding. What is screening and selection? Are they same or different? Be it microbial, animal, or plant cell fermentation, these techniques remain, when we say screening and selection.

Screening is a passive technique, and selection is an active technique. Any example?

Student: Mam, there is certain bacteria which can utilize the blood agar media some which cannot. So, in this screening process when we subject them to this same media, we can understand which are the cells which utilize the media and which are ones which cannot, so, that is the screening process.

So extrapolate it to secondary metabolites from plant cells.

We need to select the highest yielding callus line.

The callus is a mix of a number of cells. That is why we say that callus would lead to difference in with subsequent subcultures you may find that the yield keeps on varying.

Student: co culture and see the bacteria is growing

Bacteria where is the bacteria coming?

Student: Like say if the plant is producing a particular metabolite, they will defend against the bacteria that will be kind of a test organism to find out if the production is actually happening or not.

On paper this can be done, but think you need to develop a bioprocess. So, think simple which can cost you less, which can easily be implemented and then you need to take the cell lines. So, once you start growing bacteria along with plant cells; plant cells will not survive.

Student: Mam, we can just introduce some amount of stress conditions in which a secondary metabolite is produced. And the stress is subjected to all of those callus lines and we see which of them produces the most amount of secondary metabolites.

That is correct, but the only check here is that you have opted for a material which is known to produce that compound and you have a number of cell lines. So, one is you check the yield of the product and then you select the highest yielding cell line.

Other is that, if it is a pigment producing callus, then you can take that piece of callus which demonstrates that colour. Then you know from that mixture of cells which cells will be producing that callus. This is screening.

Coming on to selection where would like to only take the desired cell lines the others you do not care. So, only the ones which you want to select should survive. It will be useful when you are selecting a plant cell line. Say, if I am trying to find a cell line which is highly resistant to salinity stress, so only those which can resist will survive the others would not survive.

So, by subsequent subculturing and acclimatization you will end up having a cell line which is resistant to the stress and therefore, you can pick up the desired cell line. So, now plant cell cultures we know that they are genetically heterogeneous, meaning they can have chromosomal variations. So, that was what am I talking about when I say change in chromosomal number, arrangement.

What kind of variation? Somaclonal variation, epigenetic instability. Epigenetic instability means change in the gene expression or cellular phenotype caused by mechanism other than changes in their DNA sequence. So, genetic instability leading to product accumulation only in some population of cells is a problem which the heterogeneous callus would have. Now, productivity in such mixed population of cells is liable to be unstable, so generally when we induce a callus from an explant it is not called as a plant cell line.

Because from the wound which you create, the cell starts to grow around the wound and the cells start rapidly dividing forming a dedifferentiated mass. Now, all these cells have a different makeup and as you would grow it on a solidified medium they would multiply. So, some of the cells which are close to the medium will have more exposure to the nutrients and nutrients are more available to those cells than the cells which are at the top. So, biochemical gradients may form which may lead an ultimate change in their biosynthetic potential.

So, it is generally observed that when you begin with callus lines, you first bring it to suspension and through cell sieving. If you remember I talked about the cell sieving technique where in order to get to a synchronous culture based on the size, an assumption is made that the cells of same size and shape can be assumed to be in the same metabolic state.

So, thereby leading to a synchronous culture or by repeated subculturing, under the conditions which are preferable for the secondary metabolite of interest such that, during the subculture natural selection happens. So, you will get only the preferred cells which are capable of producing the secondary metabolites under those conditions and would continue to grow and later they would be the majority.

So, it is always the kind of cells in the callus and the number of those cells in that callus which are capable of producing your secondary metabolite which are important. So, to increase that number, it is generally observed that when you begin with the callus induction the yields will have a large variation for initial a year or so. Only after 3-4 years of subculture you will find that it comes down to a consistent product yield.

So, productivity in such mixed population of cells is liable to be unstable due to change in any factor. That is the very reason why we in lab always insist on keeping the conditions consistent you cannot take lightly the time of subculturing of the callus which is the parent material and is going to form your master cell bank.

So, the conditions in which you subculture, the time at which you subculture is important. You cannot think that if it is 30 days, let me do it in 40 days it does not matter. Everything matters, the time at which you are subculturing, the conditions you are maintaining. Say today you had put 0.8 g as inoculum, tomorrow you had put 0.6 g, it is going to vary.

So, one has to be really careful while subculturing your master cell bank. Since changes in any factor environmental or nutritional can favour the growth of a particular sub-population of cells thereby changing the composition of the culture and hence the variation in culture. Also, because it is something which can tweak the biosynthetic potential of your cell mass, it also becomes a tool for variation. So, the variation in the culture offers the opportunity of isolating from the mixed population of variant cells, the ones with high productive capacity. And thus, developing cell lines of high productivity.

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So, the rate of accumulation within the productive cells and the proportion of such cells in the culture is what governs the yield or selection of the high yielding cell line. The low production in cell culture can be due to, competition between the primary and the secondary pathways, they have the same intermediates as we have seen and then they bifurcate for secondary metabolism.

So, there will always be a competition in terms of carbon flux. Low expression of key enzymes in the pathway in rate limiting steps or gene expression of the secondary metabolism can be a limitation.

Then screening is a passive technique by which a number of cells are analysed for the desired trait. So, we generally we would look for the highest yielding cell line, we will take some part of the callus and do extraction and we will do yield analysis. Or if it is a coloured pigment you can directly view it, if it is visible to eyes. If it fluoresces under UV then to you can do it by UV light. So, whether the callus which you have obtained is producing your compound or not you can check.

Now, selection is what? Selection is an active process by which it deliberately favours only the survival of the desired variant and the wild other type of cells they will die.

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Culture systems used for screening and selection

Callus culture:

- Cell lines which accumulate high levels of colored compounds are selected with visual observation.
- The callus can be checked visually for altered pigmentation by the naked eye, for colored spots under UV lamp.
- Screening of pigments (chlorophyll, anthocyanins, napthoquinones, carotenoids, etc.) can be easily and successfully done with calli.

So, callus systems are used for screening and selection. Cell lines which accumulate high levels of coloured compounds can be selected through visual observation. The callus can be checked visually for altered pigmentation by the naked eye for coloured spots under UV lamp. Screening of pigments for example, if suppose you know from literature that greener is the callus it is somewhere linked to secondary metabolism of secondary metabolite biosynthesis. So then, you would like to select the most green callus line. So screening on the basis of pigments like chlorophyll, anthocyanins, napthoquinones, carotenoids (these are all coloured pigments), it becomes very easy to do the screening.

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Suspension culture:

- The selection can be performed with a fine, rapidly growing suspension consisting of aggregates up to 50 cells or with a rather lump culture.
- Fine suspension culture is suitable in selection for resistance. The selective agent (a pesticide or metabolite) is added to the culture medium at a concentration, which kills all sensitive cells. The toxic compound equally affects all cells and thus the sensitive ones should be eliminated. The disadvantage is, that the surviving cell population will be a mixture of cells being resistant due to various biochemical reasons.
- In the selection program for resistance in cell culture one can detect growth of resistant population only after 4-12 weeks.

Now selection can also be performed by using your sieving techniques. Sieving means using different mesh sizes and trying to reduce the mesh size for example, from 100 microns to less than 100. So, by the same size of cell, you can end up in a synchronous culture, and the selection can be performed with the fine rapidly growing suspension consisting of aggregates of 50 cells or so.

Now, fine cell suspension is suitable. Why do you think every time the stress is on fine cell suspension for selection process, how will it help than the lump culture?

Student: uniform metabolic activity.

We are talking in terms of selection, selection means?

Student: So that the size variation will not affect the selection process, and all cells will have the same metabolic activity.

That was cell sieving, now in general there can be more methods apart from cell sieving directly like for example, if you are looking for resistance. So, the rest will get killed and only those which are resistant will survive either this resistance can be salt resistance or can be against a toxic metabolite or antimetabolite resistance.

So, if suppose the others get killed and you need only those which survive it is recommended to use dilute cell suspensions. Like you were giving examples for bacteria you grow it in a solidified medium such that only the bacteria which can degrade that particular metabolite grows. Then what do you do, you pick up the colony.

Colony, so there also what is recommended to use.

Student: Solution.

Why dilute solutions? What is a callus? A heterogeneous mass of cells. So, therefore, it is preferred you bring it in suspension, and then select for a resistant cell line and allow that to grow and then multiply it to give you a fresh callus using single cell culture techniques which we have learnt before.

So, in the selection program for resistance in cell culture one can detect growth of the resistant population. But because you end up in single cells it will take time for the callus to reappear and for the culture to again come back to a rapidly growing state. So,

therefore it is said that it will take some 1 or 2 months to reach to a uniform callus, a single cell based callus line.

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Plating of cells/protoplasts:

- To avoid inherent problems in the use of callus or cell culture for selection and screening, the plating of cells or protoplasts on solid media or matrices is preferred.
- In plating of cells, the first step is to prepare single cells or small sized aggregates for plating. The cells have to be plated at low densities allowing growth of individual, clearly separated colonies that can be isolated and subcultured.
- Unlike microbial cells, cultured plant cells require a minimum inoculum density for growth.

Now, to avoid inherent problems in the use of callus or cell culture for selection and screening, the plating of cells or protoplast on solid media or matrices can be preferred. Now, in plating the cells, the first step is to prepare single cells or small size aggregates for plating. The cells have to be plated at lower densities allowing growth of individual, clearly separated colonies that can be isolated and then subcultured.

Unlike microbial cells, cultured plant cells require minimum inoculum density, we studied that plant cells will be needing minimum inoculum density for the growth to begin.

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Preparation of single cells and protoplasts

- To filter successively cells of a fine suspension culture through sieves of decreasing mesh width from 500-75 μm.
- The obtained suspension will consist of single cells and small aggregates, which most likely originated from a single cell.
- For large aggregates suspension, adding pectinase in the medium 1 or 2 days before sieving can disrupt it.
- Screening and selection should be done from a freshly prepared cell suspension from the plant than from a very old suspension culture.
- The most ideal tissue for isolation of single cell is leaf and mesophyll cells which can be mechanically or enzymaticaly isolated.
- In order to select a single cell for plating, cell suspension must be diluted to densities at which plant cells do not grow without special precautions.

Generally, plant cells communicate with each other through plasmodesmata.

So, there is cell to cell contact which is necessary, and there is communication happening. So, to facilitate and bring it to that condition minimum inoculum density is needed. Now, for preparation of single cells or protoplast we have already studied that we can successively filter cells of fine suspension culture through cell sieving techniques.

So, if you keep passing the cell suspension around 500 to 75 microns this will end up in synchronous culture. The obtained suspension will consist of single cells or small aggregates, which most likely will have originated from single cells. Why and how can you assume that? When we are using these cell sieving techniques we are starting from big size and we are ending up at a very small size 75 micron cells are less size.

So, generally 50’s or 100 cells would be there in cell aggregates. If you know the average cell size you will get to the logic why this sieving technique of 500 to 75 microns will end up in either single cells or will end up in very small aggregates which would obviously be coming from the single cells. This is because they have multiplied and formed a little bigger than the single cell.

To avoid large aggregates in suspension, adding pectinase helps. Why? This is in relation to the cell wall composition we have studied earlier.

Generally you will find that even when you induce a callus from different types of explants they will all vary not only in their biochemical makeup but they will also sometimes vary in their morphology.

Some will be very hard and compact, some will be very friable that you will just touch with a spatula and they will disperse in the medium. So, that is because of the friability which is because of the water content in the callus. So, in large aggregate suspensions, if we are using pectinase because it is preferred that the callus should be friable in nature.

Screening and selection should be done from a freshly prepared cell suspension from the plant than from a very old suspension. Why? Why should you prefer young cells? Because they will be more actively growing cells.

The most ideal tissue for isolation of single cell is the leaf and mesophyll cells which can be mechanically and enzymatically isolated.

In order to select a single cell for plating cell suspensions must be diluted to densities where the plant cells do not grow without special supplementation.

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Methods or screening and selection

- Some secondary metabolites have an intense color allowing direct visual detection.
- Altered pigmented areas of callus can easily be detected.
- Analysis of cell extract is done to know the quality of the clone.
- The clones under investigation have to be divided for subculture and chemical analysis (measuring absorption, fluorescence and color rxns.)

So, what are the methods of screening and selection? Secondary metabolites may show an intense colour, so by visual detection you can take a piece of the callus and start propagating that callus. So, you will end up in a callus line which will be giving the product of interest. Altered pigmented areas of the callus can be easily detected or direct

analysis of the cell extract is done to know the quality of the clone. The clones under investigation have to be divided for subculture and chemical analysis.