

Plant Cell Bioprocessing
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Lecture - 28
Case study – Part 1

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Today just to give you a wholesome picture of whatever you have studied up till now, we will take up a Case Study which is a collation of all the data by one single research group. How they began and how at every stage the strategies which you have studied in plant cell technology or plant cell bioprocessing are used. At every stage there could be an enhancement in the productivity up till the reactor level. The study was done for the secondary metabolite azadirachtin, which is a biopesticide.

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Pesticides- fact sheet

Use- To control the agriculture pests

Advantages- Highly effective in controlling the pest problem in almost all crop systems

Disadvantages-

- Synthetic chemicals containing Chlorine, Sulphur, Phosphate as a reactive group
- Resistant to biochemical degradation
- Development of resistance in many order of insect pests

Gurjan Prakash (2006) Production of Azadirachtin (Biopesticide) from Plant Cell Suspension Culture of *Azadirachta indica* A. Jinn. (Niem), Indian Institute of Technology Delhi

So, I will not take up the advantages disadvantages of these.

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Solution ...

Biopesticides

➤ Botanical Pesticides- > 200 Species of plants have insecticidal properties. The most promising botanicals pesticides belongs to family Meliaceae, Asteraceae, Labiateae, Cancellanceae Compositae, Solanaceae , Chenopodiaceae , Rutaceae etc.)

Family: Meliaceae
Genus: *Azadirachta*

Gurjan Prakash (2006) Production of Azadirachtin (Biopesticide) from Plant Cell Suspension Culture of *Azadirachta indica* A. Jinn. (Niem), Indian Institute of Technology Delhi

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Azadirachtin is present in a ubiquitous plant, neem. Azadirachtin is found in all the parts of the plant. However, it is collected commercially from the seeds of this tree and this seed formation happens twice in a year. Moreover, there are disadvantages as I have mentioned before, that generally ripe seeds are collected, which have maximum concentration of azadirachtin in it.


But this also has a lot of sugar content in it, because of which there are pathogenic contaminations, specially fungal pathogens. And it is why industries face problems. There is contamination with the fungal metabolites while some of these are carcinogenic like aflatoxin.

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Advantages of azadirachtin over chemical pesticides

Chemical Pesticides	Azadirachtin- As Biopesticide
Chemically synthesized	Natural product
Persist in nature for long duration	No persistence in environment
Leave hazardous residues in food, feed, water and users, highly polluting	Biodegradable, no residue, non polluting
Toxic for non-target organism	Very specific hence no toxicity against non-target organism
Destroy natural enemies & beneficial insects.	Compatible with other biological agents
Large no. of pest species has developed resistant against chemical pesticides.	Low probability of developing resistant due to its multiple mode of action.

Guram Prakash (2006). Production of Azadirachtin (Biopesticide) from Plant Cell Suspension Culture of Azadirachta indica A. Juss. (Neem). Indian Institute of Technology Delhi




It is a highly complex big molecule with lot of chiral centers.

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Azadirachtin production

Seeds	Chemical Synthesis	Plant Cell/tissue Culture
Limited availability	Structurally very complex compound so chemical synthesis is difficult	Supply under controlled conditions, independent of geographical/seasonal variations, environmental/ soil factors
Non-uniform supply		Continuous supply of uniform quality and yield
Contamination with toxic metabolites		Free from any contamination
Land consumption		Reduction in space & time

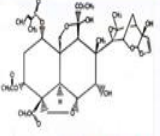
Guram Prakash (2006). Production of Azadirachtin (Biopesticide) from Plant Cell Suspension Culture of Azadirachta indica A. Juss. (Neem). Indian Institute of Technology Delhi



So, complete chemical synthesis, has not been reported yet.

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Azadirachtin



$C_{35}H_{44}O_{16}$

- Most important secondary metabolite of *Azadirachta indica* (neem tree).
- Widely recognized as most potent biopesticide from plant origin and alternative for chemical pesticides.
- Broad spectrum properties of azadirachtin to combat diseases like Malaria, Dengue, Cancer and AIDS are also being investigated.
- Mode of Action: Antifeedancy, Insect Growth Regulation, Reproduction Interruption

Gottam Prakash (2006). Production of Azadirachtin (Isoprenoid) from Plant Cell Suspension Culture of *Azadirachta indica* A. Jinn. (Neem). Indian Institute of Technology Delhi



The molecule has a wide mode of action to a number of pests known in different plant varieties. So, that is the reason why it is very looked forward and is a high in demand biopesticide in the market. Now the first reason is it has a very diverse mode of action, completely biodegradable which is a big advantage and nontoxic to other organisms. It has a specific mode of action towards pathogens. So, what they had proposed was a plant cell and tissue culture based bioprocess for *in vitro* production of azadirachtin.

In nature, you will find that neem is present everywhere but the limitation is that if you collect it from different parts of the country, you will find that the yield of azadirachtin is variable. It is season dependent, it is plant age dependent, geographical location dependent and moreover there are collection problems with the material.

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Approaches for Production of Secondary Metabolites

Plant Cell Suspension Culture
(Plant cell suspension cultures generated by transferring callus to liquid media)

Hairy Root Culture
(Hairy root cultures obtained by infection of *Agrobacterium rhizogenes*)

Guram Prakash (2006). Production of Azadirachtin (Isoprenoid) from Plant Cell Suspension Culture of *Azadirachta indica*. A. Jau. (Niem). Indian Institute of Technology Delhi

The slide features a diagram with two images on the left. The top image shows a flask with a yellowish suspension, representing plant cell suspension culture. The bottom image shows a petri dish with a hairy root culture. Arrows point from these images to their respective text descriptions. A woman is visible in the bottom right corner of the slide frame.

So, two kinds of approaches were used - plant cell suspension based and the other was hairy root culture based.

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Objectives

- I. To establish the cell suspension and hairy root culture system of *A. indica* for production of azadirachtin.
- II. To study the different strategies for enhancement of azadirachtin production in cell suspension and hairy root culture of *A. indica*.
- III. Large-scale cultivation of *A. indica* cell/hairy root culture in different kinds of bioreactors.
- IV. Development of mathematical model for the *A. indica* suspension culture and offline feeding optimization to overcome substrate limitation/inhibition in bioreactor.
- V. Azadirachtin production in bioreactors under optimized cultivation strategies under fed-batch and continuous (with cell retention) mode of cultivation.

Guram Prakash (2006). Production of Azadirachtin (Isoprenoid) from Plant Cell Suspension Culture of *Azadirachta indica*. A. Jau. (Niem). Indian Institute of Technology Delhi

The slide lists five objectives for the production of azadirachtin. A woman is visible in the bottom right corner of the slide frame.

So, for the plant cell suspension cultures the objectives which were laid down were to establish the cell suspension of *A. indica* for production of azadirachtin under *in vitro* condition. So, one had to establish a high yielding fast growing plant cell suspension culture of *Azadirachta indica*. Then second was to develop different strategies for enhancement of azadirachtin production in the cell suspension culture. This means that

you also develop strategies how to further improve the yield and productivity of azadirachtin.

Now, yield was considered as per unit biomass yield which means, inherent capacity of the cell line to produce that product, azadirachtin. And secondly, biomass also has to be maximized, that the overall productivity or the titer levels could improve because azadirachtin is an intracellular product. Then once you had a high yielding cell line, one can develop the cell suspension culture from that cell line. One can further optimize cell suspension cultures using different strategies for higher productivity, which could further improve the productivity.

Now, all these optimized conditions were integrated in a bioreactor. So, first before taking it into the reactor level, a suitable bioreactor was selected. It was based on the mass transfer and the mixing time characteristics. Ultimately it was based on substrate utilization rates, product formation rates and biomass formation rates. So, once a suitable reactor was selected, a batch kinetic study had to be established in that reactor.

They were able to see the kinetics of growth, and kinetics of product formation in that selected reactor. Once we knew the kinetics of product formation, substrate and growth, they were given a form of mathematical equation which was called as a model. Such a model could be manipulated *in silico* to develop feeding strategies to further improve productivity at the batch reactor level.

Now, for further improving productivity from batch, what is generally done is to remove the nutrient limitation which is done by feeding substrates. Now as you all know you have already studied that feeding can be done in various different ways. So, what should be the feed concentration, time of feed and the duration of feed and the way you feed such as exponential feeding strategy, constant feed strategy intermittent feeding, has to be optimized. You cannot afford to do experiments by hit and trial.

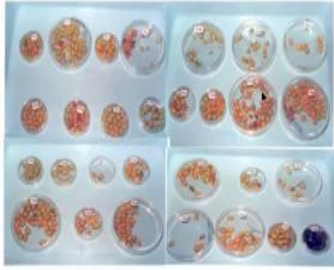
There will be n number of permutation and combinations. So, that was the reason why modeling was important. Once model was in place which was robust by fitting the batch data into those set of equations, then this model was extrapolated for fed batch and continuous cultivation. *In silico* feeding strategies were designed in fed batch and continuous cultivations and the best strategy was selected and was experimentally

verified. So, we will see how the complete steps led to enhancement in productivity, more than what is present in nature.

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Identification of elite tree (and part their off) for biopesticide production

Seeds from different parts of India (Different agroclimatic zones) were collected and azadirachtin content was measured.



Gunjan Prakash (2006). Production of Azadirachtin (Biopesticide) from Plant Cell Suspension Culture of *Azadirachta indica* A. Juss. (Neem). Indian Institute of Technology Delhi



So, what was done? I said that it is important to begin with the high yielding cell line. So, first thing is to collect the different variety of plant material from the country.

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Variability of azadirachtin content in seeds of different agroclimatic zones of India


S.No	Seeds Kernel	Aza A (mg/g)
1.	Ai-33*	0.2 ± 0.14
2.	Ai-39 [†]	0.8 ± 0.07
3.	Ai-5*	0.9 ± 0.05
4.	Ai-41 [†]	1.2 ± 0.18
5.	Ai-34*	1.3 ± 0.03
6.	Ai-10*	1.4 ± 0.04
7.	Ai-36*	1.6 ± 0.09
8.	Ai-37*	1.6 ± 0.08
9.	Ai-45 [†]	1.7 ± 0.03
10.	Ai-19*	1.8 ± 0.08
11.	Ai-17*	1.8 ± 0.08
12.	Ai-2*	1.8 ± 0.05
13.	Ai-3*	1.9 ± 0.05
14.	Ai-24*	1.9 ± 0.09

*AFRI, Jodhpur
** Ahmedabad
† Thailand

Prakash, Gunjan & Emmanuel, C. & Srivastava, Ashok. (2005). Variability of azadirachtin in *Azadirachta indica* (neem) and batch kinetics studies of cell suspension culture. *Biotechnology and Bioprocess Engineering*, 10, 198-204.

Gunjan Prakash (2006). Production of Azadirachtin (Biopesticide) from Plant Cell Suspension Culture of *Azadirachta indica* A. Juss. (Neem). Indian Institute of Technology Delhi

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15.	Ai -40*	2.1 ± 0.10
16.	Ai -31*	2.1 ± 0.11
17.	Ai -26*	2.1 ± 0.02
18.	Ai -20*	2.2 ± 0.005
19.	Ai -42*	2.3 ± 0.03
20.	Ai -25*	2.5 ± 0.07
21.	Ai -28*	2.6 ± 0.08
22.	Ai -35*	2.6 ± 0.27
23.	Ai -12*	2.8 ± 0.10
24.	Ai -6*	2.8 ± 0.06
25.	Ai -22*	2.9 ± 0.08
26.	Ai -38*	3.0 ± 0.01
27.	Ai -44*	3.2 ± 0.19
28.	Ai -4*	3.3 ± 0.05
29.	Ai -21*	3.9 ± 0.01
30.	Ai -43 [†]	5.1 ± 0.01

* Lucknow
† Tamilnadu
‡ Trivendrum

Prakash, Gunjan & Emmanuel, C. & Srivastava, Ashok. (2005). Variability of azadirachtin in *Azadirachta indica* (neem) and batch kinetics studies of cell suspension culture. *Biotechnology and Bioprocess Engineering*, 10, 198-204.

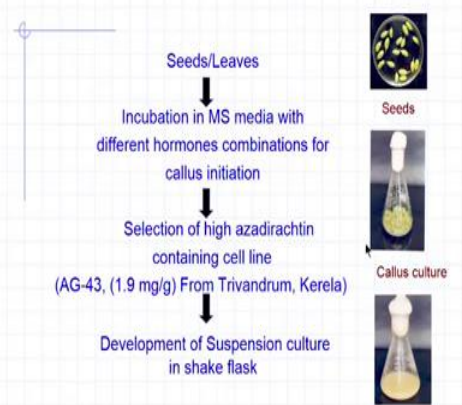
Gunjan Prakash (2006). Production of Azadirachtin (Biopesticide) from Plant Cell Suspension Culture of *Azadirachta indica*. A. Jinn. (Neem). Indian Institute of Technology Delhi



So, 30 seed varieties across the country were selected and they were screened for the azadirachtin yield in them. Once the highest yielding seed variety was found to be the Trivandrum variety, it was then taken forward to develop cell lines.

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Establishment of callus and suspension culture



Seeds/Leaves

Incubation in MS media with different hormones combinations for callus initiation

Selection of high azadirachtin containing cell line (AG-43, (1.9 mg/g) From Trivandrum, Kerela)


Development of Suspension culture in shake flask

Seeds

Callus culture

Suspension

Gunjan Prakash (2006). Production of Azadirachtin (Biopesticide) from Plant Cell Suspension Culture of *Azadirachta indica*. A. Jinn. (Neem). Indian Institute of Technology Delhi



Now, as I have mentioned before, a highest yielding plant would lead to a high yielding cell line, that was the logic behind this. Once this was done, callus was induced. Each callus line which will be induced will have a different genetic makeup and therefore, a different biosynthetic capacity. Again screening and selection of cell lines was done


based on the growth index and azadirachtin yield. And then finally, the highest yielding cell line was selected. Now once a callus line was selected, the cell line was taken forward to develop liquid culture which was suspension culture.

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To study the different strategies for enhancement of azadirachtin production in cell suspension culture of *A. indica*

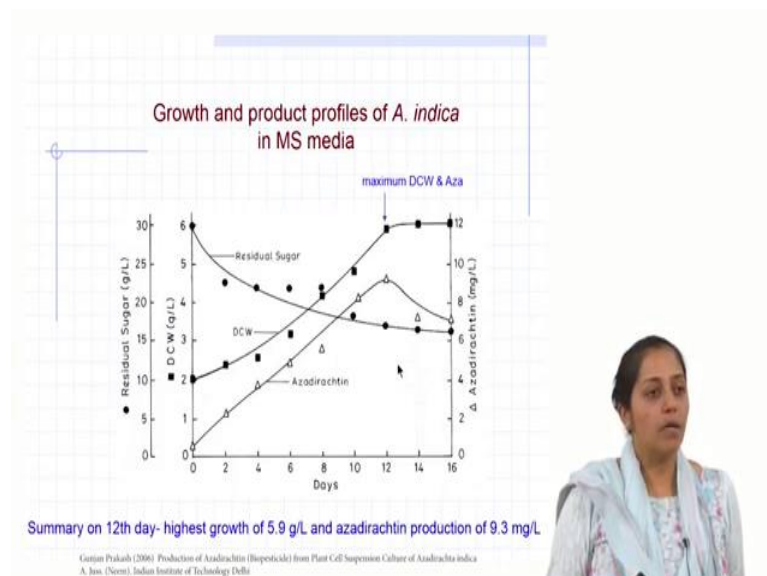
- Optimization of medium composition and culture conditions like light, temperature, pH and agitation.
- Use of biotic or abiotic elicitors that could stimulate the metabolic pathways.
- Addition of a precursor of the desired compound in the culture medium to increase the production
- Permeabilization of cells to release the secondary metabolites

Guram Prakash (2006) Production of Azadirachtin (Insecticide) from Plant Cell Suspension Culture of *Azadirachta indica* A. Jha, (Nees), Indian Institute of Technology Delhi



Now, before going on to do any kind of optimization in suspension culture, what is important? To know the reference level. Reference level means from what level I need to improve. So, for that I need to know once I have developed a suspension culture, a batch kinetics in the shake flask was plotted.

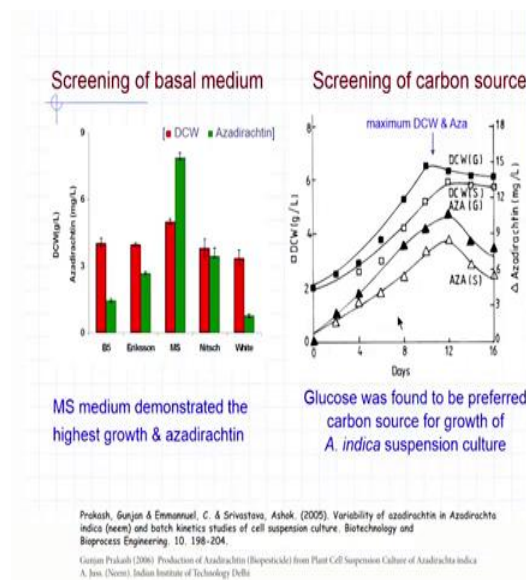
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And the day for the harvest was found from batch kinetics. Now the reference point for us is to have better productivities than what is achieved here. On the 12th day, end of the log phase, the biomass is 5.9 g/l, which is maximum biomass and azadirachtin titer, 9.3 mg/l.

Now this becomes our maximum possible azadirachtin titer in the given conditions of shake flask. Now when the suspension culture was developed, it was developed in MS media. So, a number of basal media were selected, and then suspension culture was developed in different basal media.

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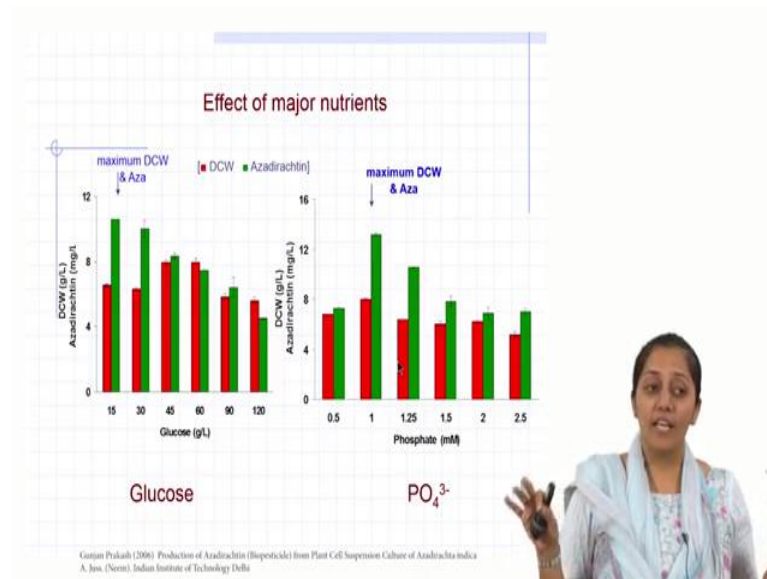
Different basal media, known compositions like B5 gamborg's medium Eriksson, MS, Nisch, White's - different predefined media for plant cell and tissue cultures were tested. So, in these media, the cell line was grown, the suspension was developed and based on the azadirachtin titer and biomass yields, MS was selected for suspension culture, for further optimization. Now in MS generally sucrose is used as carbon source in plant cell and tissue cultures.

Now, it was important because sucrose is easily available, economical than the other carbon sources. Fructose is expensive and can be only be preferred, if it is really making a remarkable difference. So, therefore, what the group did was they picked up glucose, because ultimately glucose is easily metabolisable. So, they did a kinetic study to see

how in comparison to sucrose, glucose was giving higher yields. So, they did this initial study and they found that glucose was giving better yields than sucrose.

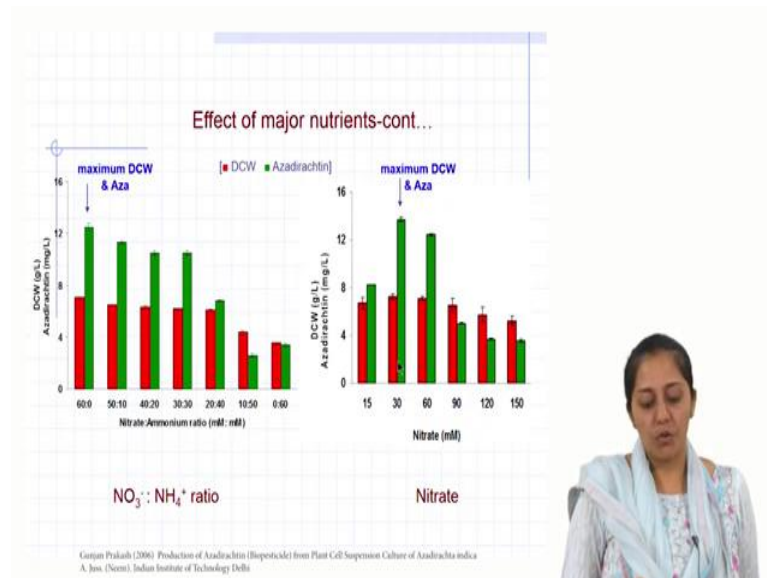
So, this kinetics on the right hand side. You can see that glucose was found to be a better carbon source than the sucrose.

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It will be nice to know what range should be selected for studying the interactive effect. So, in order to select the range for the interactive effect study using statistical tools, design of experiments, single factor experiments were carried out. Each of these major components were varied in a range and the effect on biomass and azadirachtin was observed. Based on this they could get a rough idea that where it is highest. So, that the plus minus could be further increased or decreased to get to the maximum, the optimum value using statistical design of experiments.

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So, they did for phosphate, nitrate, ammonia, glucose and they also did nitrate ammonium ratio study; because it is possible that the culture may not prefer ammonia over nitrate or may prefer nitrate over ammonia. So, then in that case, it is important to study nitrate to ammonium ratio. Interestingly what they found was that, absence of ammonia is giving the highest azadirachtin yield. So, which means while studying the interactive effect, you can even exclude ammonia or keep ammonia at very low concentrations for plus minus range.

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Plackett-Burman Design

Range of variables selected to screen the most important parameters

Level	Glucose (g/L)	Nitrate (mM)	PO ₄ ³⁻ (mM)	MgSO ₄ ·7H ₂ O (g/L)	CaCl ₂ ·2H ₂ O (g/L)	Inoculum (g/L)
Level-1	15	15	0.5	0.18	0.22	2
Level+1	60	90	2.5	0.74	0.88	8

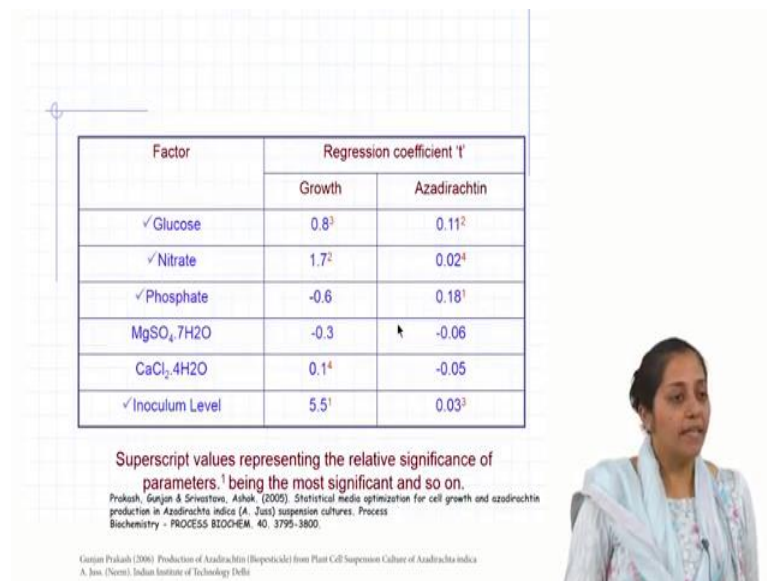
Glucose (g/L)	Nitrogen (mM)	PO ₄ ³⁻ (mM)	MgSO ₄ ·7H ₂ O (g/L)	CaCl ₂ ·2H ₂ O (g/L)	Inoculum (g/L)	Response DCW (g/l)	Aza (mg/g)
15	90	0.5	0.18	0.88	2.00	7.2	0.180
15	15	0.5	0.74	0.88	8.00	14.6	0.204
60	15	0.5	0.18	0.22	8.00	17.8	0.231
60	90	2.5	0.74	0.88	8.00	21.2	0.265
15	15	2.5	0.18	0.22	8.00	12.8	0.225
60	15	2.5	0.18	0.88	2.00	5.0	0.234
60	90	0.5	0.74	0.22	2.00	6.4	0.228
15	90	2.5	0.74	0.22	2.00	5.2	0.260

Prakash, Gunjan & Srivastava, Ashok. (2005). Statistical media optimization for cell growth and biosynthesis production in Azadirachta indica (A. Juss.) suspension cultures. Process Biochemistry - PROCESS BIOCHEM. 40, 3795-3800.

Gunjan Prakash (2006) Production of Azadirachtin (Biospeptide) from Plant Cell Suspension Culture of Azadirachta indica A. Juss. (Neem), Indian Institute of Technology Delhi

Then they also studied the rest of the major factors. Based on their single factor results, they created plus minus levels for the screening of the most significant factors in these selected major components. There are 6 major components out of which, if you design and start optimizing using statistical design of experiments, it will go up to some more than hundred experiments. So, what will be more intelligent step would be that first to see how significant each of them is and then select only the most significant top 2 or top 3 or top 4. So, this is what they did.

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Factor	Regression coefficient 't'	
	Growth	Azadirachtin
✓ Glucose	0.8 ³	0.11 ²
✓ Nitrate	1.7 ²	0.02 ⁴
✓ Phosphate	-0.6	0.18 ¹
MgSO ₄ ·7H ₂ O	-0.3	-0.06
CaCl ₂ ·4H ₂ O	0.1 ⁴	-0.05
✓ Inoculum Level	5.5 ¹	0.03 ³

Superscript values representing the relative significance of parameters, 1 being the most significant and so on.

Prakash, Gurjar & Srivastava, Ashok. (2005). Statistical media optimization for cell growth and azadirachtin production in *Azadirachta indica* (A. Juss.) suspension cultures. *Process Biochemistry* - PROCESS BIOCHEM. 40, 3795-3800.

Gurjar Prakash (2006). Production of Azadirachtin (Biphenyls) from Plant Cell Suspension Culture of *Azadirachta indica*. A. Juss. (Nem). Indian Institute of Technology Delhi

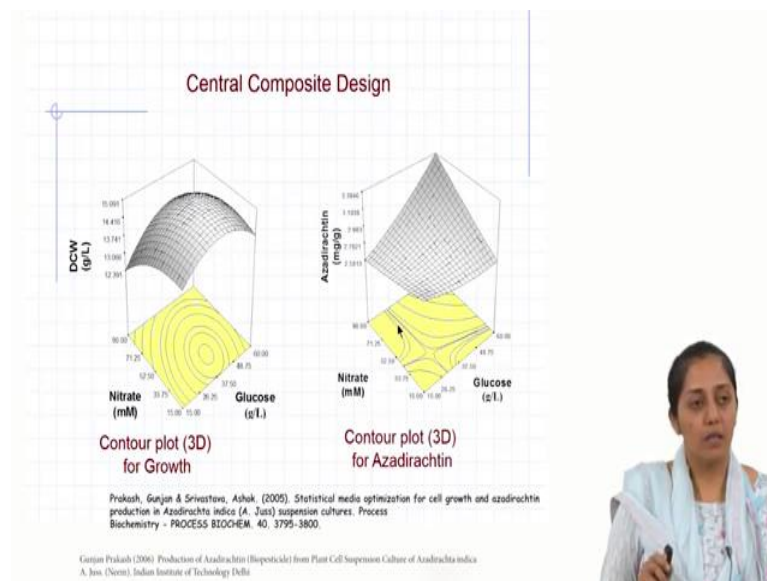
They did a plackett burman design which is a screening design in which they could able to find out which and how these 6 are impacting individually the growth and the azadirachtin yield. They could then rank these based on the ANOVA results, then the statistical analysis is done and based on that analysis you can even rank the factors based on the model which comes out and the coefficients associated with each of those main factors.

So, then based on that they could find that the inoculum level, calcium chloride, nitrate, glucose were critically affecting growth. And glucose, nitrate, phosphate and inoculum levels were affecting azadirachtin. Now our idea is to get maximum titer of azadirachtin so, but it is always recommended to do these optimizations for independent factors it should not be aliased or it should not be clubbed.

Because if you will take titer you will never be able to find how one is affecting these two independent factors, the biomass and the azadirachtin yield. So, if you will do it individually you can even design a two stage cultivation, which can give you higher productivity than a single batch; if you will do it for only titer then this will work only for batch.

Then you will have to apply other strategies for further yield or productivity enhancement. But if it is a completely non growth associated product then it is better to run it as a two stage. So, for that, in the first stage you would like to feed in nutrients which are best for growth. And in the second stage you would like to feed in nutrients which are best for the secondary product, but it depends. If it is mixed growth associated or growth associated then you can take the titers.

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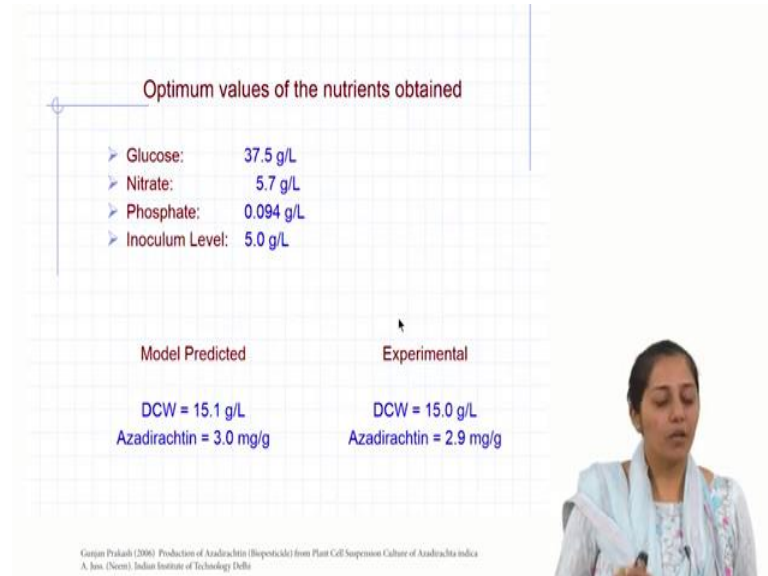


So, this was the analysis done based on the response surfaces optimization. So, the x axis and the y axis component effectively tells you, how the pairs would be affecting the growth or the azadirachtin. If you see clearly on the azadirachtin plot there is no convergence point in the design space. And the convergence point was maximum azadirachtin isn't it? That was the goal.

So, if you see even in the range selected, there is no data point which is converging. They could have added more contour plots that would give us much clear idea it is dependent on the user you can keep adding and see the convergence point. So, I am not

very sure whether the convergence point will lie within the design space, which means that there is a need to go back and see the range selected.

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But, because for us it is dependent on both the biomass as well as the secondary metabolite yield; so, you can get further to improved result from the unoptimized. They could define a media composition based on this contour analysis. See there is no 'the model' it is always 'a model'. So, you cannot claim that this is the maximum, there can always be scope for improvement. So, it is acceptable, if it is giving you better results than the reference point.


So, now the glucose, nitrate, phosphate and inoculum level were optimized. And if you can see here the biomass predicted was 15 g/l and the azadirachtin was 3 mg/g and here it is 2.9 mg/g.

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Effect of growth regulators

RUN	Growth Regulator	Conc. Tested (mg/L)	DCW (g/L)	Azadirachtin (mg/L)
Control	-	-	15.0	44.4
1.	2,4 D: Kn	4:1	14.8	37.9
2.		2:1	15.0	36.2
3.		1:0.1	15.0	36.5
4.	IBA: BA	4:1	15.2	48.7
5.		2:1	14.8	42.4
6.		1:0.1	14.8	42.1
7.	NAA: BA	4:1	15.1	43.6
8.		2:1	15.2	40.3
9.		1:0.1	15.2	40.5
10.	IAA: BA	4:1	14.8	44.5
11.		2:1	15.0	44.3
12.		1:0.1	14.7	36.4

Gurjit Prakash (2006) Production of Azadirachtin (Isoprenoid) from Plant Cell Suspension Culture of *Azadirachta indica* A. Jais. (Niem), Indian Institute of Technology Delhi




Now, then what did they do? They did auxin, cytokinin growth regulators single factor studies in different ratios. And they could find that IBA and BA were found to give maximum azadirachtin titers in this ratio.

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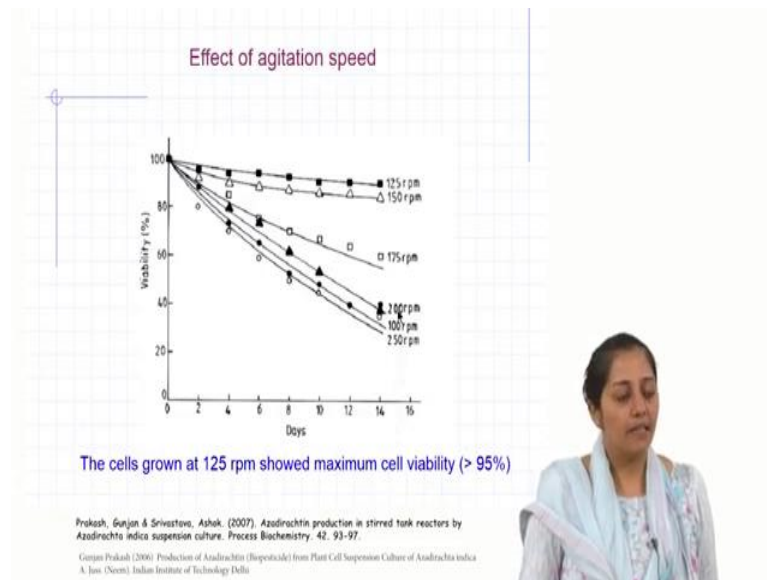
**Optimization of culture conditions
(Temperature, pH and agitation)**

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Now, see this is also improved from your reference point.

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Then they did agitation speed study. Now although mass transfer improves with agitation, the effect on the viability is also crucial, to see the overall effect on productivity or titers. So, they did a kinetic study in which they saw how the biomass is changing with time with increased RPM. So, if you can see the plot you can see beyond 125 RPM, the viability of the cell started going down. So, 125 RPM was found to be the optimum speed for maintaining the viability.

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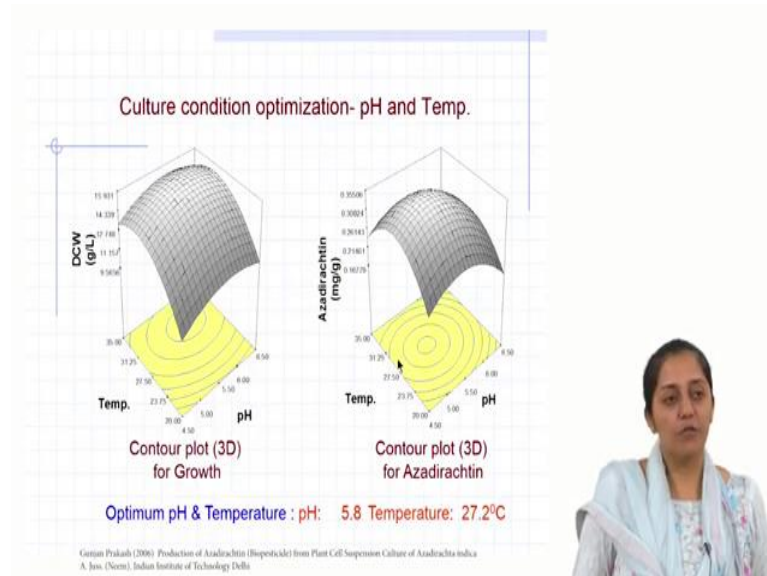
Environmental condition optimization- pH and Temperature

Experiment	pH	Temperature ^o C	DCW (g/L)	Azadirachtin Content (g/100g dry cell weight)
1.	5.5	16.8	8.12	0.116
2.	4.5	35.0	12.57	0.244
3.	6.5	35.0	12.28	0.125
4.	6.5	20.0	10.28	0.186
5.	4.5	20.0	10.28	0.277
6.	5.5	27.5	15.51	0.338
7.	5.5	27.5	15.53	0.334
8.	5.5	27.5	15.51	0.336
9.	5.5	38.1	15.19	0.210
10.	6.9	27.5	15.75	0.208
11.	4.09	27.5	11.96	0.219
12.	5.5	27.5	15.55	0.337
13.	5.5	27.5	15.51	0.339

Gunjan Prakash (2006). Production of Azadirachtin (Isoprenoid) from Plant Cell Suspension Culture of *Azadirachta indica*. A. Jinn. (Neem). Indian Institute of Technology Delhi

Then pH and temperature optimization was done, it being interactive factors, statistical design was used.

(Refer Slide Time: 18:56)



And then if you see here clearly the range seems to be fine; because they are effectively converging. And this will give you the ideal results what you expect for optimum values of pH and temperature.

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Results of medium optimization

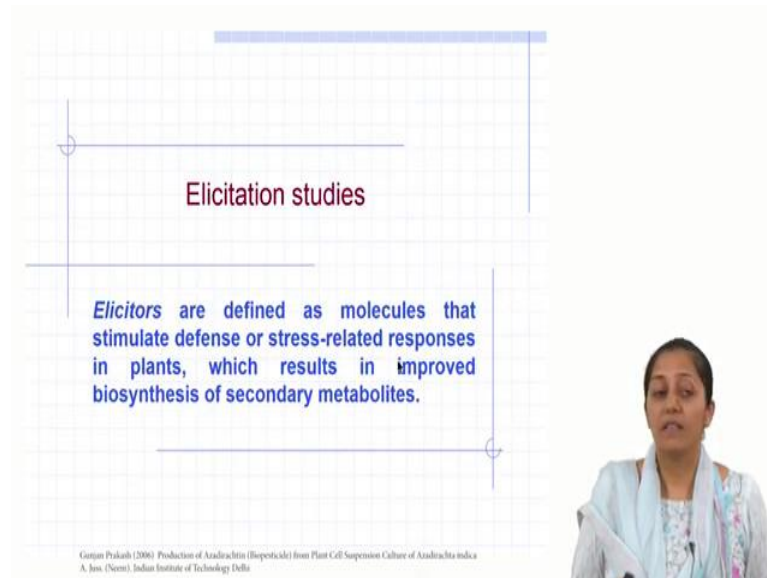
Steps	Growth [g/L]	Azadirachtin [mg/L]	Azadirachtin (mg/g)
Unoptimized medium (Shake flask)	4.9	7.8	1.59
Optimized medium (Shake flask)	15.2	47.1	3.1

Garjan Prakash (2006) Production of Azadirachtin (Biopesticide) from Plant Cell Suspension Culture of Azadirachta indica A. Juss. (Neem), Indian Institute of Technology Delhi

The table compares the results of unoptimized and optimized media. The optimized medium shows a significant increase in both growth (from 4.9 to 15.2 g/L) and Azadirachtin production (from 7.8 to 47.1 mg/L). A woman is visible in the bottom right corner of the slide.

So, now if you see the comparison to unoptimized conditions, the azadirachtin overall titre was 7.8 mg/l. But if you see the optimized conditions it had improved to 47.1 mg/l. So, this is just through simple media optimization.

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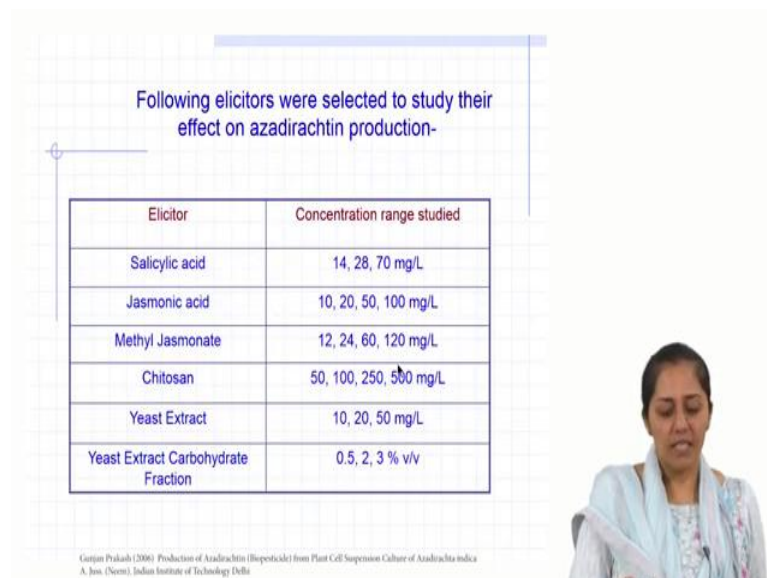
Elicitation studies

Elicitors are defined as molecules that stimulate defense or stress-related responses in plants, which results in improved biosynthesis of secondary metabolites.

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A. Jha, (Nees), Indian Institute of Technology Delhi

Now, let us see there are other strategies, which can further be used to improve the yields and productivities.

(Refer Slide Time: 19:36)



Following elicitors were selected to study their effect on azadirachtin production-

Elicitor	Concentration range studied
Salicylic acid	14, 28, 70 mg/L
Jasmonic acid	10, 20, 50, 100 mg/L
Methyl Jasmonate	12, 24, 60, 120 mg/L
Chitosan	50, 100, 250, 500 mg/L
Yeast Extract	10, 20, 50 mg/L
Yeast Extract Carbohydrate Fraction	0.5, 2, 3 % v/v

Gurjun Prakash (2006) Production of Azadirachtin (Isoprenoid) from Plant Cell Suspension Culture of *Azadirachta indica*
A. Jha, (Nees), Indian Institute of Technology Delhi


Elicitation: These are the different kinds of signaling molecules, abiotic and biotic elicitors, they tried these elicitors in different ranges.

(Refer Slide Time: 19:46)

Elicitor	DCW(g/L)	Aza(mg/g)	Aza(mg/L)
Control	15.5 ± 0.06	3.2 ± 0.03	49.7 ± 2.26
Salicylic acid (mg/L)			
SA-14	10.3 ± 0.28	4.5 ± 0.11	47.2 ± 0.13
SA-28	15.4 ± 0.06	5.5 ± 0.12	85.8 ± 1.5
SA-70	16.8 ± 0.49	8.2 ± 0.10	138.7 ± 5.86
Yeast Extract (mg/L)			
YE-10	14.6 ± 0.12	3.6 ± 0.06	53.0 ± 1.4
YE-20	16.6 ± 0.14	5.6 ± 0.11	92.9 ± 2.67
YE-50	14.1 ± 0.13	6.5 ± 0.07	91.6 ± 2.67
Yeast Extract Carbohydrate Fraction % v/v			
YECF-0.5	15.7 ± 0.14	2.8 ± 0.19	44.9 ± 2.61
YECF-2	14.7 ± 0.24	5.1 ± 0.14	75.4 ± 3.45
YECF-5	13.2 ± 0.27	5.8 ± 0.08	77.5 ± 0.5

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Contd.




Now, in different range these were all single factor experiments, they could select salicylic acid which was giving as high as 138 mg/l at that concentration.

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Jasmonic acid (mg/L)			
JA-10	16.4 ± 0.07	7.7 ± 0.09	127.9 ± 2.18
JA-20	15.5 ± 0.12	6.4 ± 0.06	99.1 ± 0.16
JA-50	18.5 ± 0.17	6.0 ± 0.05	111.8 ± 0.02
JA-100	20.1 ± 0.42	4.5 ± 0.07	90.8 ± 0.49
Methyl Jasmonate (mg/L)			
MJ-12	14.4 ± 0.10	2.9 ± 0.28	41.8 ± 3.77
MJ-24	14.5 ± 0.13	3.4 ± 0.14	49.3 ± 2.6
MJ-60	14.2 ± 0.11	3.6 ± 0.19	53.0 ± 2.54
MJ-120	14.2 ± 0.14	4.0 ± 0.06	57.3 ± 0.3
Chitosan (mg/L)			
CH-50	15.5 ± 0.21	8.9 ± 0.05	139.6 ± 2.84
CH-100	14.8 ± 0.06	7.5 ± 0.09	112.6 ± 0.88
CH-250	14.2 ± 0.11	6.9 ± 0.09	99.3 ± 0.62
CH-500	14.0 ± 0.07	3.1 ± 0.09	44.3 ± 1.04


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And also they could see that chitosan was also giving approximately the same enhancement up to 138, 140 mg/l.

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Based on the results of single elicitor addition experiment, Salicylic acid, Chitosan and Jasmonic acid were selected to study their synergistic or antagonistic interaction (if any) and the optimization of their relative concentration by Central Composite Design (CCD).



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So, then what did they do? For multiple fold enhancement, they chose the three best elicitors at the range, which they could see through the single factors. And they did an integrated study to have a synergistic effect. They again did CCD - statistical optimization.

(Refer Slide Time: 20:25)

CCD Layout for elicitor study

Run	Factor a Salicylic acid (μm)	Factor b jasmonic acid (μm)	Factor c chitosan (μm)	Dow (G/L)	Azadirachtin Mg/g
1	505	505	-327.4	12.2	8.5
2	505	-327.4	500	15.5	4.8
3	10	10	1000	15	3.2
4	10	10	10	13.5	7.3
5	505	505	505	10.4	5.4
6	505	505	505	10.2	4.8
7	1000	10	1000	10.0	17.6
8	1000	10	10	10.7	8.7
9	10	1000	10	12.5	5.2
10	505	505	505	10.3	5.5

Contd..



Surjan Prakash, Ashok K. Srivastava, Statistical elicitor optimization studies for the enhancement of azadirachtin production in bioreactor Azadirachta indica cell cultivation, Biochemical Engineering Journal, Volume 40, Issue 2, 2008, Pages 218-226, ISSN 1369-703X.

Gurjun Prakash (2006) Production of Azadirachtin (Isoprenoid) from Plant Cell Suspension Culture of Azadirachta indica A. Juss. (Neem), Indian Institute of Technology Delhi

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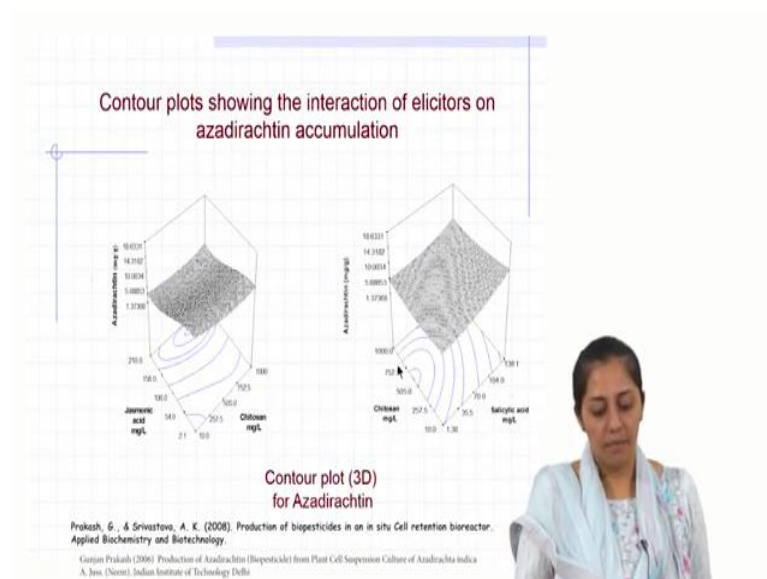
11	1337	505	505	15.5	13.2
12	505	505	505	14.7	5.7
13	505	505	505	10.3	5.5
14	10	1000	1000	12.5	9.3
15	1000	1000	1000	7.7	1.6
16	-327.4	505	505	12.7	4.3
17	1000	1000	10	16.5	6.6
18	505	505	505	10.3	6.0
19	505	505	1337.4	13.2	8.5
20	505	1337.4	505	13.6	9.6

Gunjan Prakash, Ashok K. Srivastava, Statistical elicitor optimization studies for the enhancement of azadirachtin production in bioreactor *Azadirachta indica* cell cultivation, *Biochemical Engineering Journal*, Volume 40, Issue 2, 2008, Pages 218-226, ISSN 1369-703X.

Gunjan Prakash (2006) Production of Azadirachtin (Biopesticide) from Plant Cell Suspension Culture of *Azadirachta indica* A. Jinn. (New), Indian Institute of Technology Delhi

This statistical optimization again gave them better results.

(Refer Slide Time: 20:29)



Now, for this the response would be what? It is a yield enhancement strategy. So, generally elicitor addition is well known to cause reduction in biomass. So, time of exposure to the elicitor is crucial and the response while selecting the elicitor can be yield rather than having titer. Because you would never add the elicitor on the 0 day, you would add the elicitor for an optimum exposure time such that growth is not affected by

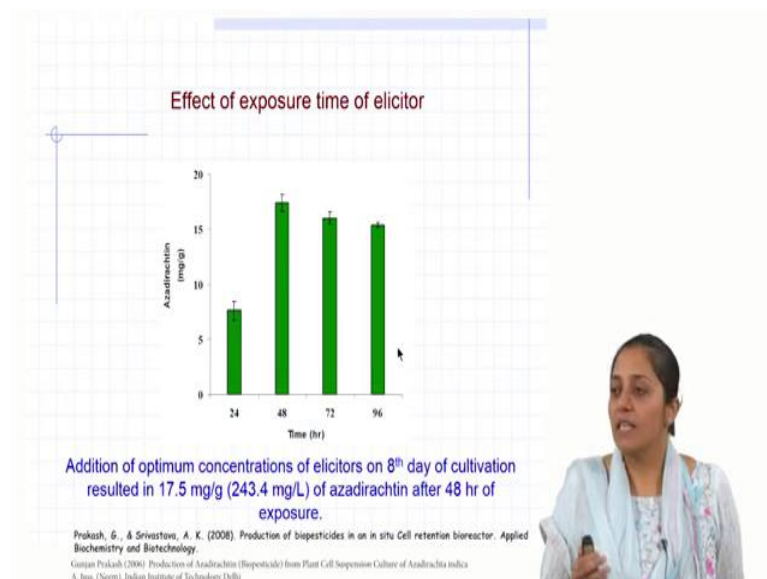
the elicitor. So, while selecting the elicitor what did they do? In this CCD design if you will see your response was azadirachtin yield.

(Refer Slide Time: 21:19)



Based on this, they could find the optimum concentrations of these three selected elicitors which could lead to maximum enhancement in azadirachtin yield. So, now it has gone up to nearly 16 mg/g.

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Now, they optimized time of addition of elicitor so that there is minimum loss in biomass. So, harvest time for the batch was 12 days if you remember. So, they added the

elicitor 48 hours, 72 hours, 96 hours, 24 hours before the harvest day. And they found that giving an exposure period of 48 hours is good enough to get the maximum yield enhancement. Later it has dipped, maybe because the viability of the cells was affected.

(Refer Slide Time: 22:18)

Precursor addition

Precursor is generally a compound, which is an intermediate, in or at the beginning of a secondary metabolite biosynthetic route, and therefore, stands a good chance of increasing the yield of the final product.

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Now, they also did precursor addition.

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Biosynthetic pathway of azadirachtin and other neem secondary metabolites

Acetate + Acetoacetyl Co-A → Hydroxymethyl glutrate

Hydroxymethyl glutrate → Mevanolate

Mevanolate → IPP

IPP → GPP

GPP → FPP

FPP → Squalene

Squalene → Azadirachtol and derivatives

Nimbolide

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Now, for precursor addition it is important to know the biosynthetic pathway. In that biosynthetic pathway, availability and concentration of the exogenously added precursor is crucial.

They chose the upstream intermediate. So, the probability of it being available for the secondary metabolism is high. Then they chose some of these precursors in the biosynthetic pathway for exogenous addition in a different range.

(Refer Slide Time: 23:01)

Precursor	Concentration range studied
Sodium Acetate	10-100 mg/L
Mevalonic acid lactone	10-100 mg/L
Isopentenyl pyrophosphate	100-1000 µg/L
Geranyl pyrophosphate	100-1000 µg/L
Squalene	10-100 mg/L

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So, this was again a single factor study.

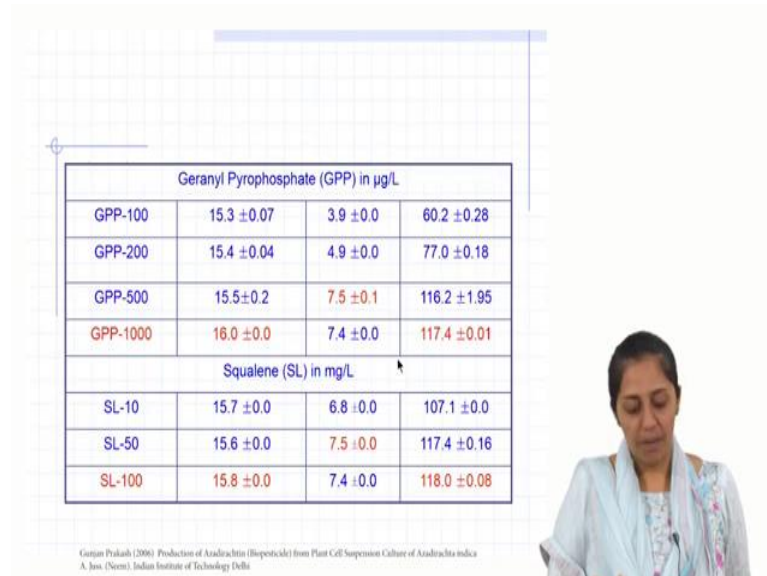
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Precursor	DCW(g/L)	Aza(mg/g)	Aza(mg/L)
Control	15.5 ± 0.06	3.2 ± 0.03	49.7 ± 2.26
Sodium Acetate (SA) in mg/L			
SA-10	16.5 ± 0.06	5.1 ± 0.01	85.5 ± 0.17
SA-50	16.8 ± 0.05	6.5 ± 0.02	110.2 ± 0.09
SA-100	16.9 ± 0.24	9.6 ± 0.02	162.7 ± 1.96
Mevalonic Acid Lactone (MAL) in mg/L			
MAL-10	15.7 ± 0.18	3.1 ± 0.0	49.6 ± 0.51
MAL-50	15.9 ± 0.31	3.9 ± 0.0	63.0 ± 1.08
MAL-100	16.0 ± 0.47	5.0 ± 0.02	80.5 ± 2.58
Isopentenyl Pyrophosphate (IPP) in µg/L			
IPP-100	15.9 ± 0.17	3.2 ± 0.01	51.3 ± 0.08
IPP-200	15.2 ± 0.08	4.3 ± 0.01	65.6 ± 0.11
IPP-500	15.7 ± 0.30	5.9 ± 0.02	92.5 ± 1.25
IPP-1000	15.9 ± 0.64	6.1 ± 0.01	96.5 ± 3.71

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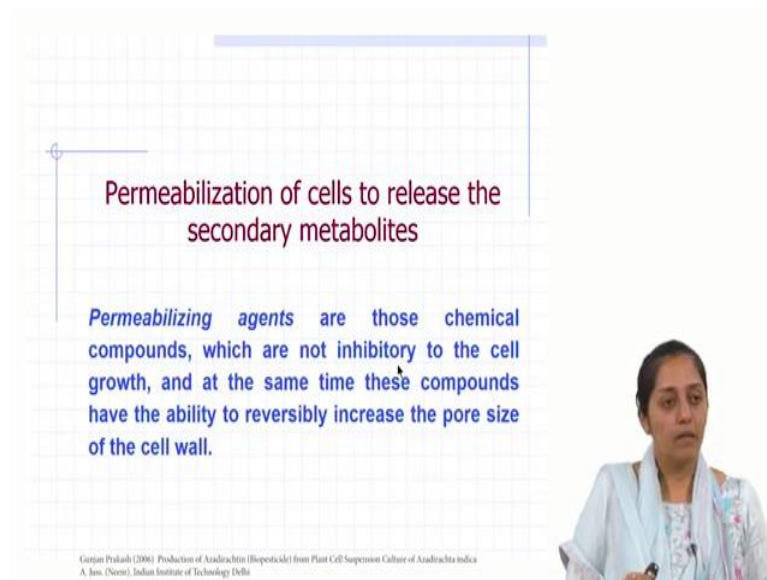
Geranyl Pyrophosphate (GPP) in µg/L			
GPP-100	15.3 ± 0.07	3.9 ± 0.0	60.2 ± 0.28
GPP-200	15.4 ± 0.04	4.9 ± 0.0	77.0 ± 0.18
GPP-500	15.5 ± 0.2	7.5 ± 0.1	116.2 ± 1.95
GPP-1000	16.0 ± 0.0	7.4 ± 0.0	117.4 ± 0.01

Squalene (SL) in mg/L			
SL-10	15.7 ± 0.0	6.8 ± 0.0	107.1 ± 0.0
SL-50	15.6 ± 0.0	7.5 ± 0.0	117.4 ± 0.16
SL-100	15.8 ± 0.0	7.4 ± 0.0	118.0 ± 0.08

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And they could chose IPP, then GPP geranyl pyrophosphate then squalene, mevalonic acid, lactone. And they could find that GPP at certain concentration level was giving more than 100 mg/l azadirachtin.

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Permeabilization of cells to release the secondary metabolites

Permeabilizing agents are those chemical compounds, which are not inhibitory to the cell growth, and at the same time these compounds have the ability to reversibly increase the pore size of the cell wall.

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So, these are all independent experiments. So, there can be two ways of optimizing; one is you can run a relay system the other is you run a parallel system and then finally, integrate. So, generally you will observe people do independent studies and then finally,

integrate as a combined study to see synergistic effects. So, then permeability enhancement was also checked.


(Refer Slide Time: 24:06)

Permeabilization studies

To promote the release of the intracellular product from the cells while maintaining the cell viability permeabilization studies were carried out.

Precursor	Concentration range studied
<i>n</i> -hexadecane	2, 5, 10, 15%
1-Dibutylphthalate	2, 5, 10, 15%
1-Decanol	2, 5, 10, 15%

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They used certain permeabilizing agents which are known in literature in concentration range which are well below that concentration which can affect the viability. But still viability was checked as the data from literature can vary among species.

(Refer Slide Time: 24:29)

Solvent (%)	DCW (g/L)	Viability (%)	Aza Yield(mg/L)		Azadirachtin Release (%)
			Intracellular	Extracellular	
Control	15.5	100	49.2	2.2	4.2
<i>n</i>-Hexadecane					
2	15.5	100	56.2	7.5	11.7
5	15.9	100	59.4	9.0	13.1
10	14.8	83.5	39.5	10.9	21.6
15	14.0	83.6	34.1	6.8	16.6
1-DNBP					
2	11.3	54.4	31.8	4.3	11.9
5	10.3	44.1	20.2	3.4	14.4
10	9.1	40.5	14.4	2.4	14.2
15	7.3	37.0	10.2	2.0	16.4
1-Decanol					
2	8.9	31.5	12.9	6.7	34.1
5	6.3	26.9	7.05	4.1	40.0
10	6.1	23.3	3.35	6.1	64.8
15	5.3	21.2	2.43	6.1	71.7

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And they could find that *n* hexadecane at 5 percent volume could give you azadirachtin release up to 13 percent. Now, why do you think other permeability enhancers which are

giving very high release have not been chosen? I see it is going up to 70 percent in decanol, but why they have chosen n hexadecane with 5 percent?

Cell viability, which will impact your biomass is affected in case of others.

(Refer Slide Time: 25:11)

Large-scale cultivation of *A. indica* suspension culture in bioreactor

Gunjan Prakash (2006) Production of Azadirachtin (Biospeckle) from Plant Cell Suspension Culture of *Azadirachta indica* A. Joshi, (NCCM) Indian Institute of Technology Delhi

The slide features a grid background with a blue header bar. The title is centered in a dark red font. A small image of a flask with yellow liquid is positioned above the title. A presenter is visible in the bottom right corner.

So, once we came to know that these are the strategies, which are leading to enhancement; then it is important to choose the right reactor for mass cultivation.

(Refer Slide Time: 25:24)

Scale up in bioreactor

- Batch cultivation (with setric impeller/ centrifugal impeller/Bubble column reactor)
- Fed batch cultivation
- Continuous cultivation with cell retention

Suspension culture

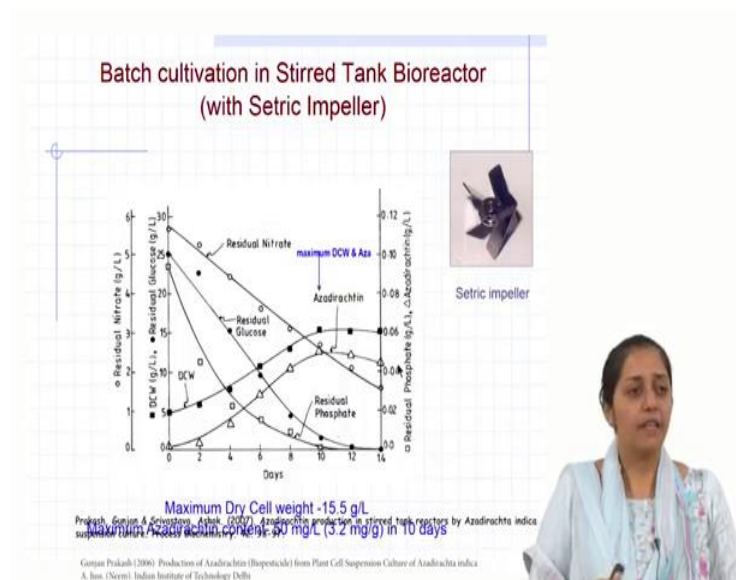
Gunjan Prakash (2006) Production of Azadirachtin (Biospeckle) from Plant Cell Suspension Culture of *Azadirachta indica* A. Joshi, (NCCM) Indian Institute of Technology Delhi

The slide features a grid background with a blue header bar. At the top, there is an image of a flask with yellow liquid labeled 'Suspension culture'. Below it, the text 'Scale up in bioreactor' is centered. Three arrows point downwards to three different cultivation methods, each accompanied by a small photograph of a bioreactor. A presenter is visible in the bottom right corner.

For that what was done? Batch cultivations were carried out. First even in the stirred tank reactor I mentioned that the shear forces can be varied by changing the impeller design. So, then they did a study in which in the same stirred tank reactor, there were different impellers which were used based on their mass transfer coefficients and mixing time characteristics.

So, they tried with setric impeller which is a low shear impeller known for plant cell cultivations. They tried with centrifugal impeller which is also well known for very low shear forces and very good mass transfer efficiency at that concentration. So, they tried these two configurations and they also did pneumatic reactors in which they used bubble column reactors.

(Refer Slide Time: 26:13)



And for this then they drew the kinetics in each of these reactors. So, first is the simple reactor, which is easy to scale up which is your stirred tank configuration well known in industry. So, stirred tank configuration setric impeller was used and the kinetics was drawn. So, what could you see as a change once it was brought from the shake flask to the reactor?

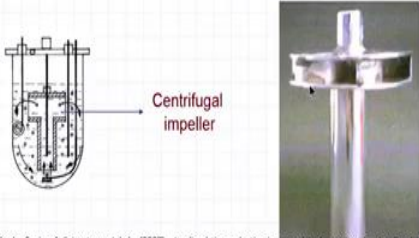
Student: Productivity

Very good. So, the productivity could be enhanced, because the kinetics was faster now and the proper reason could be the maintenance of controlled conditions.

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Batch cultivation in Centrifugal Impeller Bioreactor (with Centrifugal Impeller)

This bioreactor was designed by installing a centrifugal-pumplike impeller in a conventional stirred vessel (Wang and Zhong, 1996).



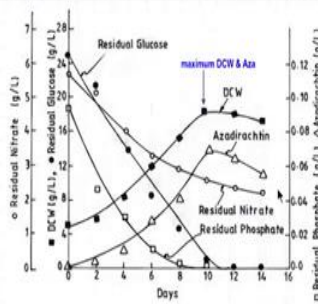
Prakash, Gunjan & Srivastava, Ashok. (2007). Azadirachtin production in stirred tank reactors by *Azadirachta indica* suspension culture. *Process Biochemistry*, 42, 93-97.

Gunjan Prakash (2006) Production of Azadirachtin (Isoprenoids) from Plant Cell Suspension Culture of *Azadirachta indica*. A. Inst. (NIRM), Indian Institute of Technology Delhi

Then centrifugal impeller was used.

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Batch kinetics in Centrifugal Impeller Bioreactor



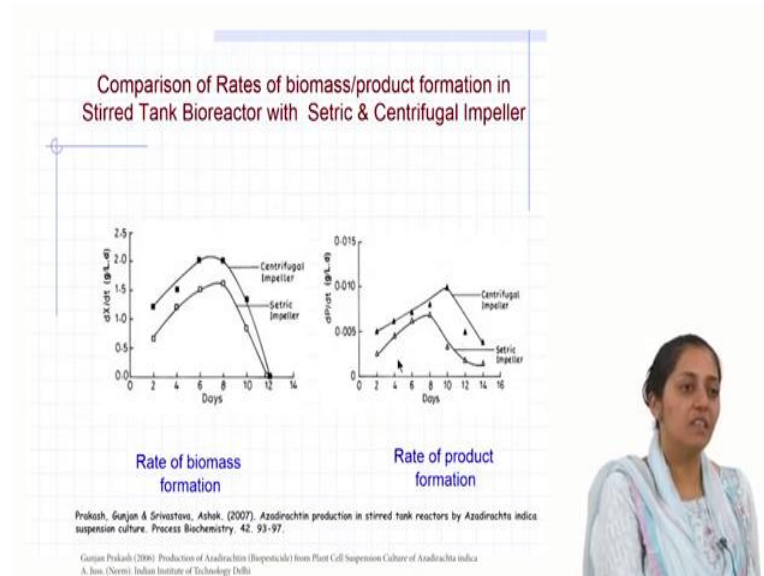
Maximum Dry Cell weight - 18.7 g/L
Maximum Azadirachtin content - 72.1 mg/L (3.8 mg/g) in 10 days

Prakash, Gunjan & Srivastava, Ashok. (2007). Azadirachtin production in stirred tank reactors by *Azadirachta indica* suspension culture. *Process Biochemistry*, 42, 93-97.

Gunjan Prakash (2006) Production of Azadirachtin (Isoprenoids) from Plant Cell Suspension Culture of *Azadirachta indica*. A. Inst. (NIRM), Indian Institute of Technology Delhi

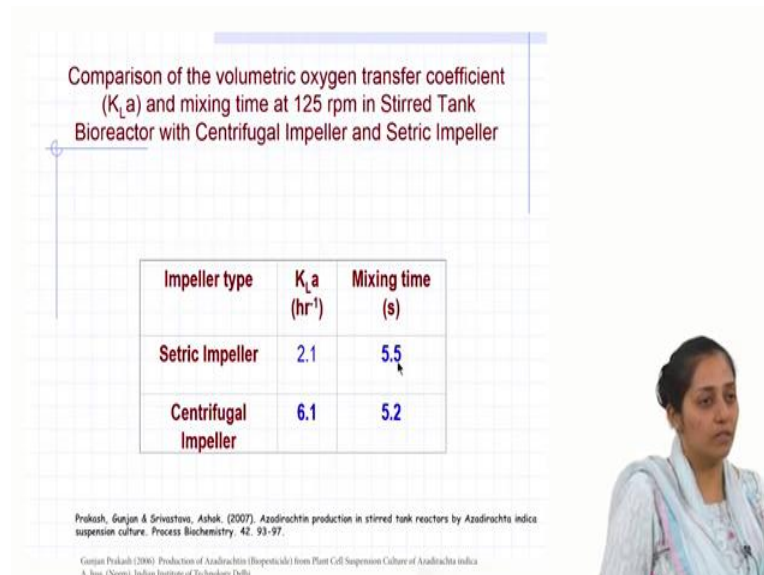
Now, again the kinetics was drawn. So, you can see that the biomass has been improved and the azadirachtin content also has improved in 10 days.

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What could be the reason? They drew biomass formation rate and product formation rate. And they could see that you can see from the figures that centrifugal impellers leads to better biomass formation rates during the cultivation period and also better product formation rates. So, they wanted to justify what could be leading to this.

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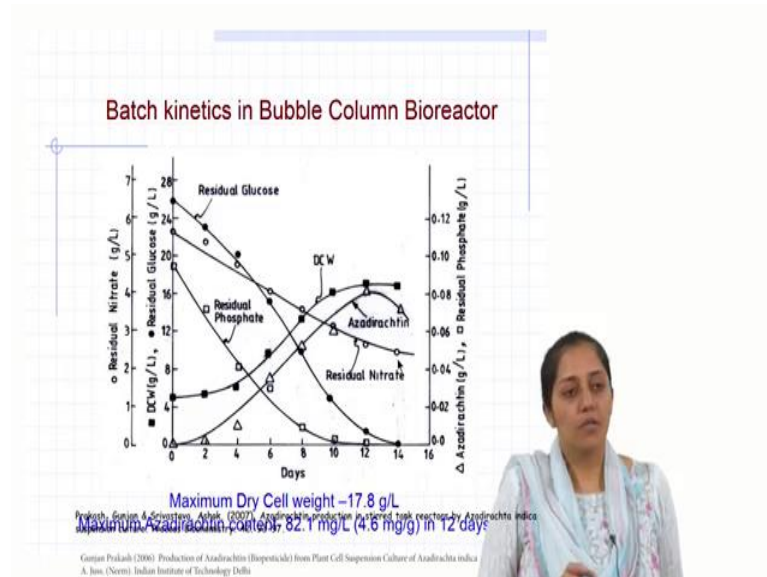


So, they calculated $k_L a$ under the given operating conditions in which the reactor was run, they calculated $k_L a$ and they also calculated the mixing time. After calculating can you see which one is better, which one is better?

Student: Centrifugal impeller

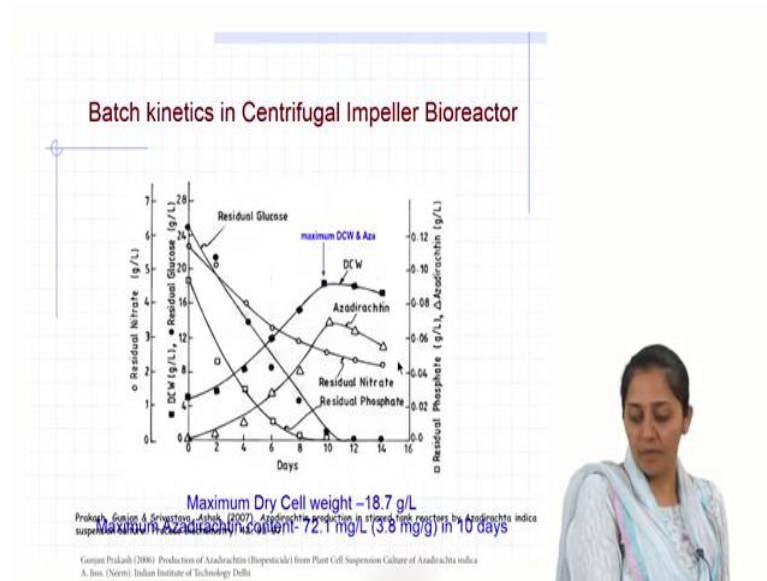
Why because $k_L a$ is better and mixing time is less

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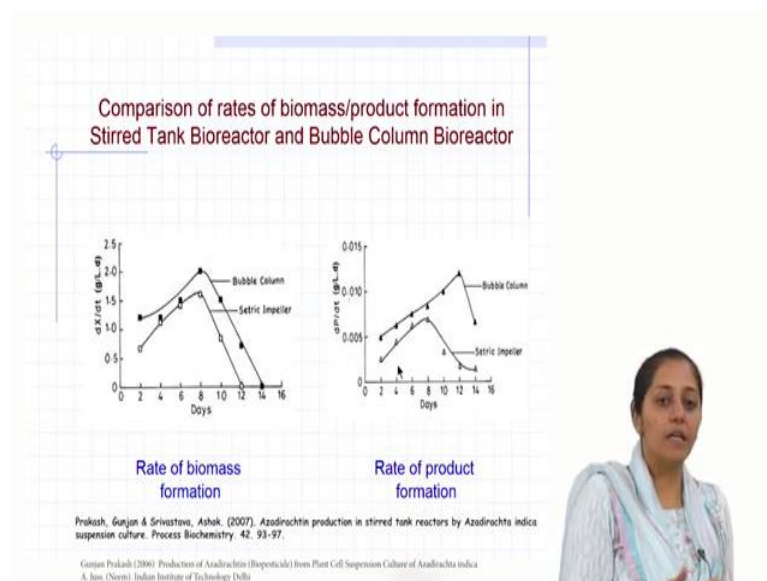


So, then pneumatically agitator reactor was used as no moving parts are there in the reactors. So, they could see that the dry cell weight was equivalent, but the growth rates have gone low. So, which means that, this may be because of better mixing because of the moving part inside the reactor in STR. So, one should prefer a stirred tank configuration rather than pneumatic reactors. So, they did a comparison with the steric impeller, in comparison to the centrifugal impeller where it had improve to 18.7 and 72 mg/l.

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So, when they did a comparison in terms of setric impeller, they could find that the biomass formation rates and the product formation rates were better. But if you compare it with the centrifugal impeller, the bubble column was lower. So, finally, a stirred tank reactor configuration with centrifugal impeller was chosen for further studies.