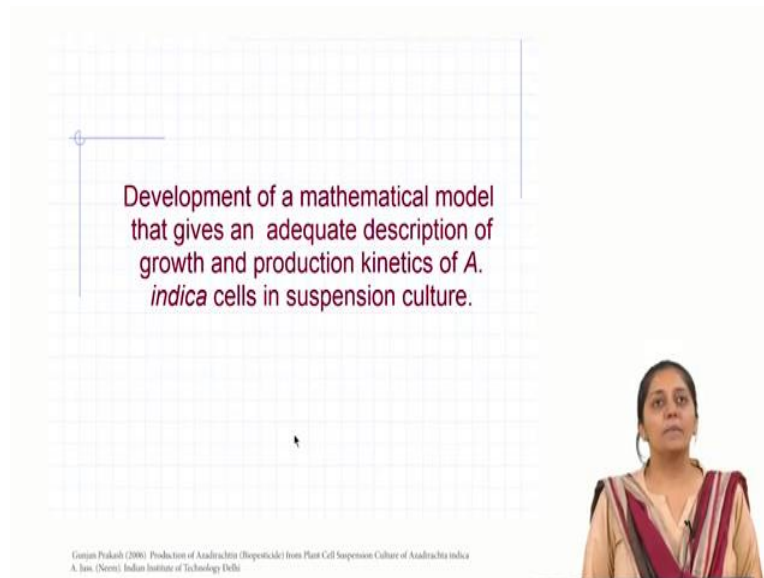


Plant Cell Bioprocessing
Dr. Smita Srivastava
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Lecture - 29
Case study – Part 2

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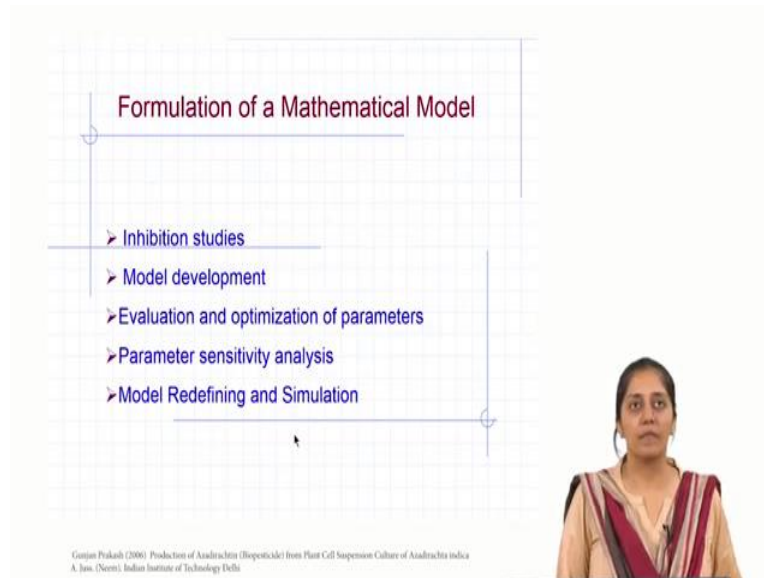
Welcome back, we were discussing the azadirachtin case study. The authors have selected the high yielding cell line for azadirachtin production followed by optimization of the culture conditions for maximum productivity of azadirachtin. They have also implemented yield enhancement strategies like, precursor addition and elicitor addition to improve the yield of azadirachtin in the cell line.

Then they went on to establish batch kinetics in different types of reactors such as stirred tank reactor and bubble column reactor. They also tried different impellers in stirred tank reactor, thereby changing the mass transfer characteristics and the mixing time to check their effect on the batch kinetics.

So, after a suitable reactor configuration was chosen for maximum productivity of azadirachtin in batch cultivation, they also developed nutrient feeding strategies in the reactor to further enhance the yield and productivity of azadirachtin in the reactors. Now, for doing this, one way is to do hit and trial with respect to time of addition of the feed, the concentration of the feed in the reactor and the manner in which the feed is done.

Other way can be the use of a more rational way of simulating and selecting nutrient feeding strategy based on a kinetic model. They developed a mathematical model that gives an adequate description of growth and production kinetics of *A. indica* cells in the suspension culture.

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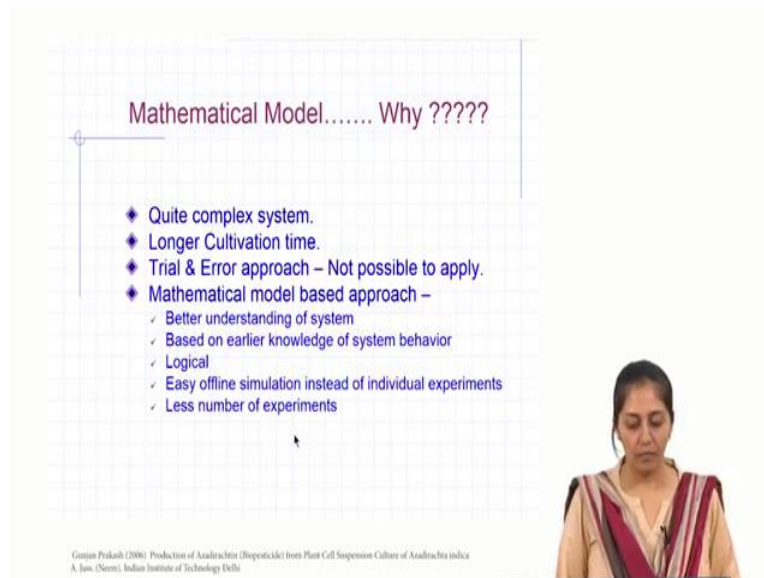


Now, when they formulated the mathematical model, the following points were kept in mind. One is, a separate substrate inhibition study with respect to the critical substrates in the medium was performed to see if beyond a certain concentration the substrates were inhibiting the growth rate of the culture.

Then they incorporated this inhibition effect in to their batch growth kinetics and then developed the model which was a description of substrate utilization kinetics, product formation kinetics and biomass (growth) kinetics. They simulated these equations, optimized and evaluated the parameters of the model equation.

Then they did parameter sensitivity analysis in the model to drop out the insignificant parameters to reduce the number of parameters in the model thereby making the model simpler for simulation. So, this is model redefining and then finally, this redefined model was used to simulate nutrient feeding strategies under fed batch or continuous cultivations.

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Mathematical Model..... Why ?????

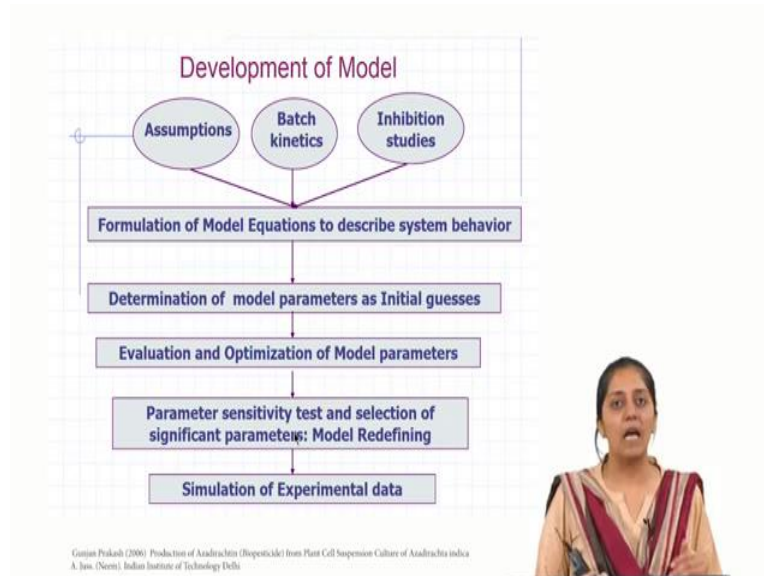
- ◆ Quite complex system.
- ◆ Longer Cultivation time.
- ◆ Trial & Error approach – Not possible to apply.
- ◆ Mathematical model based approach –
 - ✓ Better understanding of system
 - ✓ Based on earlier knowledge of system behavior
 - ✓ Logical
 - ✓ Easy offline simulation instead of individual experiments
 - ✓ Less number of experiments

Ganjan Prakash (2008). Production of Anadrachin (Biospecific) from Plant Cell Suspension Culture of Anadrachin indica A. Jain (Neeem), Indian Institute of Technology Delhi

Now, before we see the case study let us understand what is a mathematical model. So, because it is a quite complex system and plant cells take longer cultivation times, the general experimentation is more of a hit and trial approach. Hence mathematical model approach can give you a better understanding of the system.

Now, it is based on previous knowledge of the system behavior and it is therefore, more logical. Easy offline simulation can be done instead of doing hit and trial experiments and thereby the number of experiments which one would take to reach to the optimum or enhanced productivities would be less.

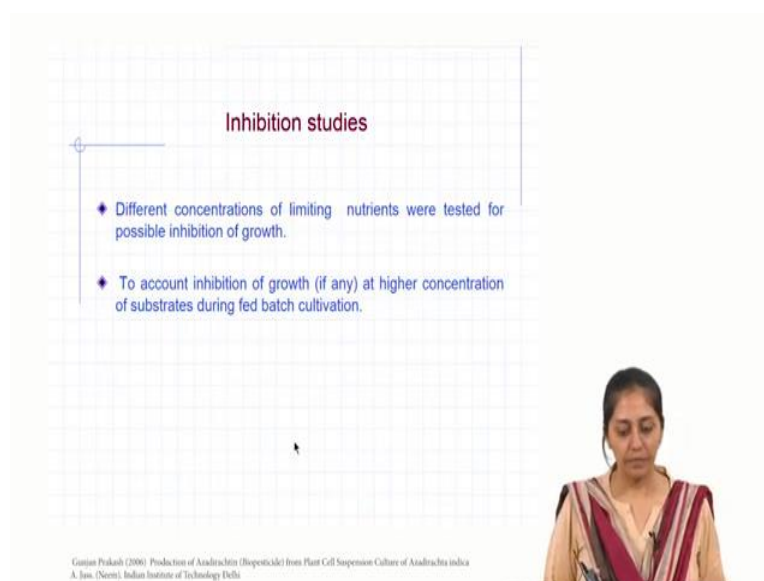
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So, what they did while developing the model? They began with certain assumptions; they had the batch kinetic data and the inhibition data in batch.

Now, they formulated the model equations to describe the system behavior. Then, they determined the model parameters based on initial guesses. Then further these model parameters were evaluated using an iterative process and parameter sensitivity analysis was done to select the significant parameter thereby redefining the model. And finally, the redefined model was simulated to predict the nutrient concentrations or the feeding strategies in the fed batch cultivation and also in a continuous cultivation.

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So, before beginning to develop the mathematical model, they did inhibition studies, in which, different concentrations of the limiting nutrients were tested for possible inhibition in growth of the culture. So, this was done to account for inhibition of growth at higher concentrations of substrate which will be fed during the fed batch cultivation.

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Mathematical equation for inhibition

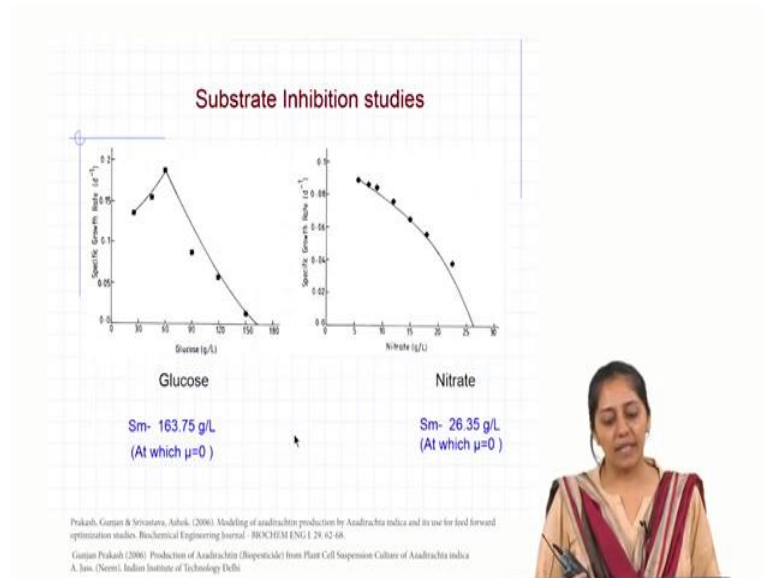
<p>◆ For growth: Monod's Kinetics</p> $\mu = \mu_m \left[\frac{S}{S + K_s} \right]$ <p>Where - μ = Specific growth rate μ_{max} = Maximum specific growth rate S = Substrate concentration K_s = Saturation constant</p>	<p>◆ For inhibition: Inhibition Kinetic equations:-</p> $\mu = \mu_m \left[1 - \left(\frac{S}{S_m} \right)^a \right]; \mu = \mu_m \left[\frac{K_i}{K_i + S} \right]$ <p>Where - S_m = Maximum inhibitory concentration of substrate at which $\mu = 0$ a = Exponent term for inhibition K_i = Inhibition constant</p>
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Gautam Prakash (2006) Production of Anadrachin (Biopesticide) from Plant Cell Suspension Culture of Anadrachin indica A. Jain (Nereis) Indian Institute of Technology Delhi



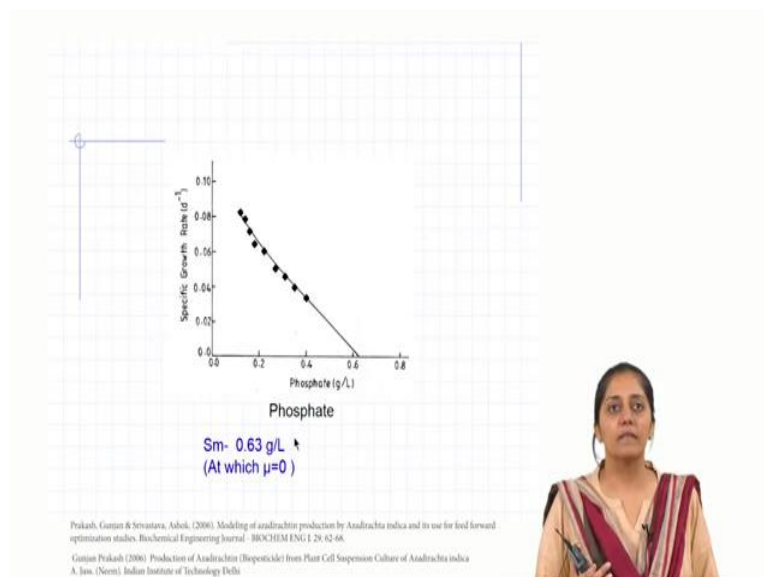
So, the different model equations which were incorporated to account for growth and for inhibition were as follows. If you can see on the slide for growth they picked up Monod's model and for defining inhibition they picked up Luong's model and also the asymptotic inhibition kinetics.

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They tried to fit the data for substrate inhibition for different rate limiting substrates.

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The substrate inhibition studies were carried out with respect to glucose, nitrate and phosphate.

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Model assumptions

- Major nutrients; carbon, nitrate, ammonium and phosphate were the limiting nutrients.
- Rest of the nutrients were available in excess in the culture broth.
- Culture environment i.e. temperature and pH remains constant throughout the cultivation.

Prakash, Ganjan & Srivastava, Ashok. (2006). Modeling of anadrachin production by Anadrachia indica and its use for fed forward optimization studies. Biochemical Engineering Journal - BIOCHEM ENG 1 29-42-06.
Ganjan Prakash (2006). Production of Anadrachin (Biopesticide) from Plant Cell Suspension Culture of Anadrachia indica. A. Jee. (Neem). Indian Institute of Technology Delhi



The model assumptions were that the major nutrients were carbon, nitrate, ammonium and phosphate. Rest of the nutrients were available in excess in the culture broth and the culture environment parameters like temperature and pH remained constant throughout the cultivation.

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Model Equations

The specific growth rate was expressed as a function of two limiting nutrients (Glucose & nitrate) by Monod's relationship and as a function of phosphate by Sigmoidal kinetics:

$$\mu = \mu_m \left[\frac{S_1}{S_1 + K_{S_1}} \right] \left[\frac{S_2}{S_2 + K_{S_2}} \right] \left[\frac{(S_3)^n}{(S_3)^n + (K_{S_3})^n} \right]$$

S₁- Glucose
S₂- Nitrate
S₃-Phosphate


Inhibition model equation:

$$\mu = \mu_m \left[\frac{K_i}{K_i + S} \right]$$

Overall biomass growth in reactor:

$$\mu = \mu_m \left[\frac{S_1}{S_1 + K_{S_1}} \right] \left[\frac{S_2}{S_2 + K_{S_2}} \right] \left[\frac{(S_3)^n}{(S_3)^n + (K_{S_3})^n} \right] \left[\frac{K_{i_1}}{K_{i_1} + S_1} \right] \left[\frac{K_{i_2}}{K_{i_2} + S_2} \right] \left[\frac{K_{i_3}}{K_{i_3} + S_3} \right]$$

Prakash, Ganjan & Srivastava, Ashok. (2006). Modeling of anadrachin production by Anadrachia indica and its use for fed forward optimization studies. Biochemical Engineering Journal - BIOCHEM ENG 1 29-42-06.
Ganjan Prakash (2006). Production of Anadrachin (Biopesticide) from Plant Cell Suspension Culture of Anadrachia indica. A. Jee. (Neem). Indian Institute of Technology Delhi



This was the model equation used which had the limiting terms with respect to all the limiting nutrients taken up in the model which are glucose, nitrate and phosphate. Then they also incorporated the inhibition terms with respect to the same three substrates to account for substrate inhibition during fed batch cultivation.

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Rate of product formation (q_p) expressed as function of both growth and non-growth components:

$$q_p = \frac{1}{X} \frac{dP}{dt} = K_1 \mu + K_2$$


The specific substrate consumption rate (q_{s_i}) was represented:

For Glucose $q_{s_1} = - \left[\frac{1}{Y_{X/S_1}} \mu + m_{s_1} \right]$

For Nitrate $q_{s_2} = - \left[\frac{1}{Y_{X/S_2}} \mu + m_{s_2} \right]$

For Phosphate $q_{s_3} = - \left[\frac{1}{Y_{X/S_3}} \mu + m_{s_3} \right]$

Prakash, Ganjan & Srivastava, Ashok. (2006). Modeling of anadrachin production by Anadrachin indica and its use for feed forward optimization studies. Biochemical Engineering Journal. BIOCHEM ENG 1 29, 62-68.
Ganjan Prakash (2006). Production of Anadrachin (Biospeptide) from Plant Cell Suspension Culture of Anadrachin indica A. Jain (Nees), Indian Institute of Technology Delhi



Then Luedeking-Piret model was used to define product formation kinetics. The last three equations demonstrate the specific substrate utilization rates, where m_s , m_{s1} , m_{s2} , and m_{s3} stand for the maintenance coefficients with respect to each of the three critical nutrients. And $Y_{x/s}$ stands for the yield coefficients with respect to substrates and μ is the specific growth rate of the culture.

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Evaluation and Optimization of Model Parameters


- A non-linear regression technique (Bard, 1974) assisted by a computer program (Votruba, 1982; Volesky & Votruba, 1992) was used to minimize deviation between model & experiment [$\Delta_j = (Y_{model} - Y_{experiment})$] for all points (n) and process variables (m).
- Calculation of model predictions was done by solving set of differential equations of the model using an integration program based on the Runge-Kutta Method of 4th order.
- Original method of Rosenbrock (Rosenbrock, 1960) was used to find out minimum value of objective function

The minimization criteria used in the program was:
(Sum of Square of Weighed Residue- SSWR)

$$SSWR = \sum_{i=1}^n \sum_{j=1}^m \frac{\Delta_j^2}{W_j^2}$$

Where:-
l = Number of experimental data points
j = Number of variables
 W_j = Weight of each variable (usually the maximum value of each variable)
 Δ_j = Difference between the model and experimental value

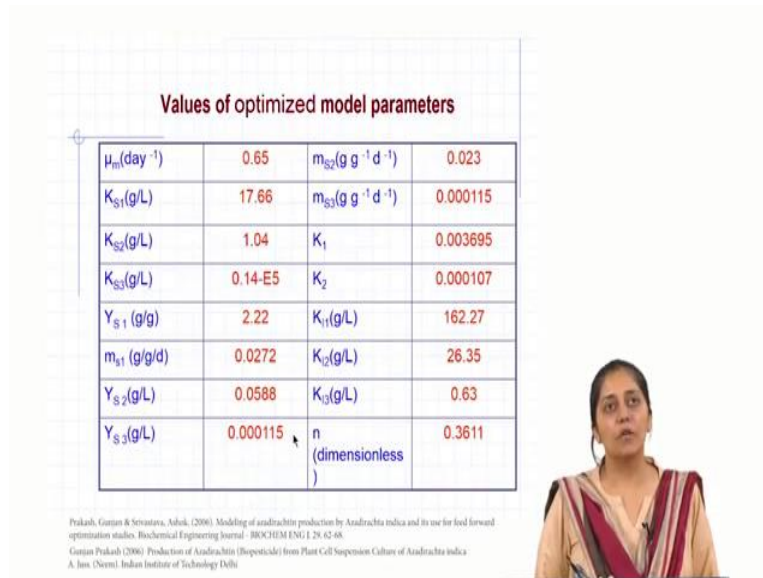
Prakash, Ganjan & Srivastava, Ashok. (2006). Modeling of anadrachin production by Anadrachin indica and its use for feed forward optimization studies. Biochemical Engineering Journal. BIOCHEM ENG 1 29, 62-68.
Ganjan Prakash (2006). Production of Anadrachin (Biospeptide) from Plant Cell Suspension Culture of Anadrachin indica A. Jain (Nees), Indian Institute of Technology Delhi



A non-linear regression technique was then applied which was assisted by a computer program to minimize the deviation between the model and the experimental values for all the data points

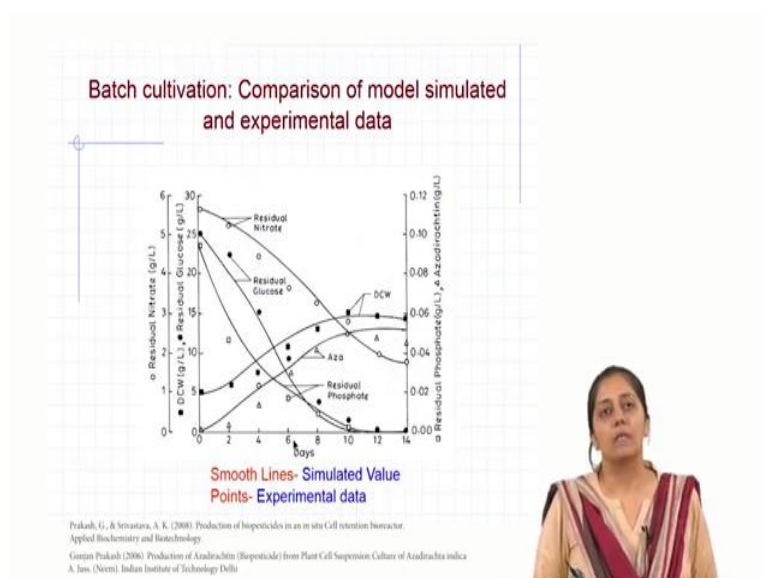
and process variables. So, an objective function which was called as sum of squares of weighed residues was used to simulate the model. So, this was based on original method of Rosenbrock, to find the minimum value of the objective function.

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These were the optimized parameter values of the model.

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Now, this picture shows that how the fitting was done using the batch kinetic data with the model.

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Extrapolation of model and development of feeding strategies

- Extrapolation for fed-batch cultivation
- Extrapolation for continuous cultivation
- Development of offline feeding strategies
- Experimental verification

Gurjun Prakash (2006) Production of Anabactin (Biopesticide) from Plant Cell Suspension Culture of Anadrachta indica A. Juss. (Niem). Indian Institute of Technology Delhi

Now, the batch model was extrapolated for fed batch cultivation and continuous cultivation to develop offline feeding strategies and then finally, the selected feeding strategy which was giving maximum productivity was experimentally verified.

(Refer Slide Time: 08:53)

Extrapolation of batch model for fed-batch/continuous cultivation

$$F = \frac{dV}{dt} = F_1 + F_2 + F_3$$

$$\frac{dX}{dt} = \mu X - \left[\frac{F}{V} \right] X$$

$$\frac{dS_1}{dt} = \left[\frac{\mu X}{Y_{x/s1}} + m_{s1} X \right] + \left[\frac{F_1 S_{01}}{V} \right] - \left[\frac{F S_1}{V} \right]$$

$$\frac{dS_2}{dt} = \left[\frac{\mu X}{Y_{x/s2}} + m_{s2} X \right] + \left[\frac{F_2 S_{02}}{V} \right] - \left[\frac{F S_2}{V} \right]$$

$$\frac{dS_3}{dt} = \left[\frac{\mu X}{Y_{x/s3}} + m_{s3} X \right] + \left[\frac{F_3 S_{03}}{V} \right] - \left[\frac{F S_3}{V} \right]$$

$$\frac{dP}{dt} = (K_1 \mu + K_2) X - \frac{F}{V} P$$

F = Total Flow rate
 F1 = Flow rate for glucose
 F2 = Flow rate for nitrate
 F3 = Flow rate for Phosphate

Dilution term (F/V) was not incorporated for cell growth and product formation in continuous cultivation due to retention of cells and intracellular product in the bioreactor by spin filter.

Prakash, Gurjun & Srivastava, Ashok. (2006). Modeling of anabactin production by Anadrachta indica and its use for feed forward optimization studies. Biotechnological Engineering Journal - BIOCHEM/ENG 1, 29, 42-48.
 Gurjun Prakash (2006) Production of Anabactin (Biopesticide) from Plant Cell Suspension Culture of Anadrachta indica A. Juss. (Niem). Indian Institute of Technology Delhi

In order to extrapolate the batch model for fed batch or continuous cultivation, they did feeding of all three critical nutrients at a constant feed rate. And, then they defined the substrate utilization rates and the product formation rates as in the batch model for all the three critical substrates.


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Parameters optimized during development of offline strategies

- ◆ Initial working volume (L)
- ◆ Start of feeding (d)
- ◆ Feed rate (L/d)
- ◆ Concentrations of substrates in feed (g/L)

The strategy resulted in maximum overall volumetric productivity (mg/L.D) with minimum residual substrate concentration possible (g/L)

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



Now, being a fed batch cultivation, the dilution terms were added to the model. Then the parameters in this model were simulated for developing nutrient feeding strategies offline, where parameters including initial working volume, start of feeding, feed rate, and concentration of substrate in the feed were varied. The strategy which resulted in maximum overall volumetric productivity with minimum residual substrate concentration in the medium after a given time was chosen for experimental validation

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feeding strategy selected for fed-batch cultivation (optimized offline)

Batch cell cultivation in bioreactor till 8th day


From 8th to 12th day
Start Feeding –
Glucose (500 g/L) @ 0.05 L/day
Phosphate (1.0 g/L) @ 0.05 L/day

From 13th to 14th day
Stop glucose feeding, Start feeding –
Nitrate (35.0 g/L) @ 0.05 L/day
Phosphate (0.5 g/L) @ 0.05 L/day

The strategy predicted a total of 22.2 g/L DCW & 90 mg/L Azadirachtin content in 14 days.

Cultus Suspension culture (Inoculum)
Inoculation
Batch Cultivation
Feeding
Fed-batch Cultivation
Harvest

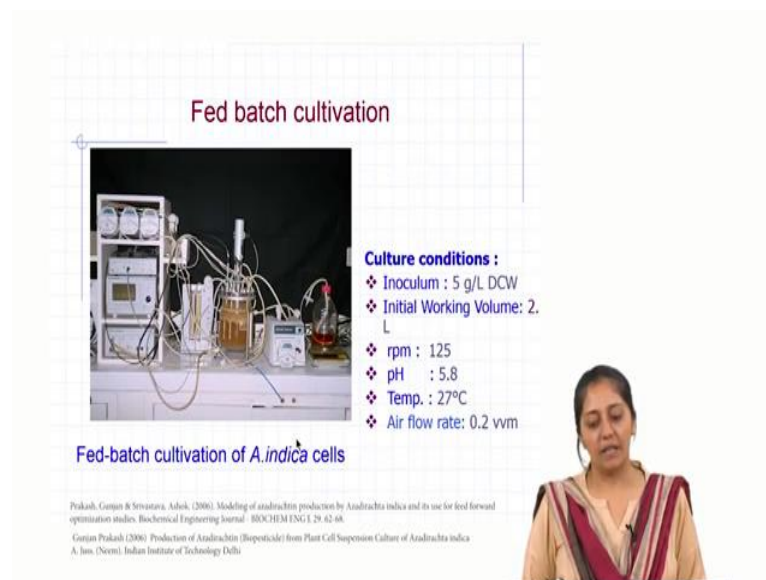
Garjan Prakash (2006) Production of Azadirachtin (Biopesticide) from Plant Cell Suspension Culture of *Azadirachta indica* A. Jais (Neem), Indian Institute of Technology Delhi



So, the feeding strategy which was selected for fed batch cultivation was as follows: Batch cultivation was carried out in the reactor till 8th day. Then from 8th till 12th day glucose feeding of 500 g/l and phosphate of 1 g/l were done at a rate of 0.05 liters per day in the reactor. Then from 13th to 14th day the glucose feeding was stopped and nitrate additional feed started at 35 g/l and the phosphate feed was continued, but at a reduced concentration of 0.5 g/l with the flow rate of all the feeds at 0.05 g/l.

So, if you notice the glucose feeding was stopped, the phosphate feeding was reduced and the nitrate feeding was started. This strategy as predicted by the model gave a total of nearly 22 g/l of biomass and 90 mg/l of azadirachtin in 14 days.

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Fed batch cultivation

Culture conditions :

- ❖ Inoculum : 5 g/L DCW
- ❖ Initial Working Volume: 2. L
- ❖ rpm : 125
- ❖ pH : 5.8
- ❖ Temp. : 27°C
- ❖ Air flow rate: 0.2 vvm

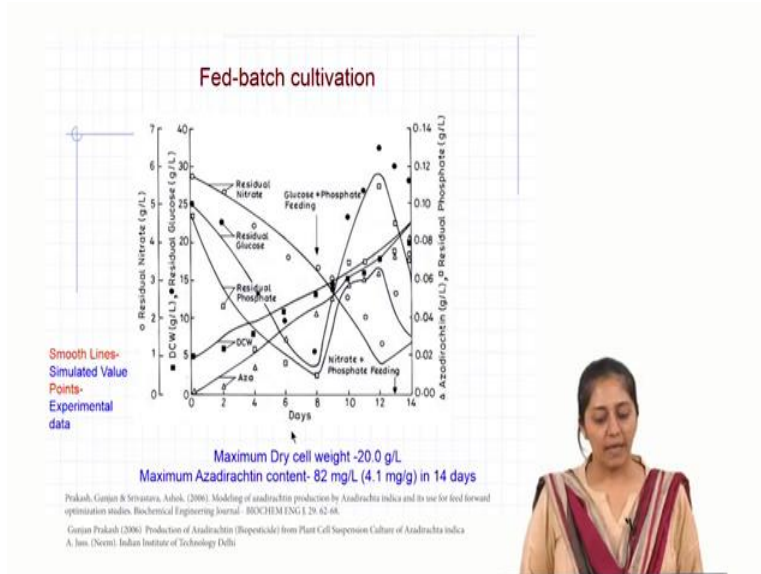
Fed-batch cultivation of *A. indica* cells

Prakash, Ganjan & Srivastava, Ashok. (2006). Modeling of azadirachtin production by *Azadirachta indica* and its use for feed forward optimization studies. *Biochemical Engineering Journal*. BIOCHEM ENG 1, 29-42-66.

Ganjan Prakash (2006) Production of Azadirachtin (Biospesticide) from Plant Cell Suspension Culture of *Azadirachta indica* A. Ind. (Neem). Indian Institute of Technology Delhi

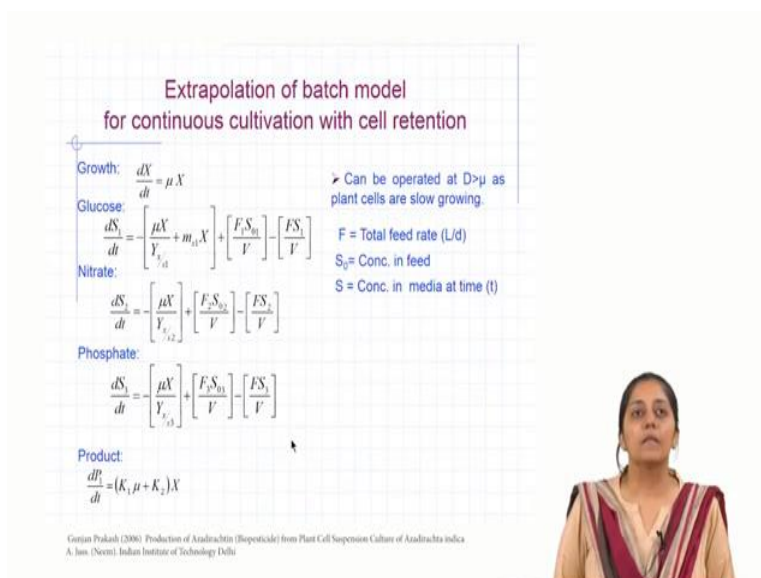
So, these were the conditions which were used for the fed batch cultivation and then the fed batch cultivation was experimentally validated.

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So, if you see the kinetics, the plot shows that as the feeding has started from 8th day you can see the residual nitrate, the residual phosphate and glucose concentrations getting high and then gradually the biomass and the azadirachtin feed increasing. Now, the dry cell weight and the azadirachtin is increasing continuously up till 14 days. Residual nitrate was becoming limiting around 12th day, so possibly that is the reason why the feeding was again started for the nitrate on day 12.

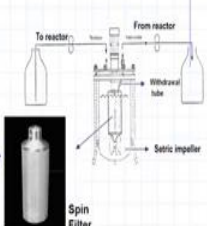
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Then the batch model was further used to develop nutrient feeding strategies in continuous cultivation, where again the same model parameters were used to design equations for the continuous cultivation.

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continuous cultivation with cell retention (Spin filter)
(optimized offline)



Batch cell cultivation in bioreactor till 8th day

MS medium salts with glucose (75 g/L), nitrate (10 g/L) and phosphate (0.5 g/L) @ 0.5 L/day in the annular space of the bioreactor outside the filter till 26th day. Medium was withdrawn at the same rate from within the spin filter

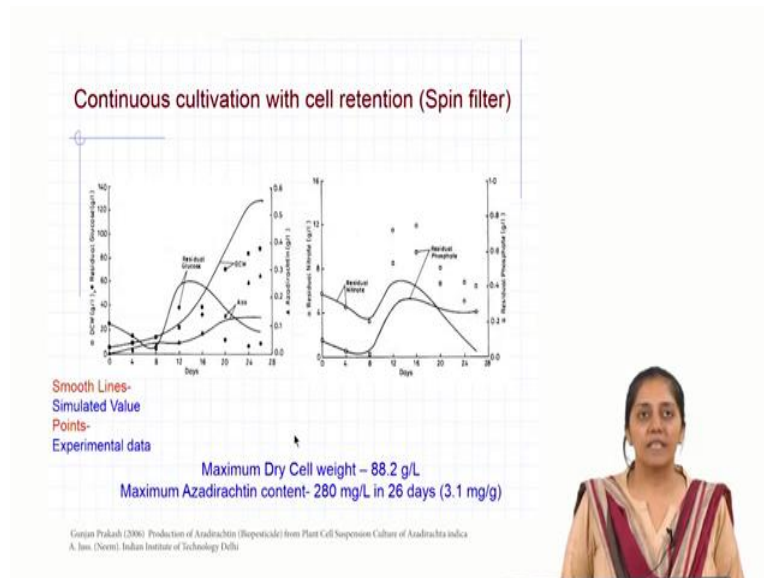
➤ Model predicted a 139.1 g/L DCW & 130 mg/L Azadirachtin in 26 days.

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

And then the model was simulated and experimentally validated. One of the strategies, which could give high azadirachtin productivities, in fed batch or in batch was chosen for experimental validation in continuous cultivation. The MS medium salts with glucose of 75 g/l, the nitrate feed of 10 g/l and phosphate of 0.5 g/l at a flow rate of 0.5 liters per day was chosen for the experimental study.

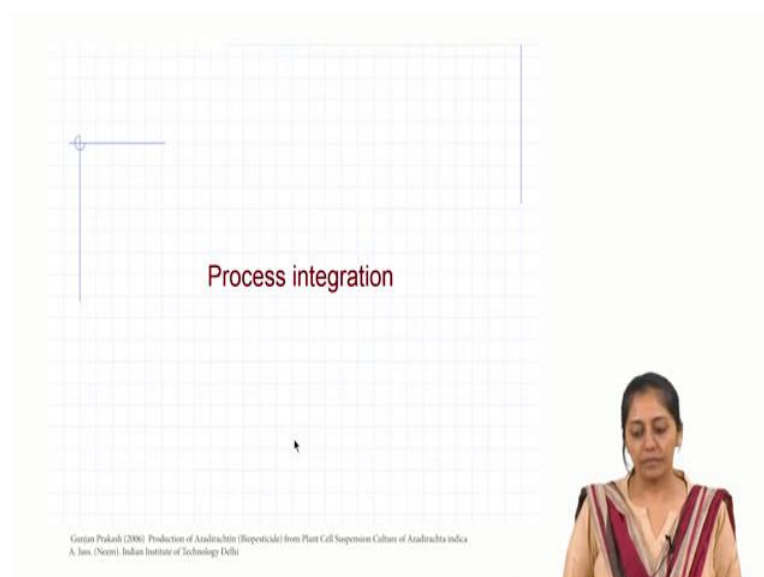
This continuous reactor which was run was a cell retention reactor set up, in which the feed was sent in the annular space of the reactor outside the filter till the 26th day and the medium was withdrawn at the same rate from inside the spin filter. The model in this case predicted nearly 140 g/l of biomass and 130 mg/l of azadirachtin in 26 days.

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So, if you see the plots you can clearly see that as the feeding was done in the continuous reactor with cell retention it led to increase continuous increase in the biomass and also in the azadirachtin productivity. So, experimentally the biomass which could be achieved was around 88 g/l and the azadirachtin concentration or titers which could be achieved now were at a level of 280 mg/l in 26 days.

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After, achieving and designing nutrient feeding strategies for continuous enhancement in the productivity of azadirachtin,

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Elicitor addition in Stirred Tank Bioreactor

Cells in bioreactor (batch mode) were treated with the optimum concentration of elicitors [salicylic acid (137.31 mg/L), jasmonic acid (2.9 mg/L) and chitosan (16.55 mg/L)] after 8 days of cultivation.



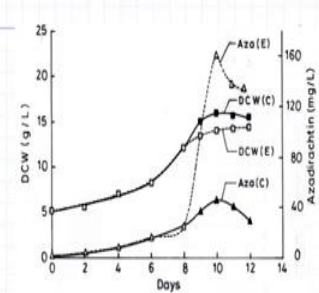
Garjan Prakash (2006) Production of Azadirachtin (Isoprenoid) from Plant Cell Suspension Culture of *Azadirachta indica* A. In: (Nees) Indian Institute of Technology Delhi

the authors went on to do process integration in which they added the elicitor and precursor selected from the shake flask studies to further enhance the yield of azadirachtin and thereby the productivity. So, the cells and bioreactors in the batch mode, were treated with optimum concentration of elicitors after 8 days of cultivation.


So, these elicitors were chosen from the previous study of optimization with elicitors. So, salicylic acid, jasmonic acid and chitosan were selected at their optimum concentrations based on the design of experiments and statistical optimization done.

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Growth and azadirachtin production in bioreactor with elicitor addition



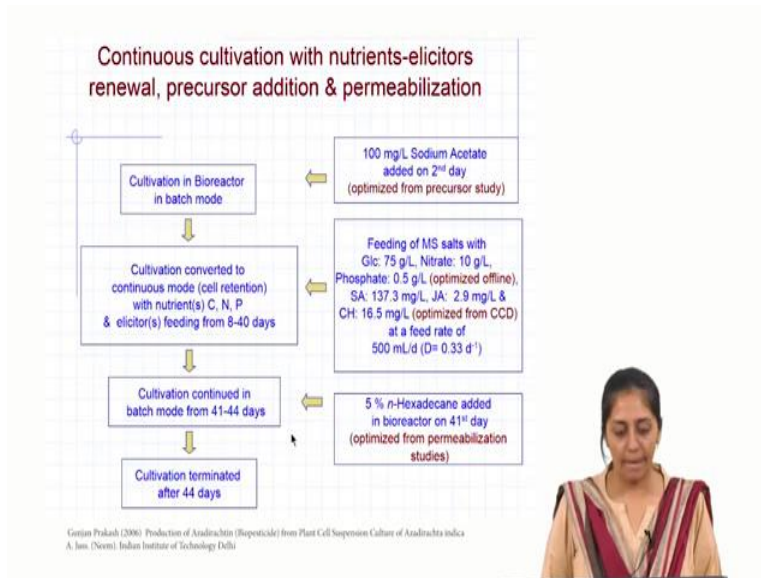
Parameter	Condition	Value	Time
Maximum Dry Cell weight	(C)	15.5 g/L	10 days
Maximum Dry Cell weight	(E)	14.0 g/L	10 days
Maximum Aza content	(C)	50 mg/L (3.2 mg/g)	10 days
Maximum Aza content	(E)	161.1 mg/L (11.5 mg/g)	10 days



Prakash, G., & Srivastava, A. K. (2006). Production of isoprenoids in an in situ Cell retention bioreactor. --Applied Biochemistry and Biotechnology.
Garjan Prakash (2006) Production of Azadirachtin (Isoprenoid) from Plant Cell Suspension Culture of *Azadirachta indica* A. In: (Nees) Indian Institute of Technology Delhi

Then these elicitors were added on 8th day and growth and azadirachtin production in the reactor was studied. And after addition the biomass was nearly the same which was 14 g/l, while the azadirachtin content increased to 11.5 mg/g in 10 days which was much higher than that obtained in the batch reactor in the absence of elicitor.

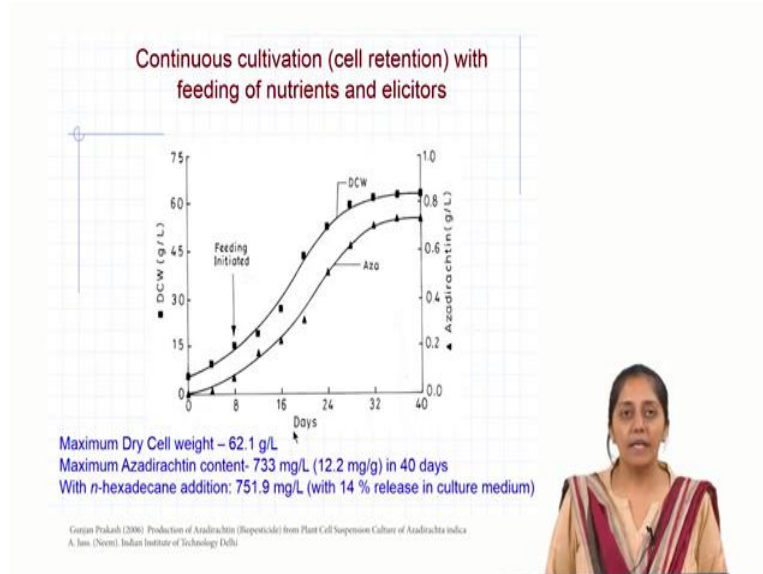
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Then continuous cultivation with nutrient addition, elicitor addition, precursor addition, and permeabilization was all done together to see an integrated effect where the cells were grown in bioreactor in batch mode. Then the cultivation was converted to continuous mode with cell retention and nutrient addition of carbon, nitrogen and phosphate with elicitor feeding from 8th till 40 days. And the cultivation was continued thereafter in batch mode till 44 days.

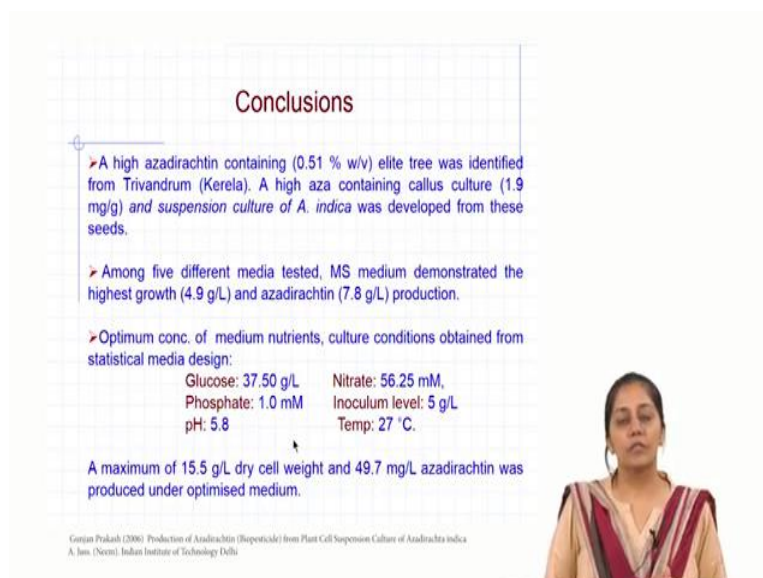
So, if you see on the right hand side, sodium acetate was used as a precursor which was added on second day, then feeding of the MS salts with glucose, nitrate and phosphate in the feed, which was optimized offline was done from 8th day till 40th day. Along with it, the addition of salicylic acid, jasmonic acid and chitosan was also done which was optimized previously using central composite design at a feed rate of 500 ml/day. Then permeability enhancers were added on 41st day when the reactor was continued in batch mode till 44th day to enable the release of the product from the cells where 5 % n-hexadecane was added in the reactor.

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So, what did they achieve? The maximum dry weight which could be achieved was now 62 g/l and the maximum azadirachtin concentration or titers achieved were more than 730 mg/l. Now, after addition of hexadecane 751 mg/l of azadirachtin was obtained thus from the final harvest, while 14 % release in the culture medium was observed.

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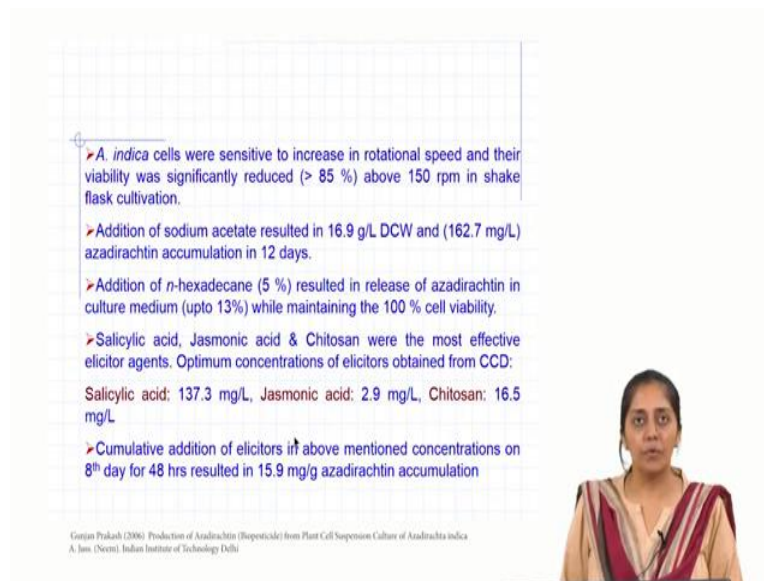


So, this is a summary of what the authors did in this case study, they began with a high azadirachtin containing tree based cell line. They developed callus culture and suspension culture. Among five different media tested, Murashige and Skoog medium demonstrated highest growth and azadirachtin production.

Then optimum concentration of the medium nutrients, culture conditions was obtained using statistical design of experiments which will minimize the number of hit and trial. And then after this they were able to achieve 15 g/l of biomass and nearly 50 mg/l of azadirachtin under optimum conditions in shake flask.

The cells were found to be sensitive to shear with increase in the rotational speed where the viability were found to drop more than 85 % above 150 rpm in shake flask.

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- *A. indica* cells were sensitive to increase in rotational speed and their viability was significantly reduced (> 85 %) above 150 rpm in shake flask cultivation.
- Addition of sodium acetate resulted in 16.9 g/L DCW and (162.7 mg/L) azadirachtin accumulation in 12 days.
- Addition of *n*-hexadecane (5 %) resulted in release of azadirachtin in culture medium (upto 13%) while maintaining the 100 % cell viability.
- Salicylic acid, Jasmonic acid & Chitosan were the most effective elicitor agents. Optimum concentrations of elicitors obtained from CCD:
Salicylic acid: 137.3 mg/L, Jasmonic acid: 2.9 mg/L, Chitosan: 16.5 mg/L
- Cumulative addition of elicitors in above mentioned concentrations on 8th day for 48 hrs resulted in 15.9 mg/g azadirachtin accumulation

Geetanjali Prakash (2006) Production of Azadirachtin (Biopesticide) from Plant Cell Suspension Culture of *Azadirachta indica*
A. Jain (NCCM), Indian Institute of Technology Delhi

Addition of sodium acetate was done as a precursor where azadirachtin productivity was improved to 162 mg/l in 12 days. Then further permeability enhancers were optimized and hexadecane 5 % was chosen which could result in 13% release of azadirachtin in the medium while maintaining the viability up as 100 %.


Then elicitors were chosen and their concentrations were optimized using statistical design of experiments where salicylic acid, jasmonic acid and chitosan were chosen. Then cumulative addition of these elicitors on 8th day for 48 hours before harvest led to the yield increase up to nearly 16 mg/g.

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► Cultivation in Bioreactor

Cultivation	DCW	Aza (mg/L)	Aza (mg/g)	Aza (mg/L.d)
Batch- Steric impeller	15.5	50	3.2	5.0
Batch- Centrifugal impeller	18.7	72.1	3.8	7.2
Batch- Bubble column reactor	17.8	82	4.6	6.8
Fed-batch cultivation	20.0	82	4.1	5.8
Continuous cultivation (Cell retention)	88.8	280	3.1	10.7
Batch cultivation (elicitor addition)	14.0	161.1	11.5	16.1
Continuous cultivation (nutrients-elicitor renewal, precursor addition, permeabilization)	61.4	751.9	12.2	17.4

Genjan Prakash (2006) Production of Anadrachin (Biphenoxide) from Plant Cell Suspension Culture of *Anadrachia indica* A. In: (Niem) Indian Institute of Technology Delhi



So, if you see a snapshot of how the productivity improved from different modes of cultivations:- when they did batch with steric impeller the productivity was 5 mg/l./d. When they did batch with a centrifugal impeller which had improved mass transfer characteristics and mixing time characteristics the productivity improved to 7.2 mg/l./d.


Then they did continuous cultivation with cell retention device where the productivity was found to further improve and then batch cultivation with elicitor addition was seen to increase the productivity to very high levels almost doubling or three folds rather. Then, continuous cultivation with an integrated study which included addition of elicitor, precursor, permeability enhancers and nutrient feeds led to the maximum enhancement in productivity up to 17 mg/l./d

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Comparison with literature data

References	Culture System	Explant	Azadirachtin content
Allan et al. 1994	Callus culture	Leaves	7 µg/g DCW
Kearney et al. 1994	Callus/Suspension/Shoot culture	Leaves Shoot	Not detected
Wewetzer 1998	Callus culture	Leaves Bark	64 µg/g DCW 44 µg/g DCW
Veeresham et al. 1998	Callus culture	Leaves Flowers	26.8 mg/g DCW 24.6 mg/g DCW
Srividhya et al. 1998	<i>In vitro</i> roots	Roots	0.004 mg/g DCW
Allan et al. 2002	Hairy root culture	Leaves	27 µg/g DCW
Balaji et al. 2003	Suspension culture (Shake flask + precursor)	Flower	45 mg/L
Present study	Suspension culture Precursor addition + (continuous feeding of nutrients and elicitors)	Seed kernel	12.2 mg/g DCW

Prakash, G., Bhawan, S. S., & Srivastava, A. K. (2002). Production of azadirachtin from plant tissue culture: State of the art and future prospects. *Biotechnology and Bioprocess Engineering*
Ganjan Prakash (2006). Production of Azadirachtin (Biopesticide) from Plant Cell Suspension Culture of *Azadirachta indica*. A. Jain. (Noida): Indian Institute of Technology Delhi



So, if you compare literature what they found was that they could achieve maximum yields of azadirachtin in the biomass by using this rational and a more systematic manner of optimization.

So, I hope this gives you an overall picture of how in plant cell bioprocessing different strategies can be implemented to achieve maximum enhancements in productivities of plant secondary metabolites.