Plant Cell Bioprocessing
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Lecture - 29
Case study – Part 2

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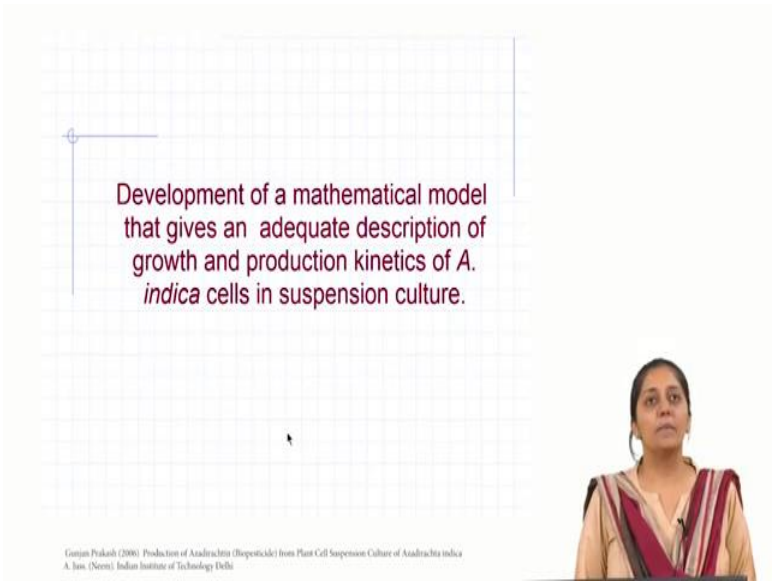

Welcome back, we were discussing the azadirachtin case study. The authors have selected the high yielding cell line for azadirachtin production followed by optimization of the culture conditions for maximum productivity of azadirachtin. They have also implemented yield enhancement strategies like, precursor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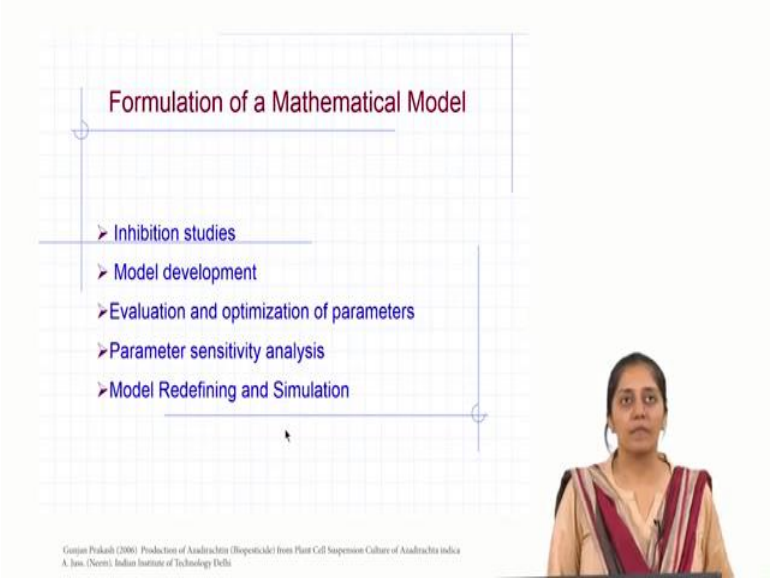

Now, when they formulated the mathematical model, the following points were kept in mind. One is, a separate substrate inhibition study with respect to the critical substrates in the medium was performed to see if beyond a certain concentration the substrates were inhibiting the growth rate of the culture.

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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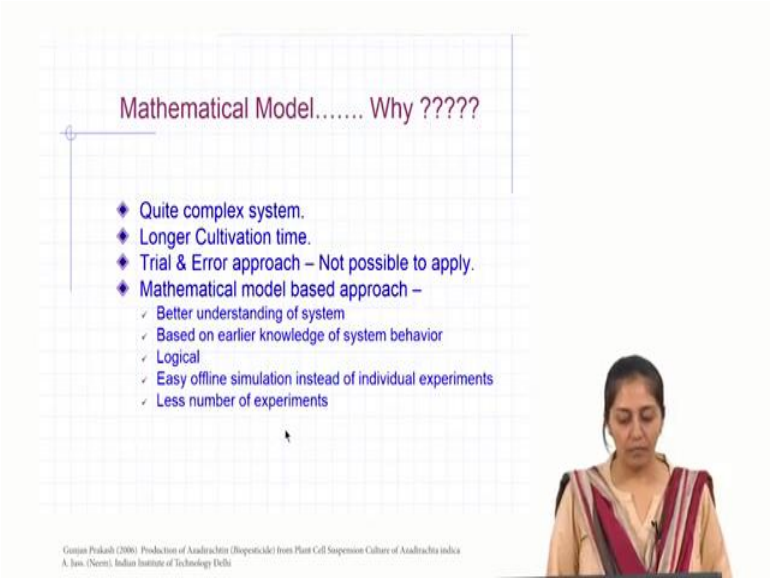

Now, before we see the case study let us understand what is a mathematical model. So, because it is a quite complex system and plant cells take longer cultivation times, the general experimentation is more of a hit and trial approach. Hence mathematical model approach can give you a better understanding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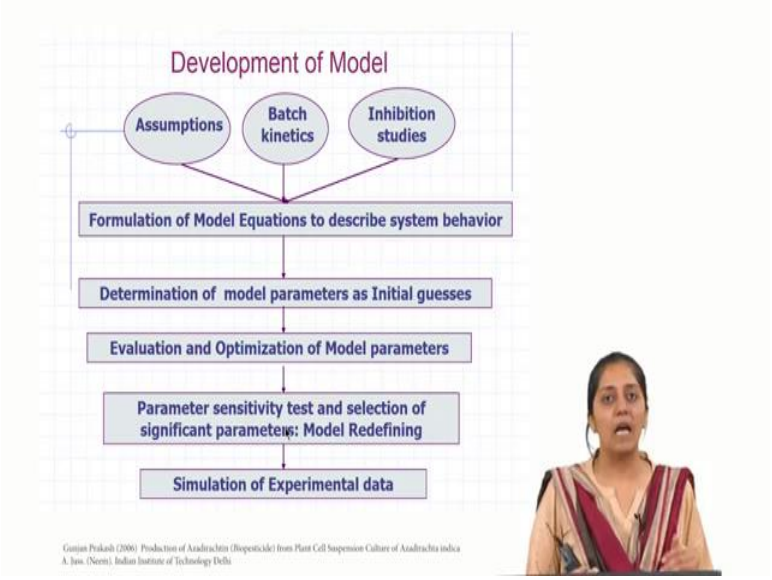

So, what they did while developing the model? They began with certain assumptions; they had the batch kinetic data and the inhibition data in batch.

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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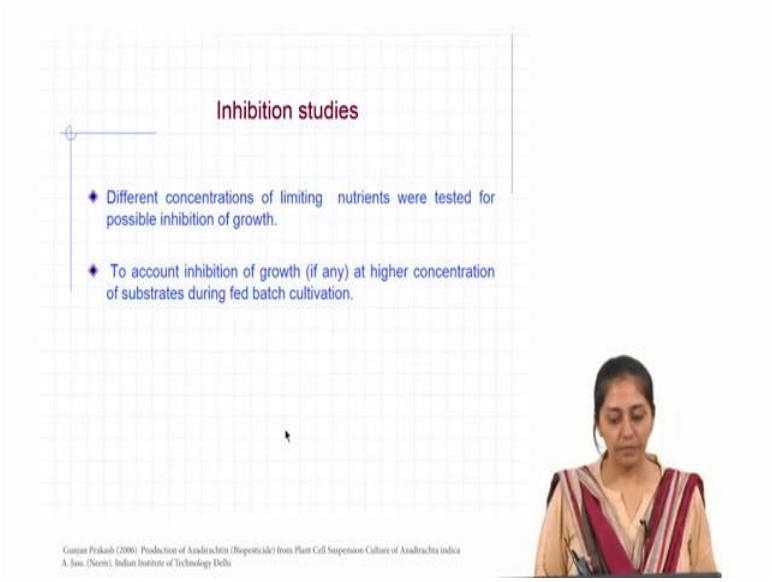

So, before beginning to develop the mathematical model, they did inhibition studies, in which, different concentrations of the limiting nutrients were tested for possible inhibition in growth of the culture. So, this was done to account for inhibition of growth at higher concentrations of substrate which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

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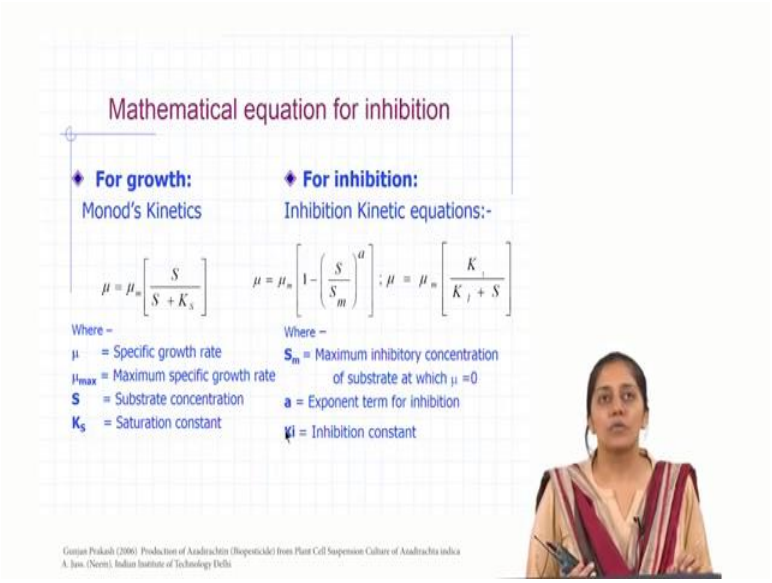

So, the different model equations which were incorporated to account for growth and for inhibition were as follows. If you can see on the slide for growth they picked up Monod's model and for defining inhibition they picked up Luong's model and also the asymptotic inhibition kinetics.

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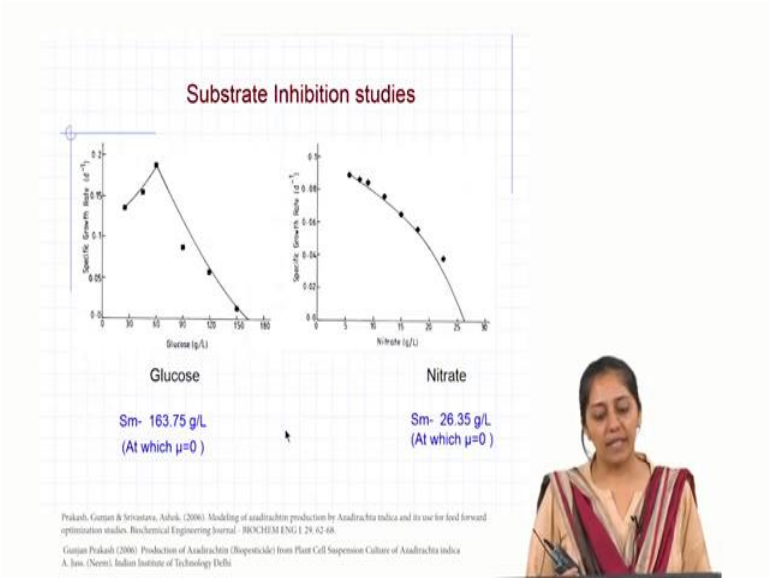

They tried to fit the data for substrate inhibition for different rate limiting substrates.

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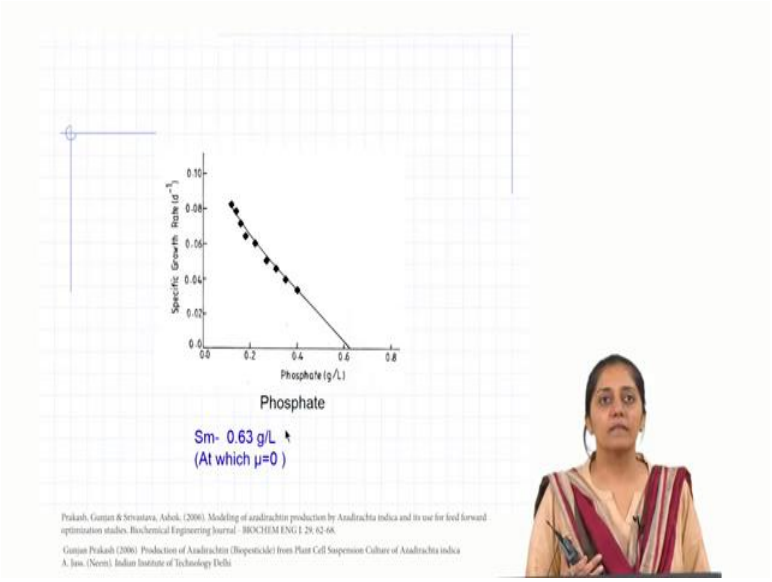

The substrate inhibition studies were carried out with respect to glucose, nitrate and phosphate.

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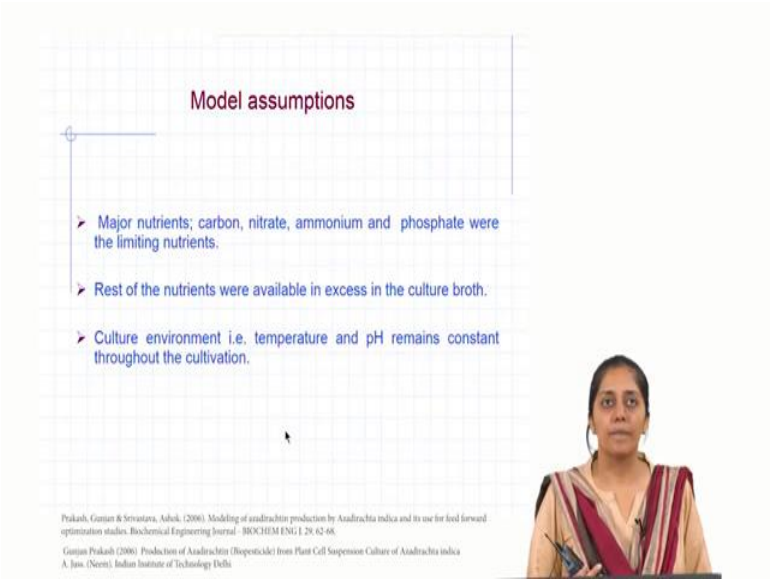

The model assumptions were that the major nutrients were carbon, nitrate, ammonium and phosphate. Rest of the nutrients were available in excess in the culture broth and the culture environment parameters like temperature and pH remained constant throughout the cultivation.

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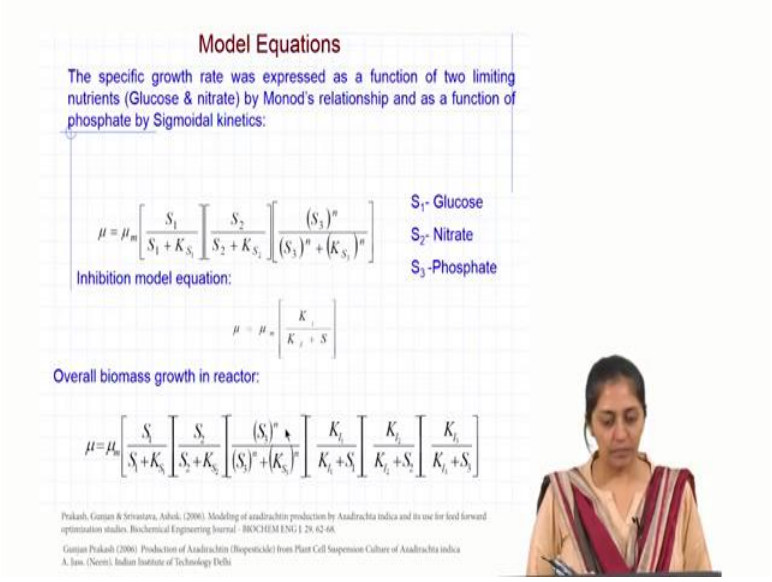

This was the model equation used which had the limiting terms with respect to all the limiting nutrients taken up in the model which are glucose, nitrate and phosphate. Then they also incorporated the inhibition terms with respect to the same three substrates to account for substrate inhibition during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

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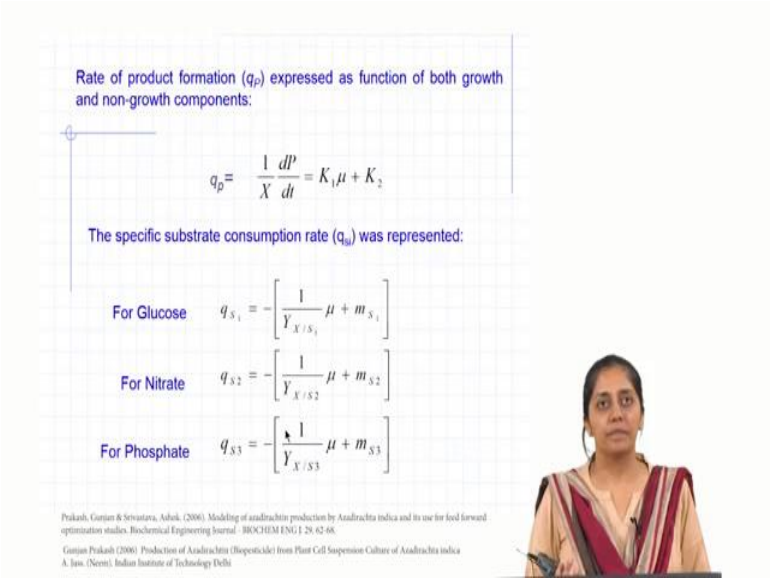

Then Luedeking-Piret model was used to define product formation kinetics. The last three equations demonstrate the specific substrate utilization rates, where m_s, m_{s1}, m_{s2}, and m_{s3} stand for the maintenance coefficients with respect to each of the three critical nutrients. And Y x/s stands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

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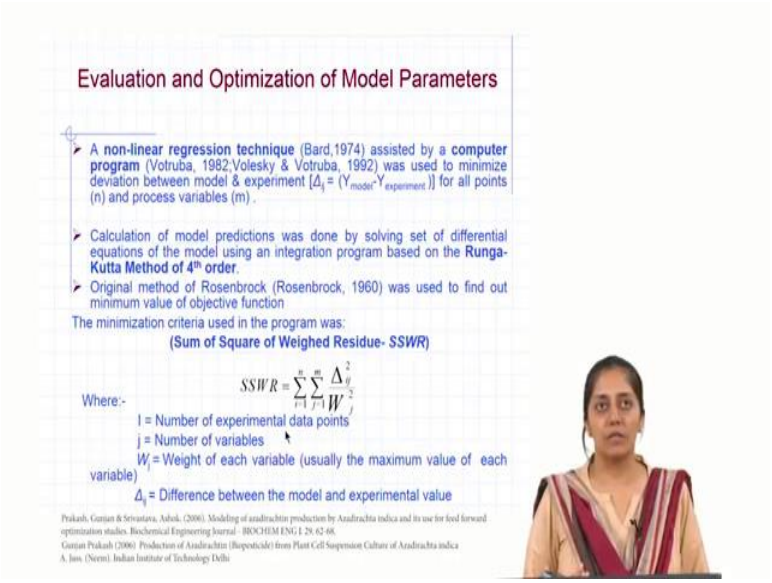

A non-linear regression technique was then applied which was assisted by a computer program to minimize the deviation between the model and the experimental values for all the data points

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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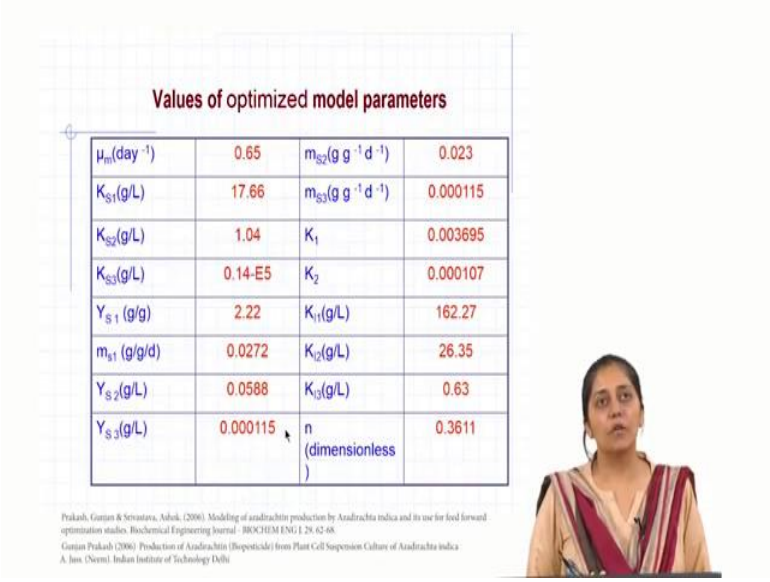

These were the optimized parameter values of the model.

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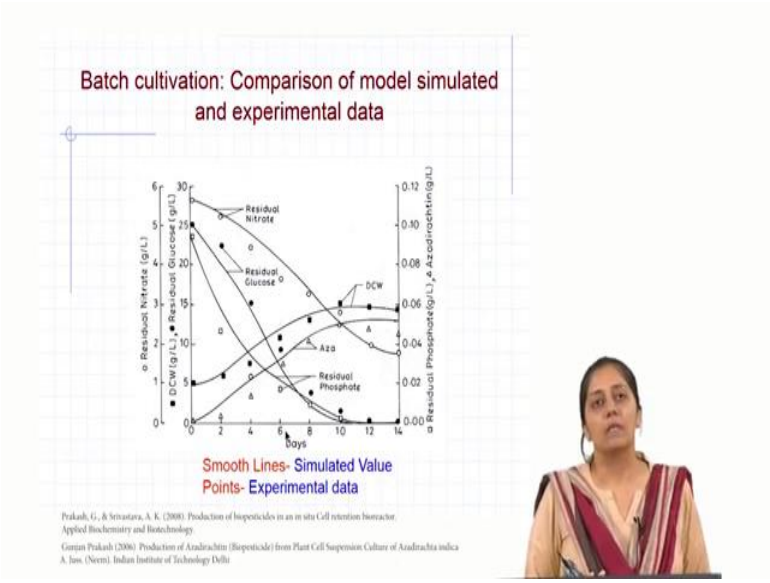

Now, this picture shows that how the fitting was done using the batch kinetic data with the model.

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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.

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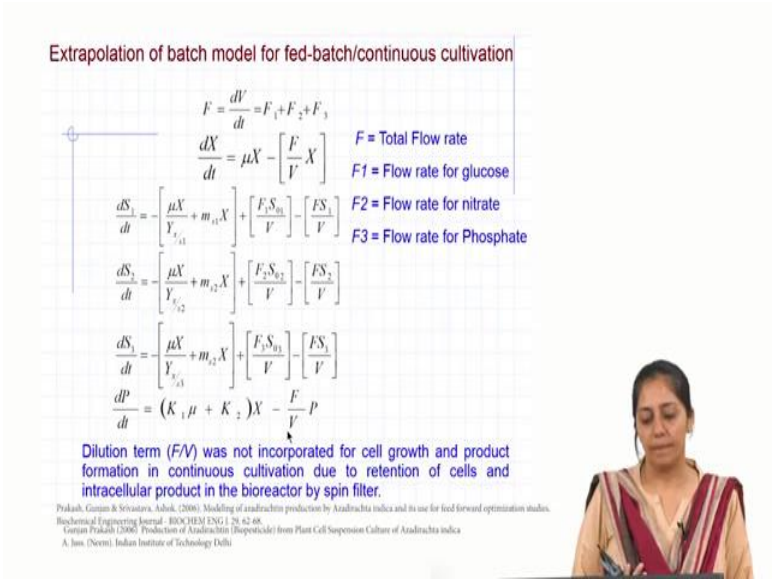

In order to extrapolate the batch model for fed batch or continuous cultivation, they did feeding of all three critical nutrients at a constant feed rate. And, then they defined the substrate utilization rates and the product formation rates as in the batch model for all the three critical substrates.

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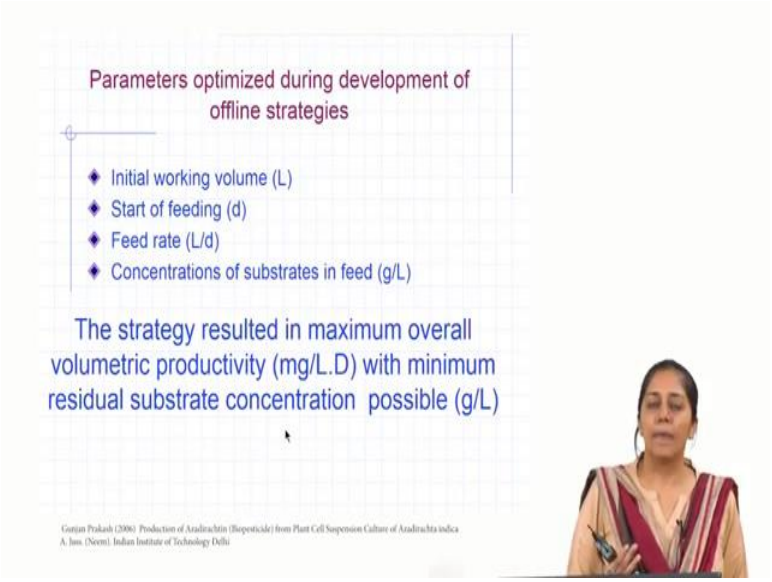

Now, being a fed batch cultivation, the dilution terms were added to the model. Then the parameters in this model were simulated for developing nutrient feeding strategies offline, where parameters including initial working volume, start of feeding, feed rate, and concentration of substrate in the feed 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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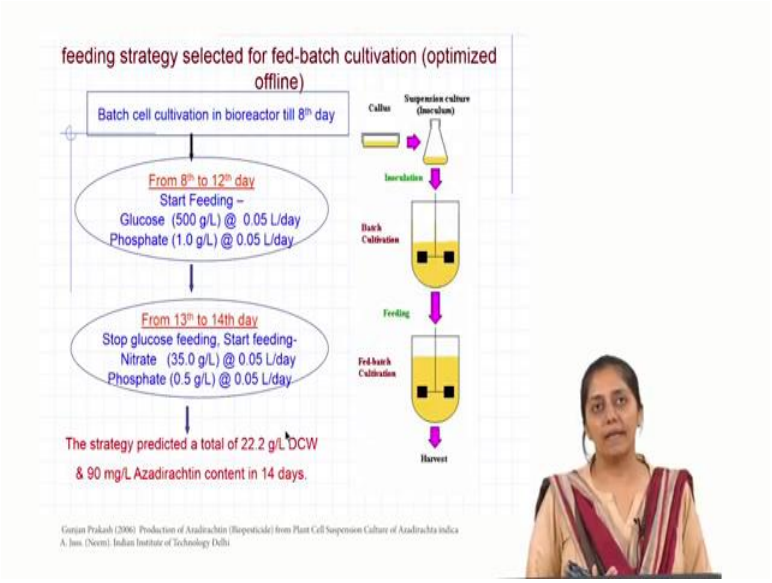

So, the feeding strategy which was selected for fed batch cultivation was as follows: Batch cultivation was carried out in the reactor till 8th day. Then from 8th till 12th day glucose feeding of 500 g/l and phosphate of 1 g/l were done at a rate of 0.05 liters per day in the reactor. Then from 13th 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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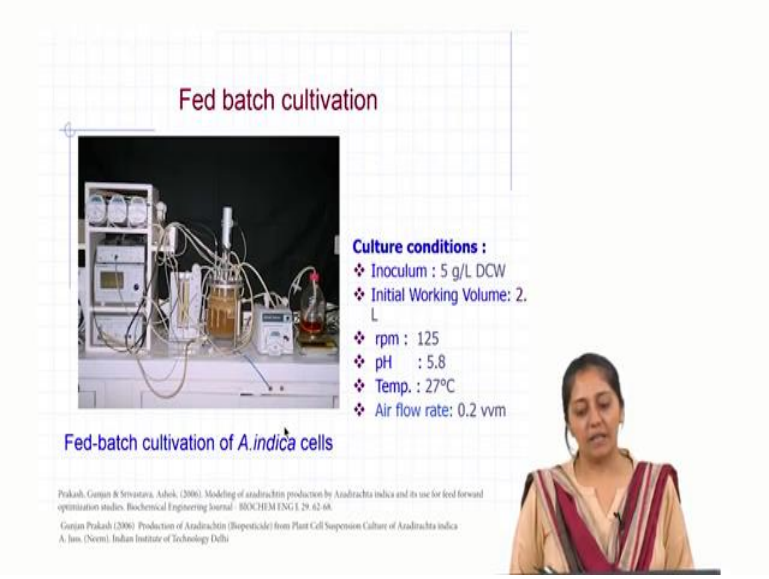

So, these were the conditions which were used for the fed batch cultivation and then the fed batch cultivation was experimentally validated.

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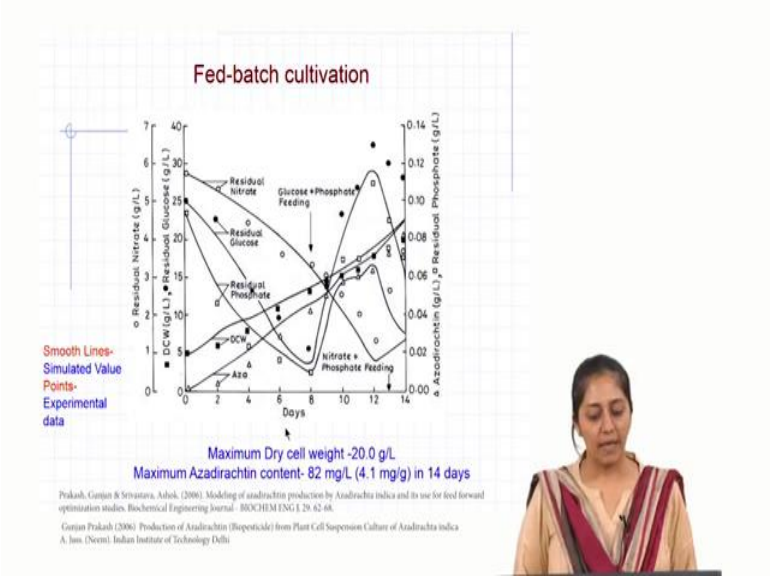

So, if you see the kinetics, the plot shows that as the feeding has started from 8th day you can see the residual nitrate, the residual phosphate and glucose concentrations getting high and then gradually the biomass and the azadirachtin feed increasing. Now, the dry cell weight and the azadirachtin 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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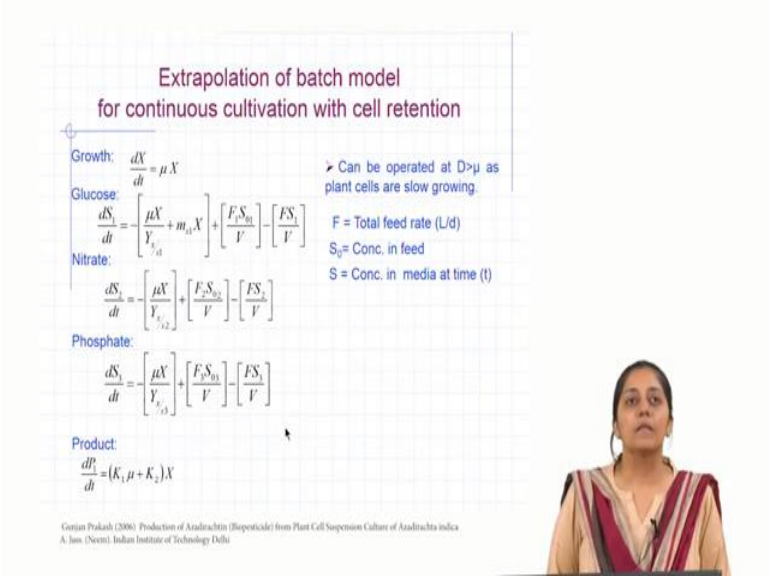

Then the batch model was further used to develop nutrient feeding strategies in continuous cultivation, where again the same model parameters were used to design equations for the continuous cultivation.

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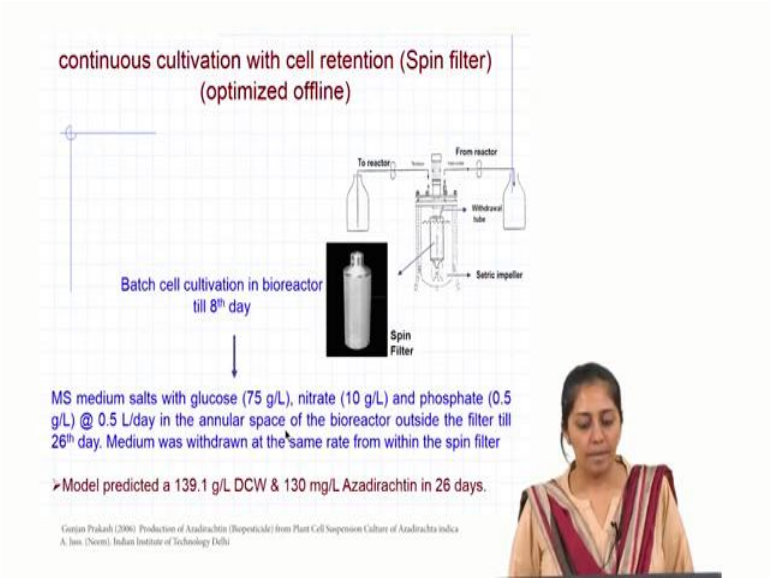

And then the model was simulated and experimentally validated. One of the strategies, which could give high azadirachtin productivities, in fed batch or in batch was chosen for experimental validation in continuous cultivation. The MS medium salts with glucose of 75 g/l, the nitrate feed of 10 g/l and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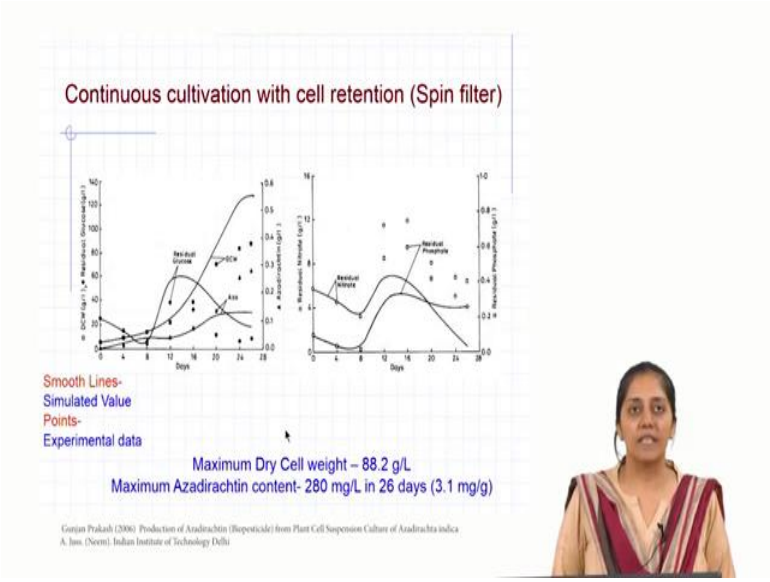

So, if you see the plots you can clearly see that as the feeding was done in the continuous reactor with cell retention it led to increase continuous increase in the biomass and also in the azadirachtin productivity. So, experimentally the biomass which could be achieved was around 88 g/l and the azadirachtin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

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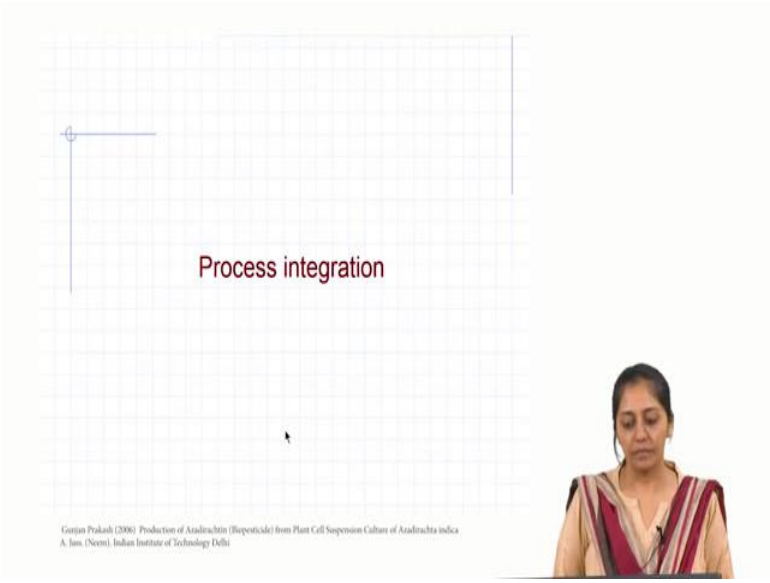

After, achieving and designing nutrient feeding strategies for continuous enhancement in the productivity of azadirachtin,

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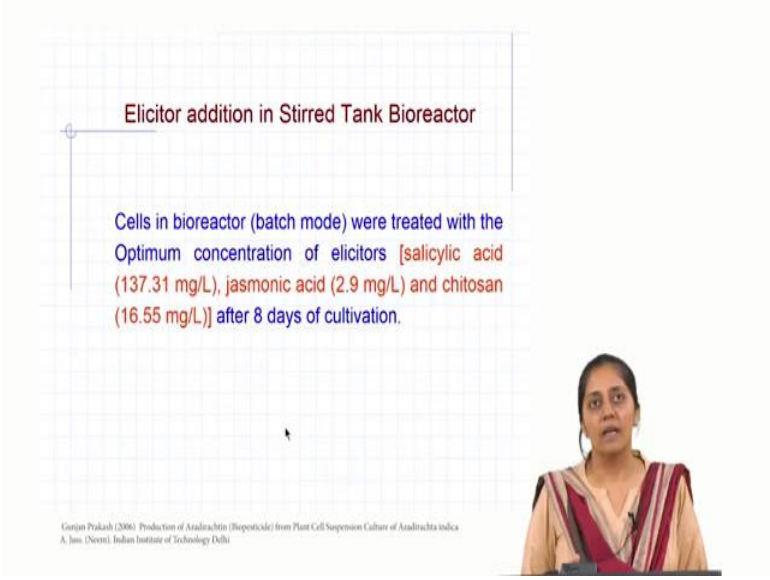

the authors went on to do process integration in which they added the elicitor and precursor selected from the shake flask studies to further enhance the yield of azadirachtin and thereby the productivity So, the cells and bioreactors in the batch mode, were treated with optimum concentration of elicitors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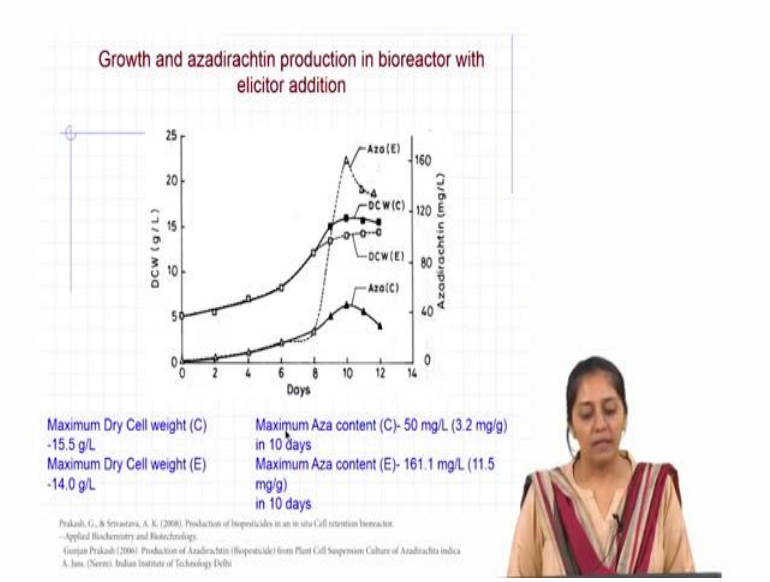

Then these elicitors were added on 8th day and growth and azadirachtin production in the reactor was studied. And after addition the biomass was nearly the same which was 14 g/l, while the azadirachtin content increased to 11.5 mg/g in 10 days which was much higher than that obtained in the batch reactor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

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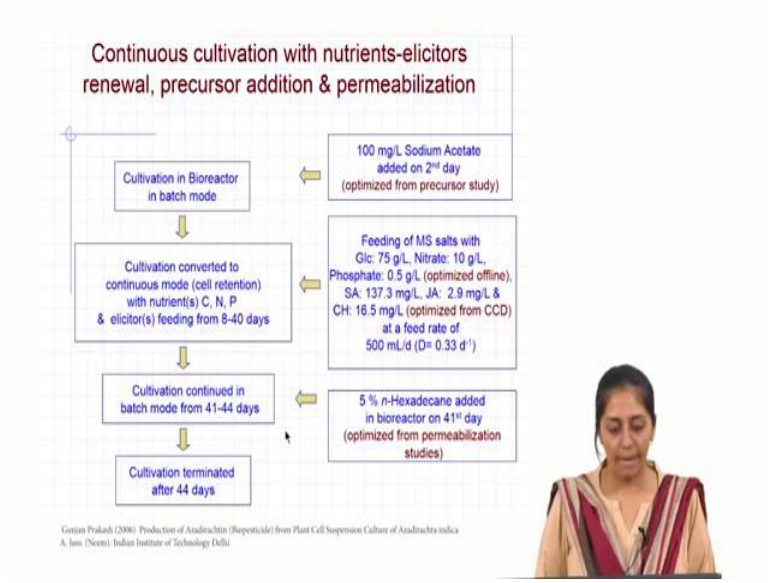

Then continuous cultivation with nutrient addition, elicitor addition, precursor addition, and permeabilization was all done together to see an integrated effect where the cells were grown in bioreactor in batch mode. Then the cultivation was converted to continuous mode with cell retention and nutrient 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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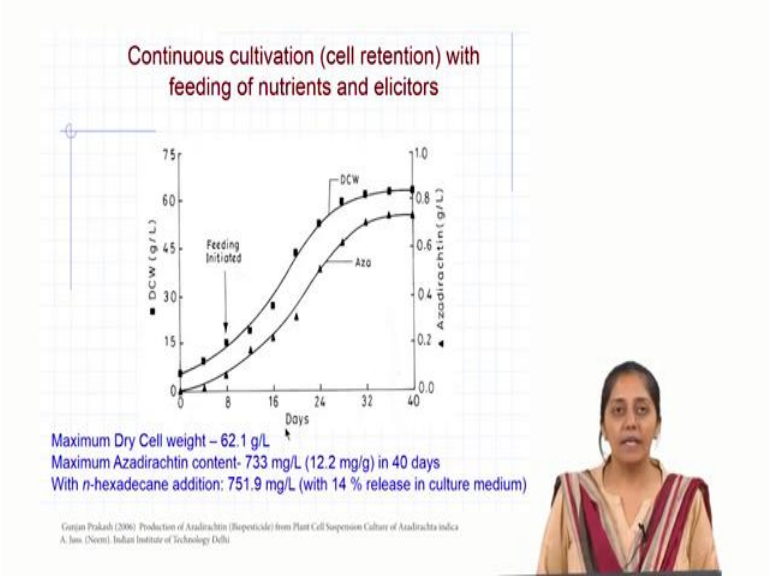

So, what did they achieve? The maximum dry weight which could be achieved was now 62 g/l and the maximum azadirachtin concentration or titers achieved were more than 730 mg/l. Now, after addition of hexadecane 751 mg/l of azadirachtin was obtained thus from the final harvest, while 14 % release in the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

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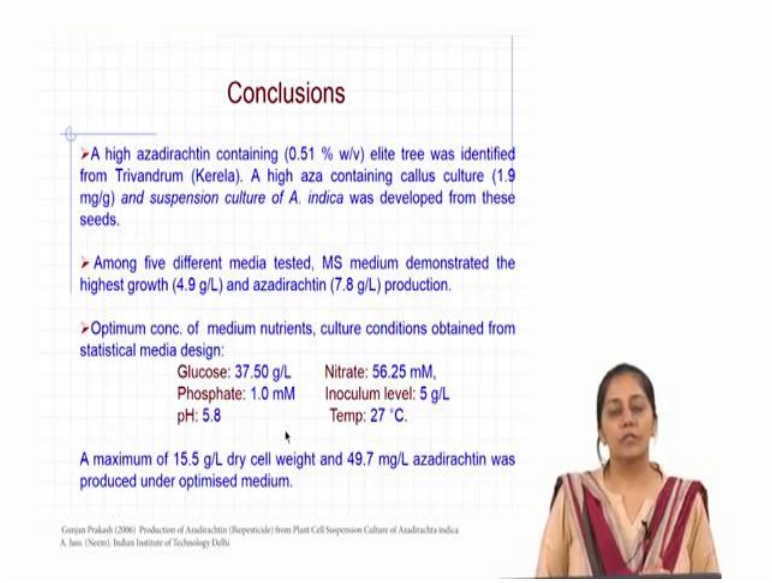

So, this is a summary of what the authors did in this case study, they began with a high azadirachtin containing tree based cell line. They developed callus culture and suspension culture. Among five different media tested, Murashige and Skoog medium demonstrated highest growth and azadirachtin production.

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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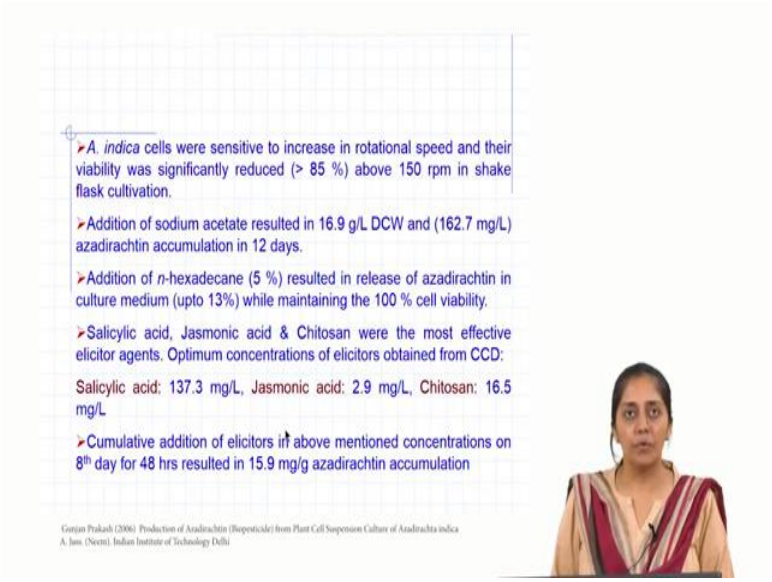

Addition of sodium acetate was done as a precursor where azadirachtin productivity was improved to 162 mg/l in 12 days. Then further permeability enhancers were optimized and hexadecane 5 % was chosen which could result in 13% release of azadirachtin in the medium while maintaining the viability up as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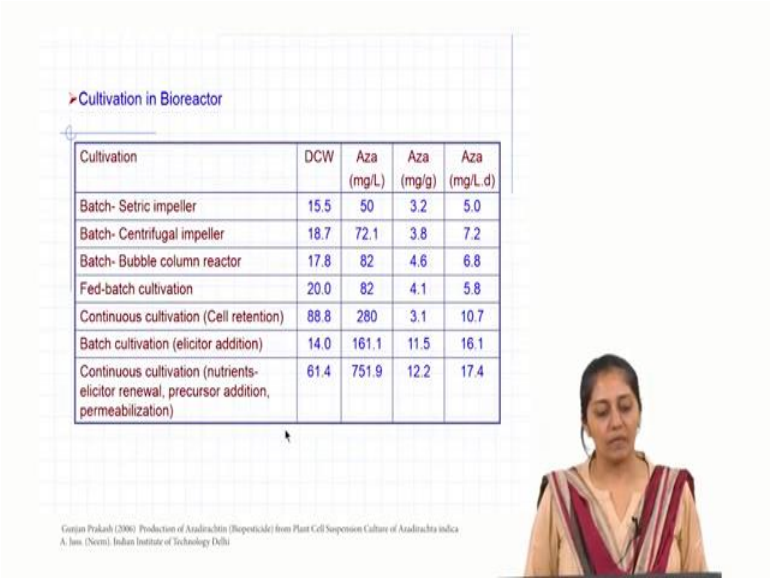

So, if you see a snapshot of how the productivity improved from different modes of cultivations:- when they did batch with steric impeller the productivity was 5 mg/l./d. When they did batch with a centrifugal impeller which had improved mass transfer characteristics and mixing time characteristics the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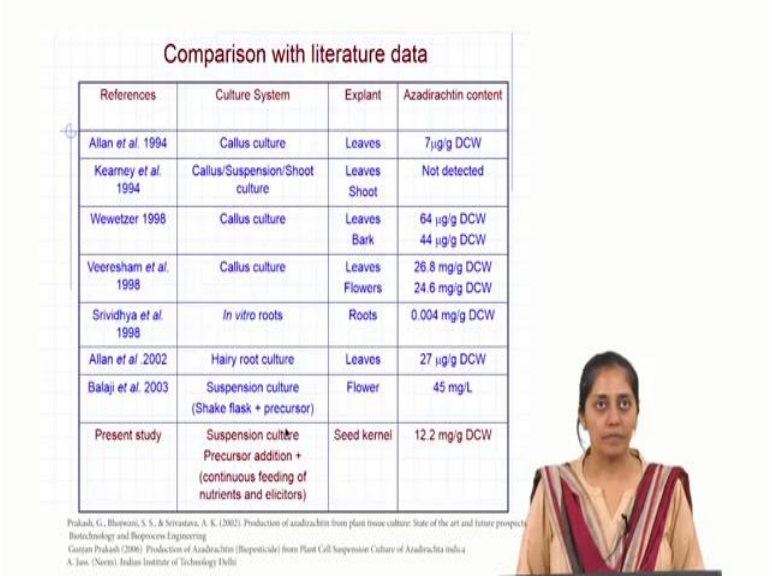

So, if you compare literature what they found was that they could achieve maximum yields of azadirachtin in the biomass by using this rational and a more systematic manner of optimization.

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.