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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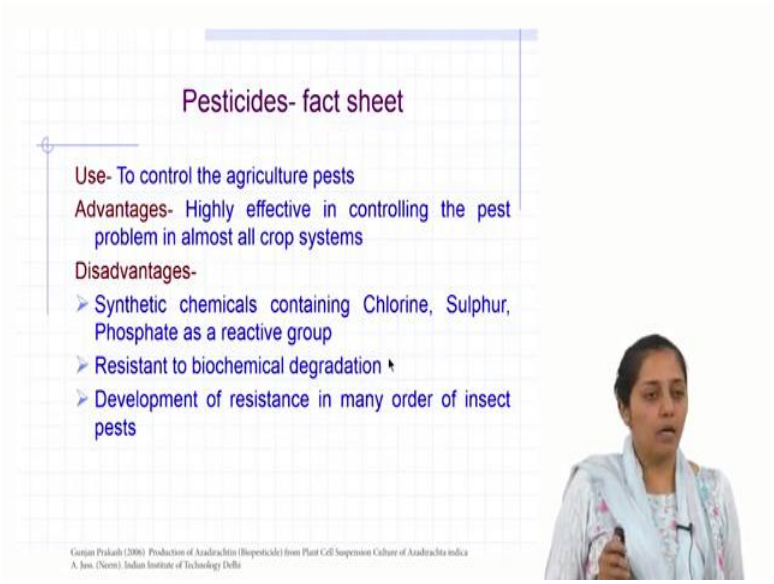

So, I will not take up the advantages disadvantages of these.

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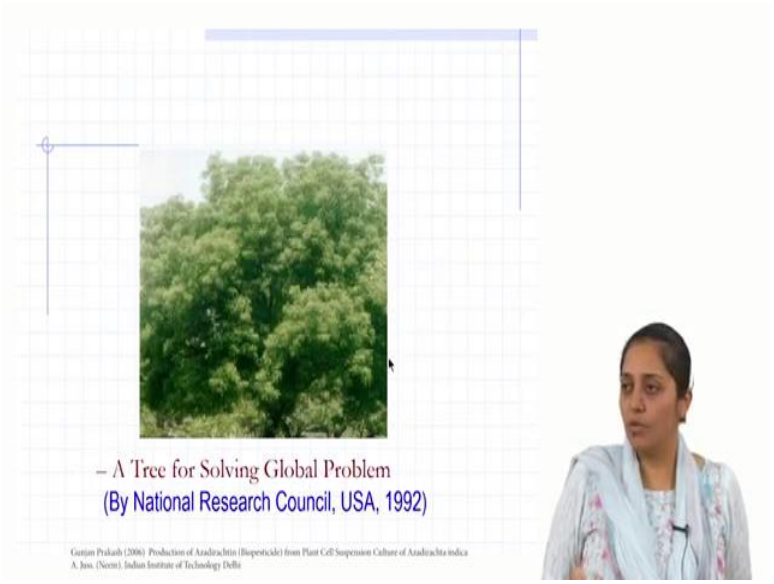

Azadirachtin is present in a ubiquitous plant, neem. Azadirachtin is found in all the parts of the plant. However, it is collected commercially from the seeds of this tree and this seed formation happens twice in a year. Moreover, there are disadvantages as I have mentioned before, that generally ripe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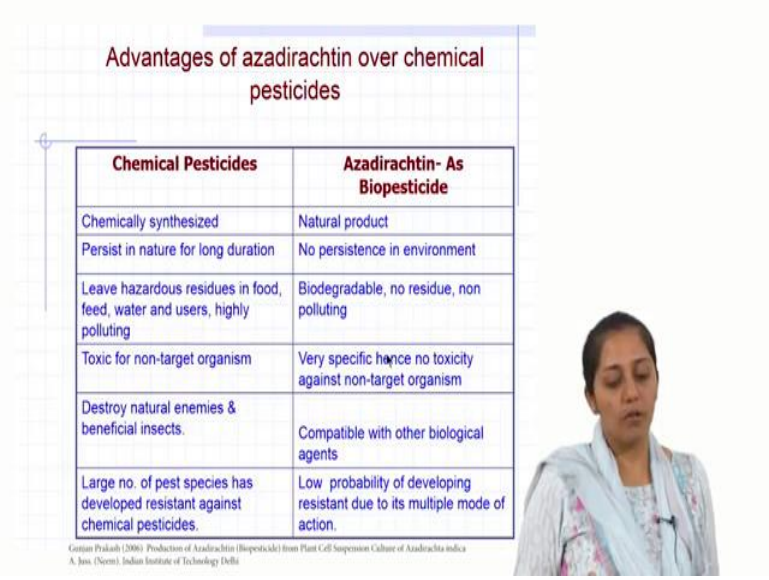

It is a highly complex big molecule with lot of chiral centers.

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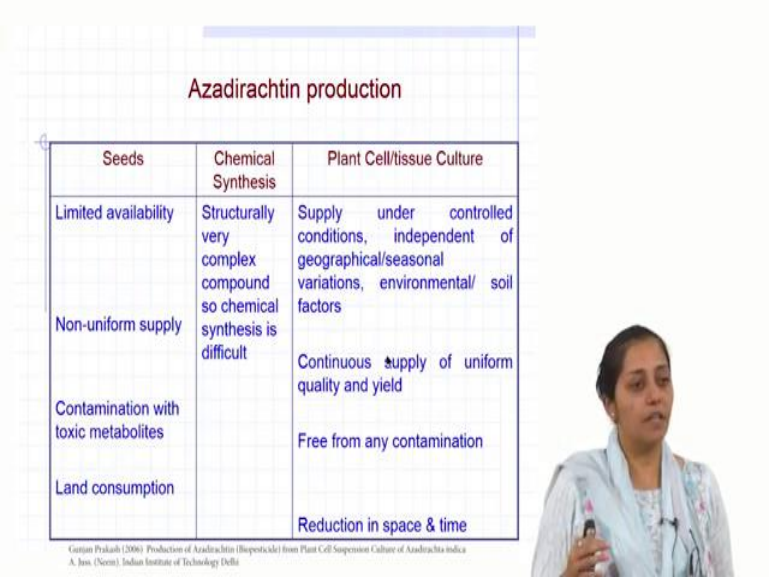

So, complete chemical synthesis, has not been reported yet.

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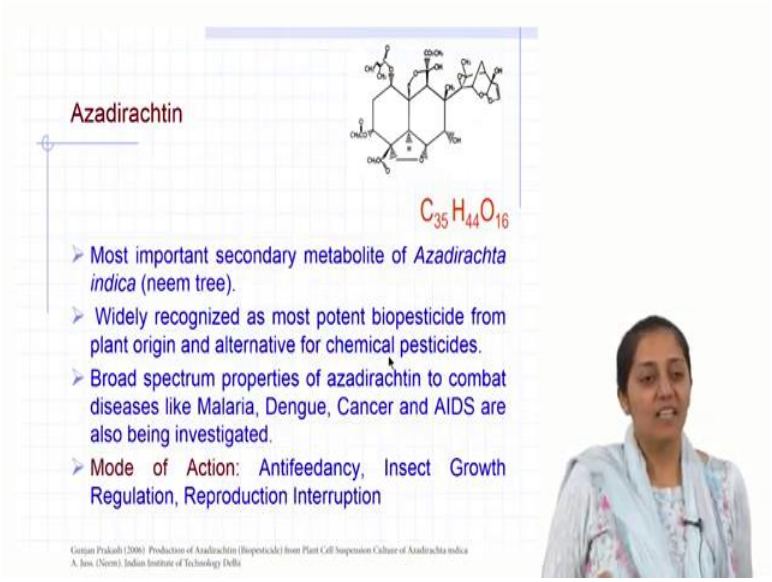

The molecule has a wide mode of action to a number of pests known in different plant varieties. So, that is the reason why it is very looked forward and is a high in demand biopesticide in the market. Now the first reason is it has a very diverse mode of action, completely biodegradable which is a big 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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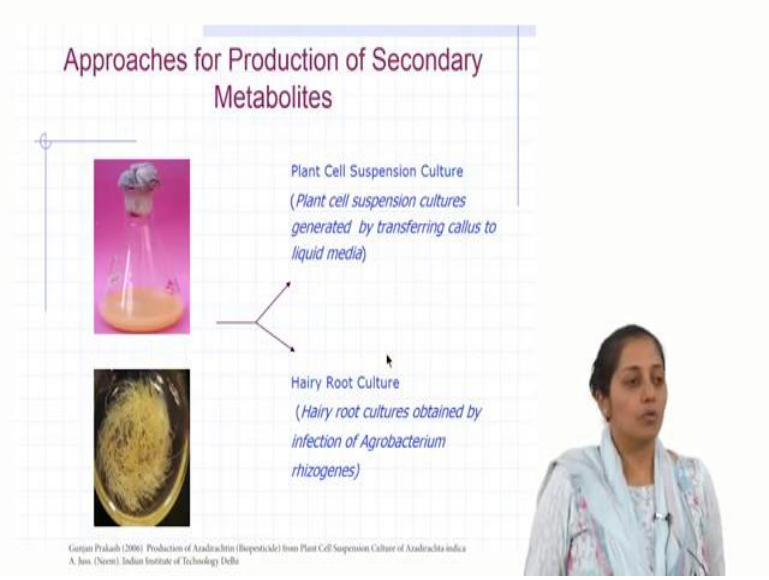

So, two kinds of approaches were used - plant cell suspension based and the other was hairy root culture based.

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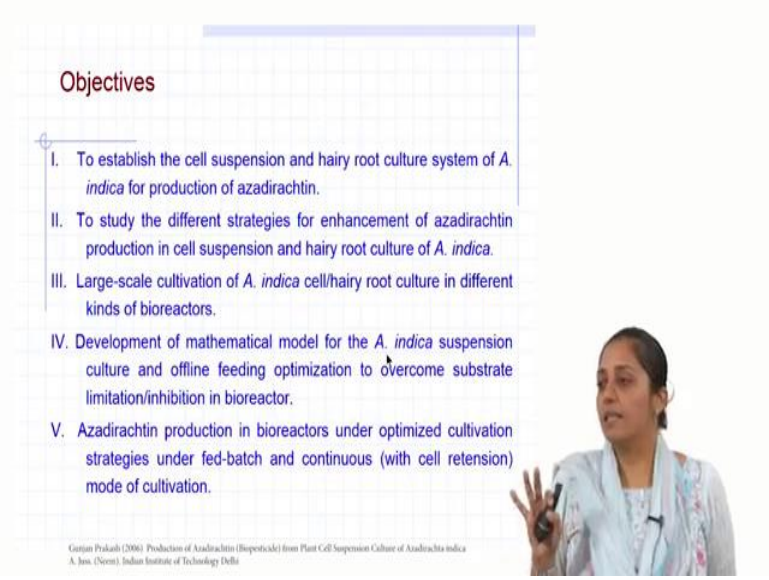

So, for the plant cell suspension cultures the objectives which were laid down were to establish the cell suspension of *A. indica* for production of azadirachtin under *in vitro* condition. So, one had to establish a high yielding fast growing plant cell suspension culture of *Azadirachta indica*. Then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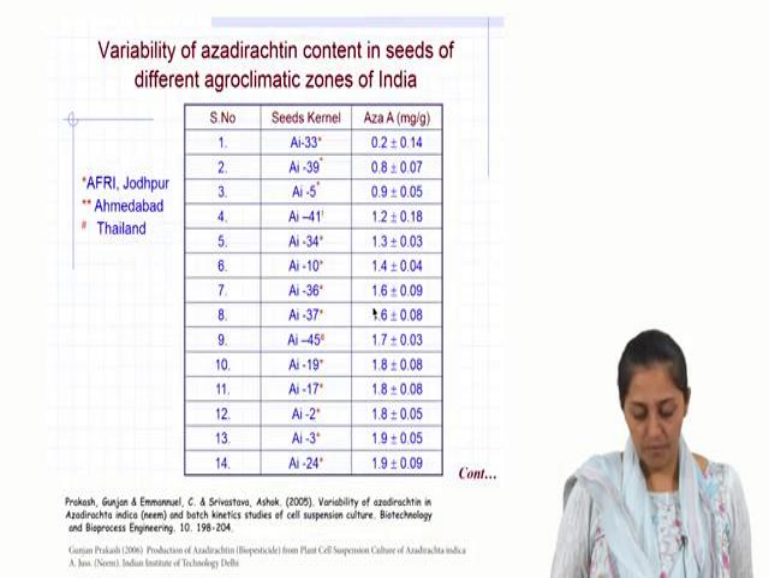

S.No	Seeds Kernel	Aza A (mg/g)
1.	Ai-33*	0.2 ± 0.14
2.	Ai -39**	0.8 ± 0.07
3.	Ai -5**	0.9 ± 0.05
4.	Ai –41#	1.2 ± 0.18
5.	Ai -34*	1.3 ± 0.03
6.	Ai -10*	1.4 ± 0.04
7.	Ai -36*	1.6 ± 0.09
8.	Ai -37*	1.6 ± 0.08
9.	Ai –45#	1.7 ± 0.03
10.	Ai -19*	1.8 ± 0.08
11.	Ai -17*	1.8 ± 0.08
12.	Ai -2*	1.8 ± 0.05
13.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

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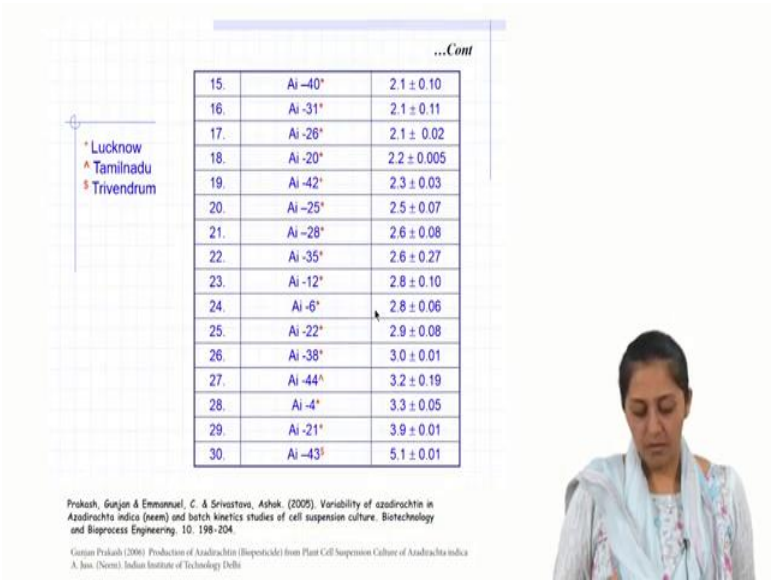

So, 30 seed varieties across the country were selected and they were screened for the azadirachtin yield in them. Once the highest yielding seed variety was found to be the Trivandrum variety, it was then taken forward to develop cell lines.

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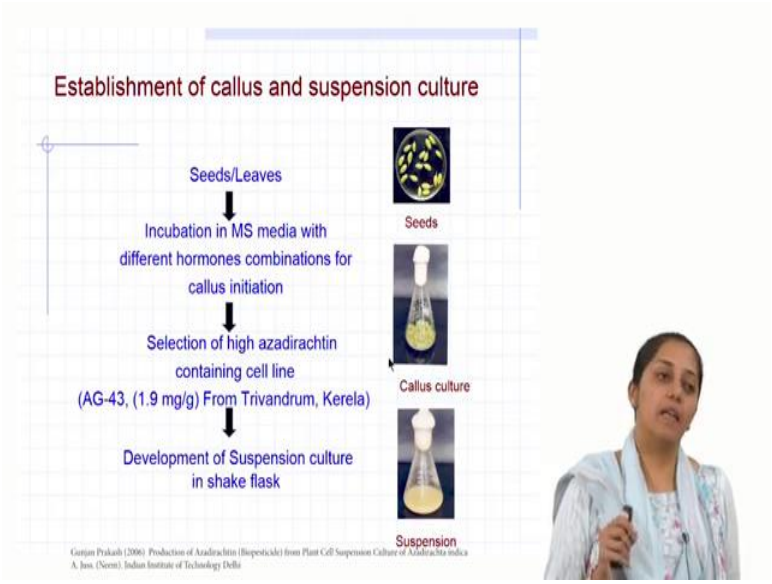

Now, as I have mentioned before, a highest yielding plant would lead to a high yielding cell line, that was the logic behind this. Once this was done, callus was induced. Each callus line which will be induced will have a different genetic makeup and therefore, a different biosynthetic capacity. Again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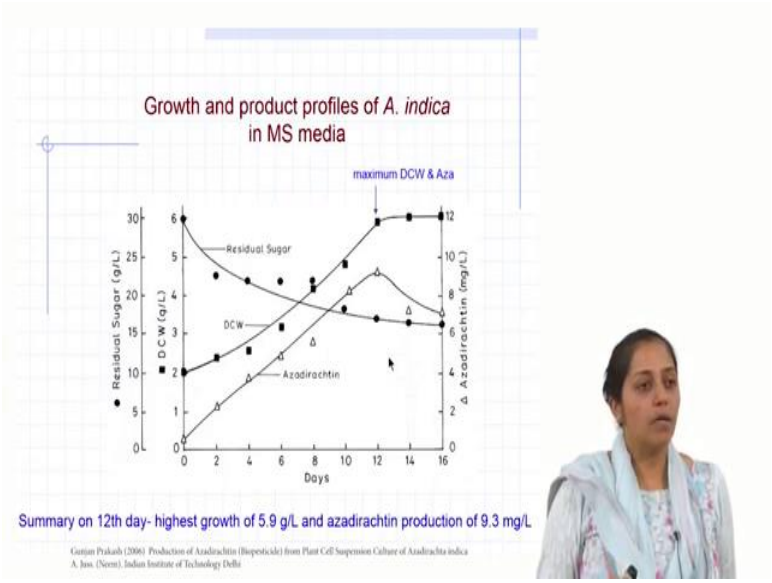

And the day for the harvest was found from batch kinetics. Now the reference point for us is to have better productivities than what is achieved here. On the 12th day, end of the log phase, the biomass is 5.9 g/l, which is maximum biomass and azadirachtin titer, 9.3 mg/l.

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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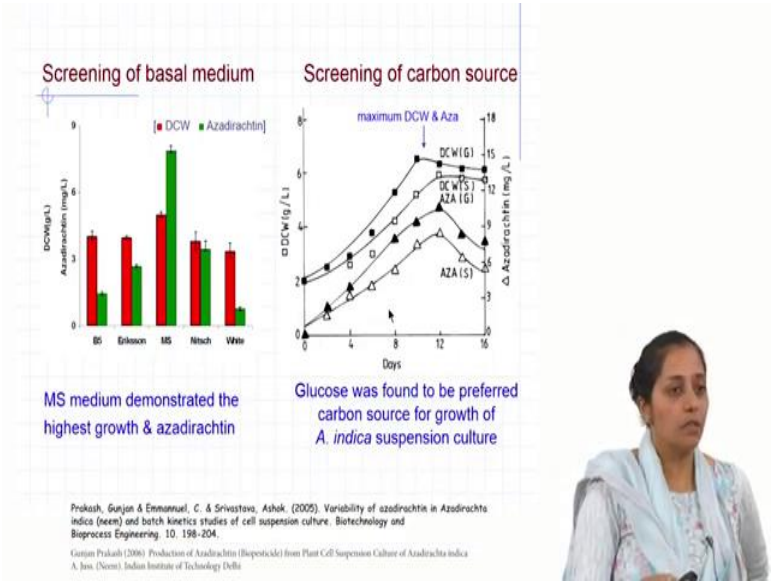

Different basal media, known compositions like B5 gamborg's medium Eriksson, MS, Nisch, White's - different predefined media for plant cell and tissue cultures were tested. So, in these media, the cell line was grown, the suspension was developed and based on the azadirachtin titer and biomass yields, 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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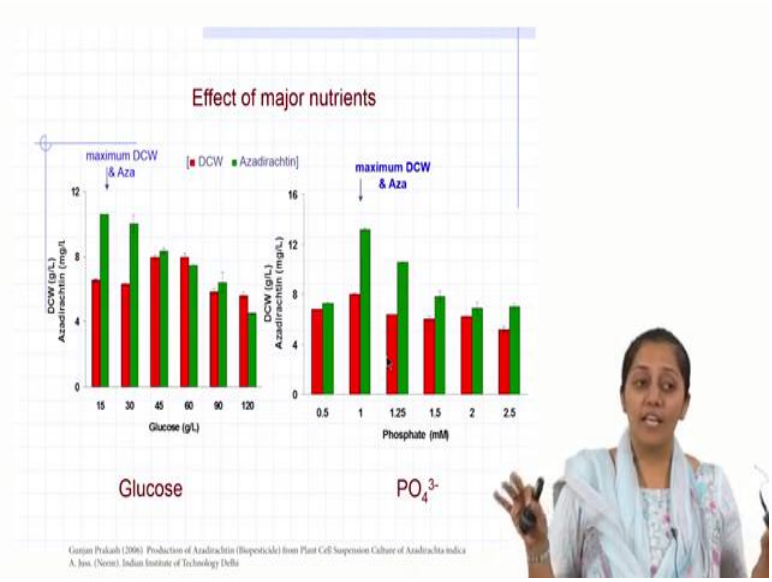

It will be nice to know what range should be selected for studying the interactive effect. So, in order to select the range for the interactive effect study using statistical tools, design of experiments, single factor experiments were carried out. Each of these major components were varied in a range 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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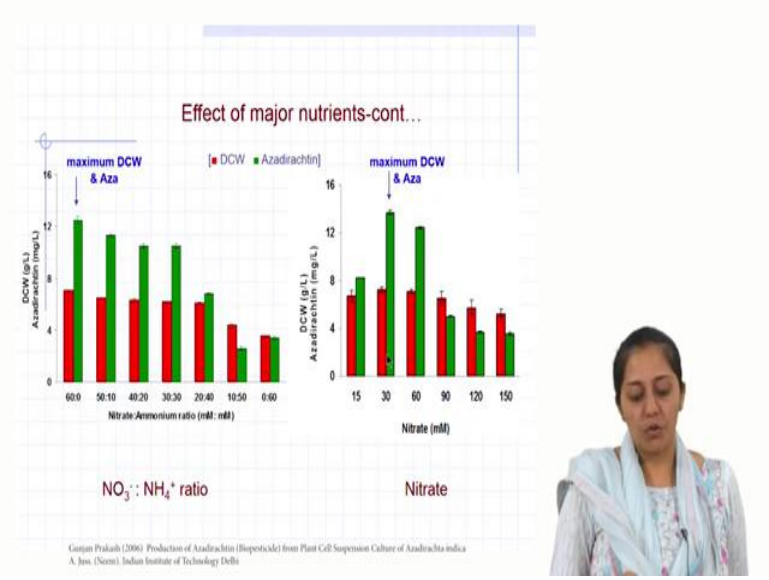

So, they did for phosphate, nitrate, ammonia, glucose and they also did nitrate ammonium ratio study; because it is possible that the culture may not prefer ammonia over nitrate or may prefer nitrate over ammonia. So, then in that case, it is important to study nitrate to ammonium ratio. Interestingly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

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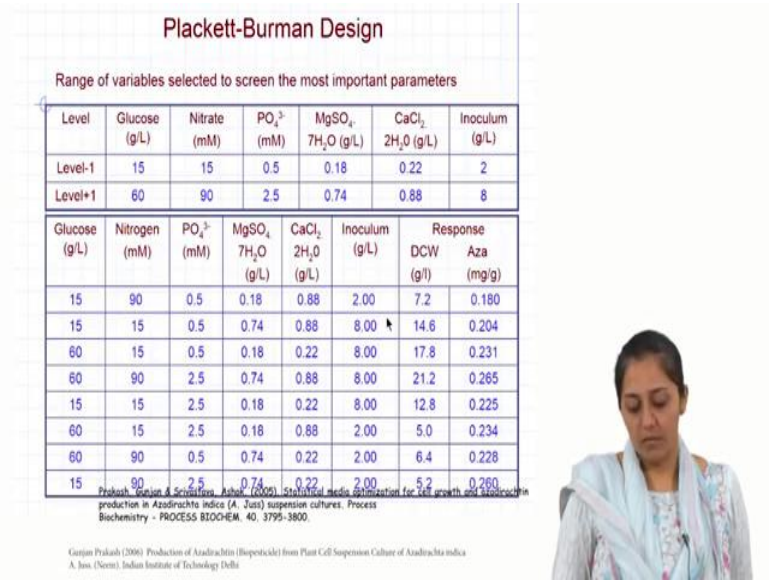

Then they also studied the rest of the major factors. Based on their single factor results, they created plus minus levels for the screening of the most significant factors in these selected major components. There are 6 major components out of which, if you design and start optimizing using statistical 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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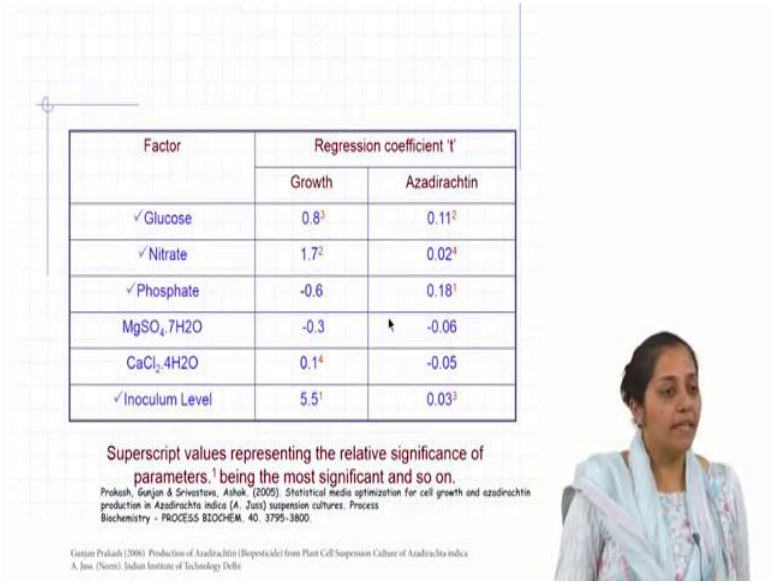

They did a plackett burman design which is a screening design in which they could able to find out which and how these 6 are impacting individually the growth and the azadirachtin yield. They could then rank these based on the ANOVA results, then the statistical analysis is done and based on that analysis 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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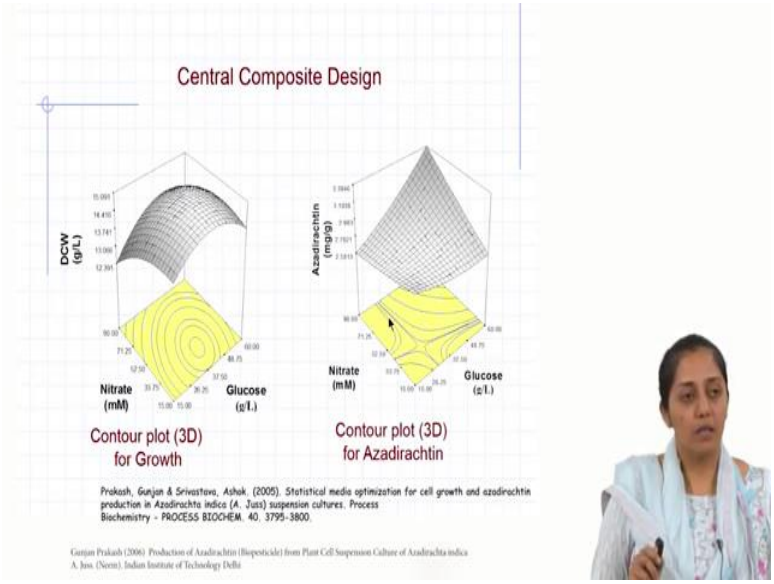

So, this was the analysis done based on the response surfaces optimization. So, the x axis and the y axis component effectively tells you, how the pairs would be affecting the growth or the azadirachtin. If you see clearly on the azadirachtin plot there is no convergence point in the design space. And 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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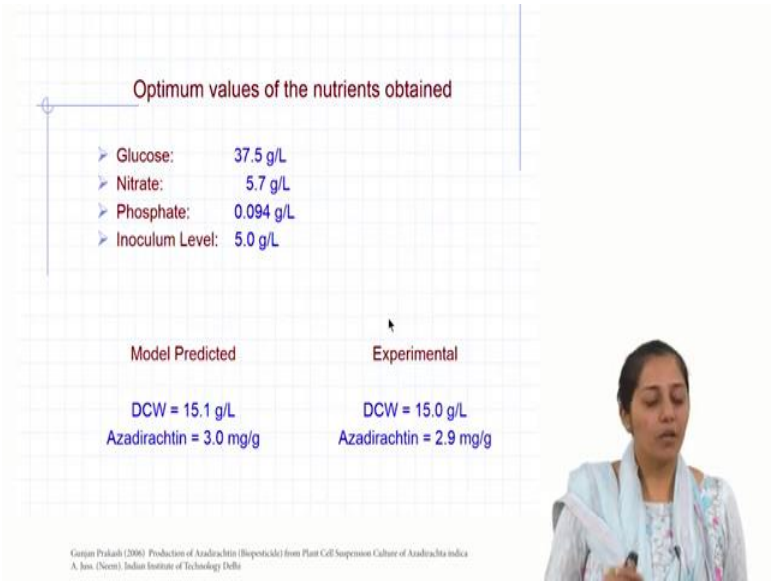

But, because for us it is dependent on both the biomass as well as the secondary metabolite yield; so, you can get further to improved result from the unoptimized. They could define a media composition based on this contour analysis. See there is no ‘the model’ it is always ‘a model’. So, you cannot 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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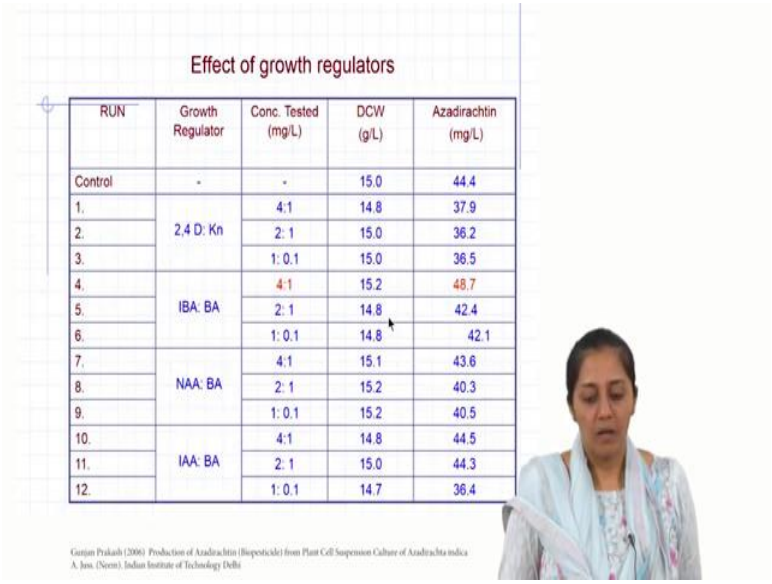

Now, then what did they do? They did auxin, cytokinin growth regulators single factor studies in different ratios. And they could find that IBA and BA were found to give maximum azadirachtin titers in this ratio.

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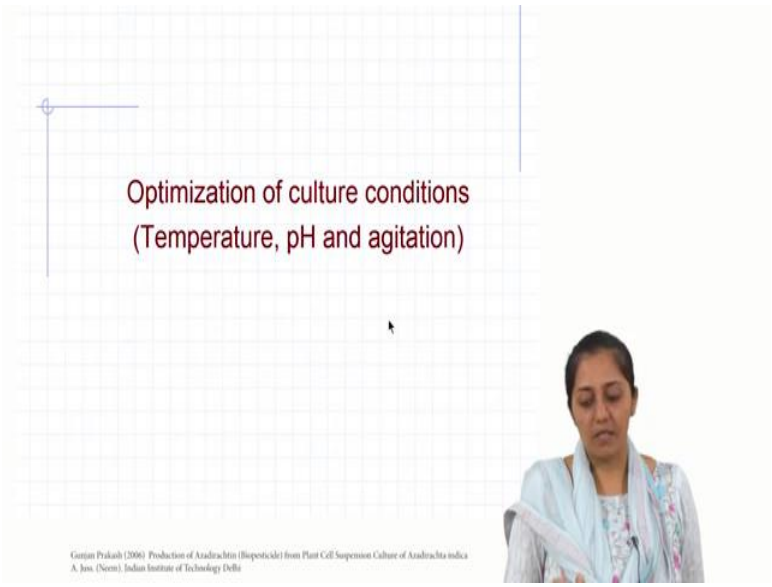

Now, see this is also improved from your reference point.

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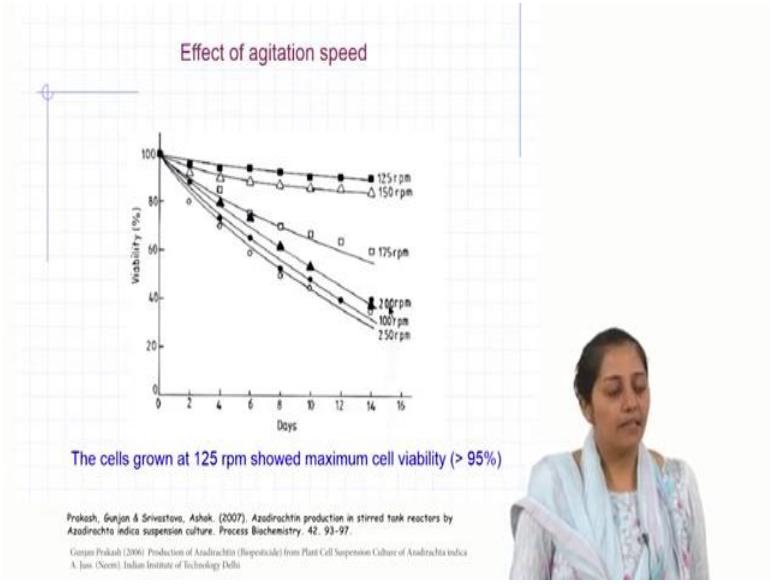

Then they did agitation speed study. Now although mass transfer improves with agitation, the effect on the viability is also crucial, to see the overall effect on productivity or titers. So, they did a kinetic study in which they saw how the biomass is changing with time with increased RPM. So, if you 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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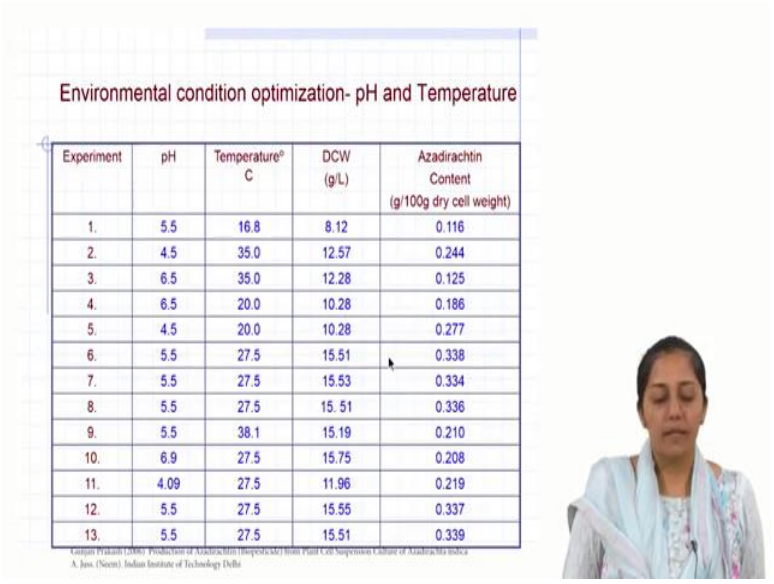

Experiment	pH	Temperature° C	DCW (g/L)	Azadirachtin Content (g/100g dry cell weight)
1.	5.5	16.8	8.12	0.116
2.	4.5	35.0	12.57	0.244
3.	6.5	35.0	12.28	0.125
4.	6.5	20.0	10.28	0.186
5.	4.5	20.0	10.28	0.277
6.	5.5	27.5	15.51	0.338
7.	5.5	27.5	15.53	0.334
8.	5.5	27.5	15. 51	0.336
9.	5.5	38.1	15.19	0.210
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

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

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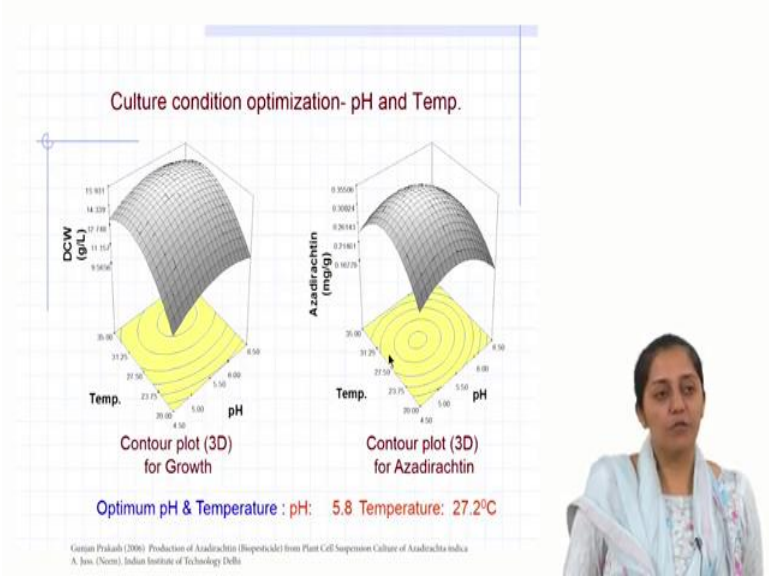

And then if you see here clearly the range seems to be fine; because they are effectively converging. And this will give you the ideal results what you expect for optimum values of pH and temperature.

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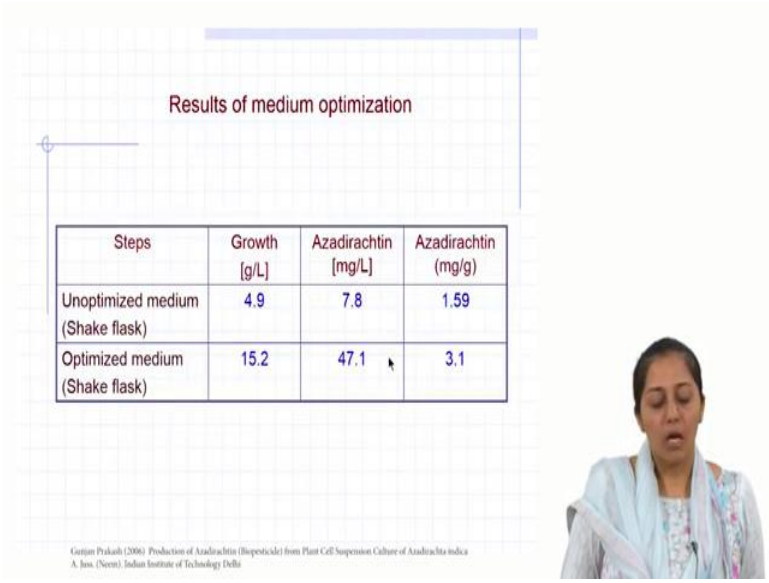

So, now if you see the comparison to unoptimized conditions, the azadirachtin overall titre was 7.8 mg/l. But if you see the optimized conditions it had improved to 47.1 mg/l. So, this is just through simple media optimization.

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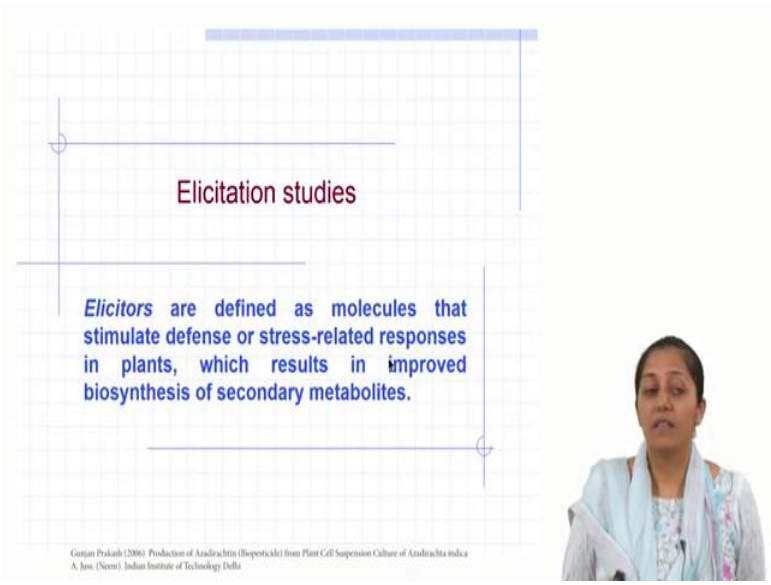

Now, let us see there are other strategies, which can further be used to improve the yields and productivities.

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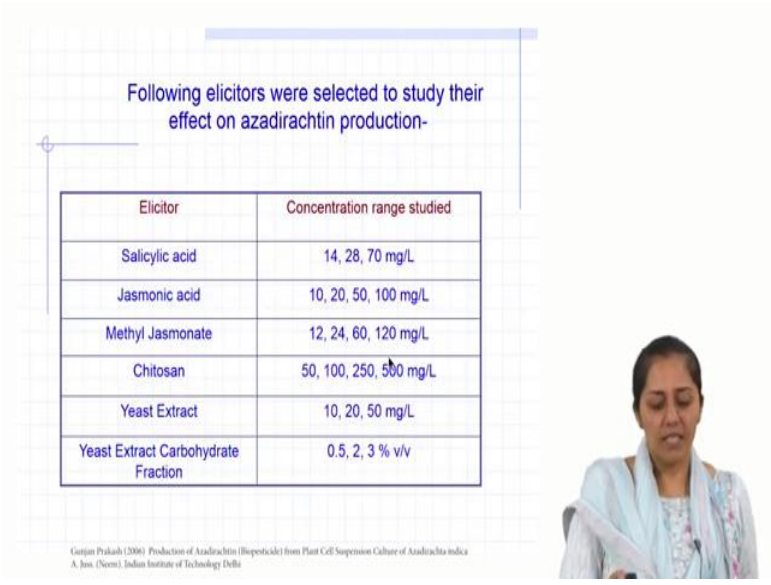

Elicitation: These are the different kinds of signaling molecules, abiotic and biotic elicitors, they tried these elicitors in different ranges.

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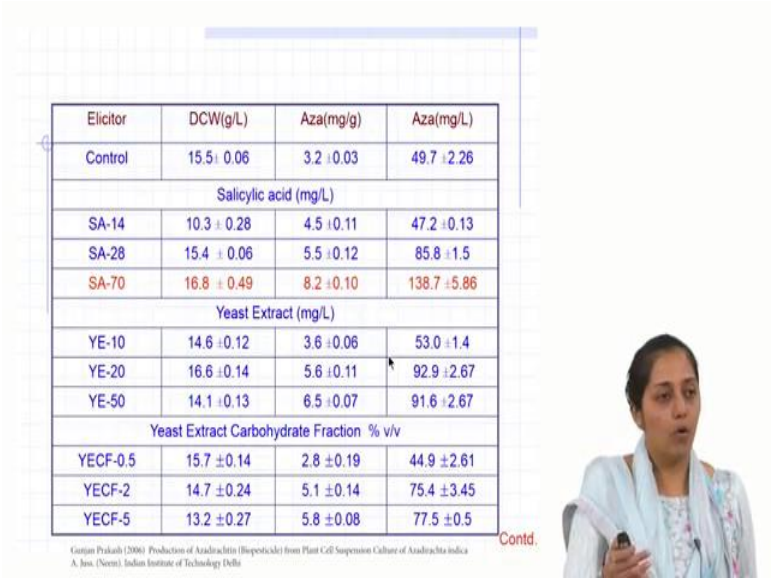

Now, in different range these were all singe factor experiments, they could select salicylic acid which was giving as high as 138 mg/ l at that concentration.

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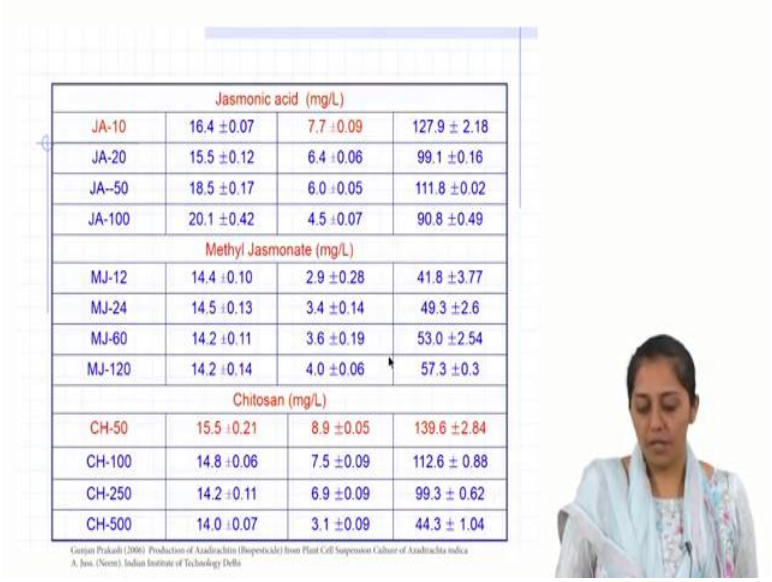

And also they could see that chitosan was also giving approximately the same enhancement up to 138, 140 mg/l.

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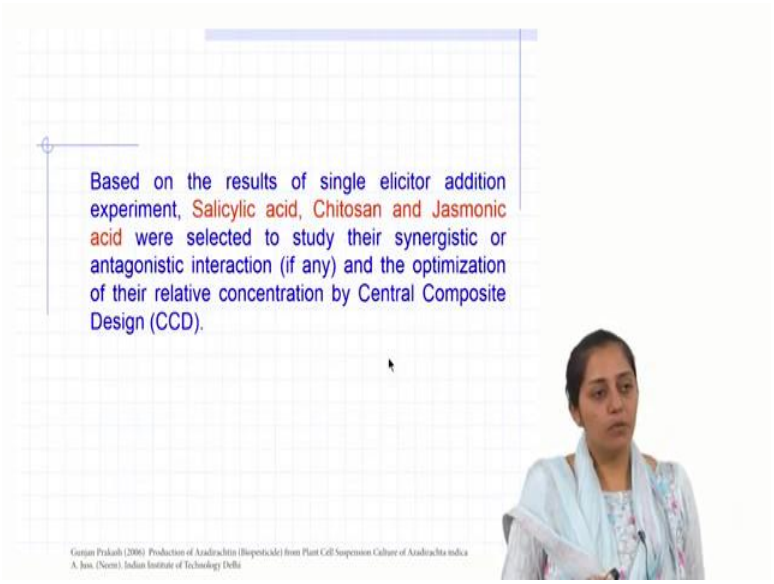

So, then what did they do? For multiple fold enhancement, they chose the three best elicitors at the range, which they could see through the single factors. And they did an integrated study to have a synergistic effect. They again did CCD - statistical optimization.

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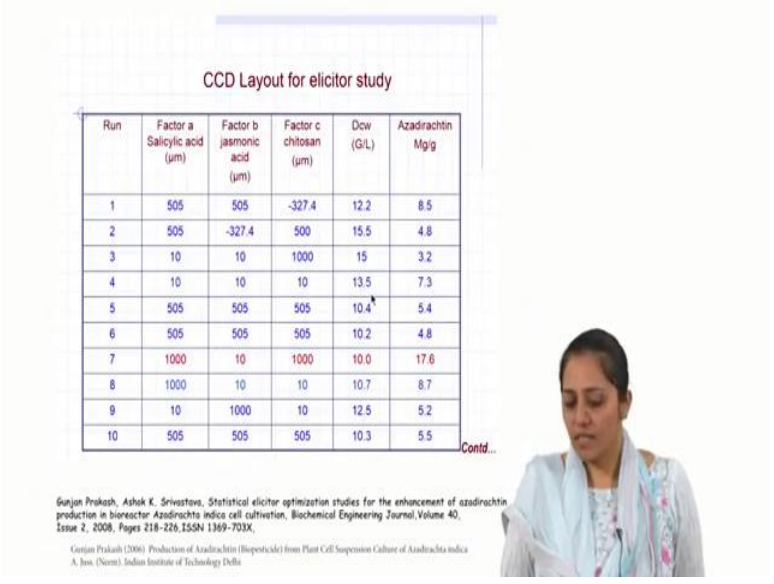

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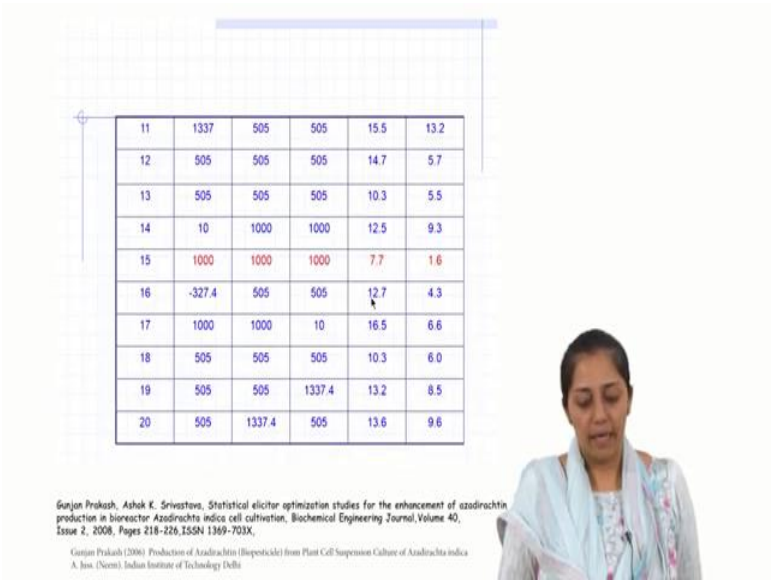

This statistical optimization again gave them better results.

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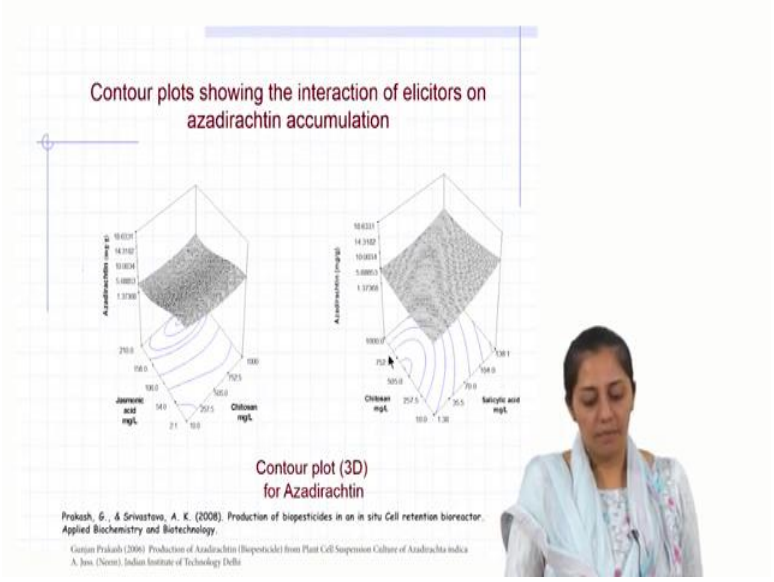

Now, for this the response would be what? It is a yield enhancement strategy. So, generally elicitor addition is well known to cause reduction in biomass. So, time of exposure to the elicitor is crucial and the response while selecting the elicitor can be yield rather than having titer. Because you would 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.

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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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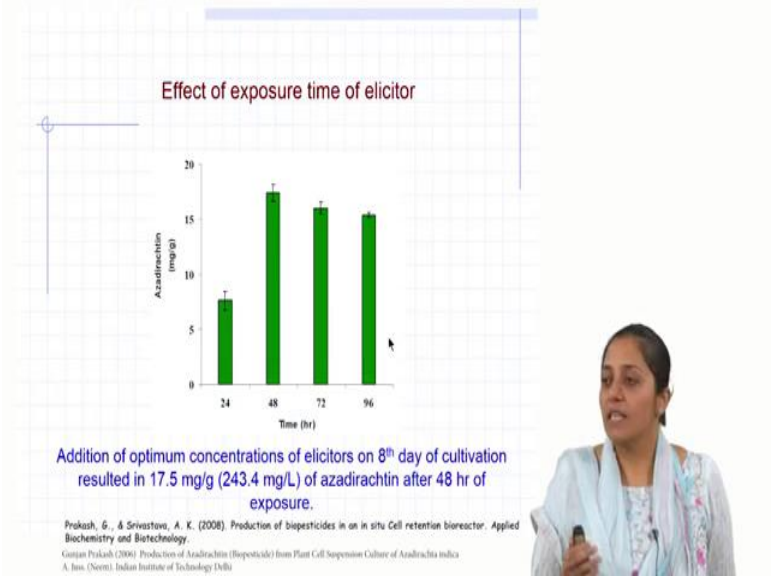

Now, they optimized time of addition of elicitor so that there is minimum loss in biomass. So, harvest time for the batch was 12 days if you remember. So, they added the

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.

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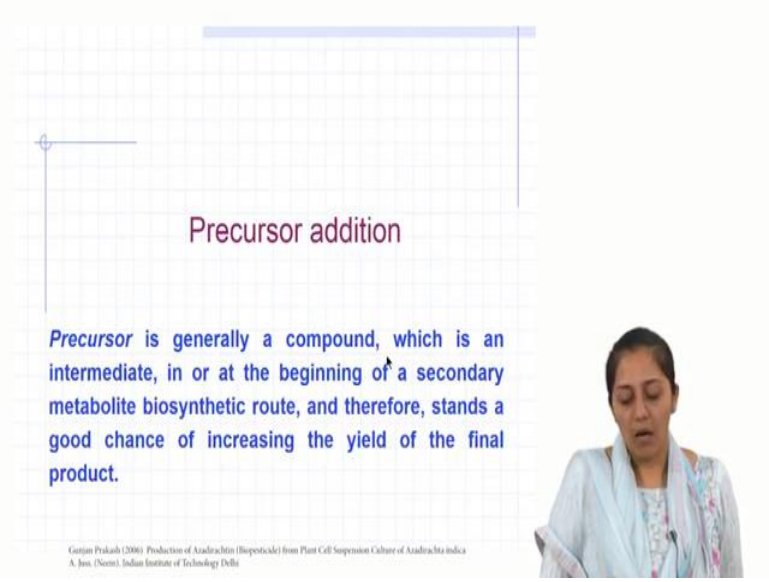

Now, they also did precursor addition.

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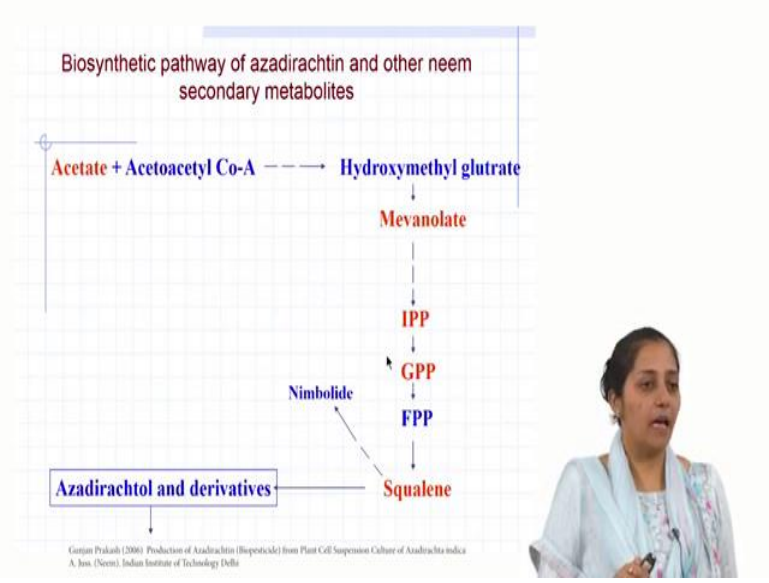

Now, for precursor addition it is important to know the biosynthetic pathway. In that biosynthetic pathway, availability and concentration of the exogenously added precursor is crucial.

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.

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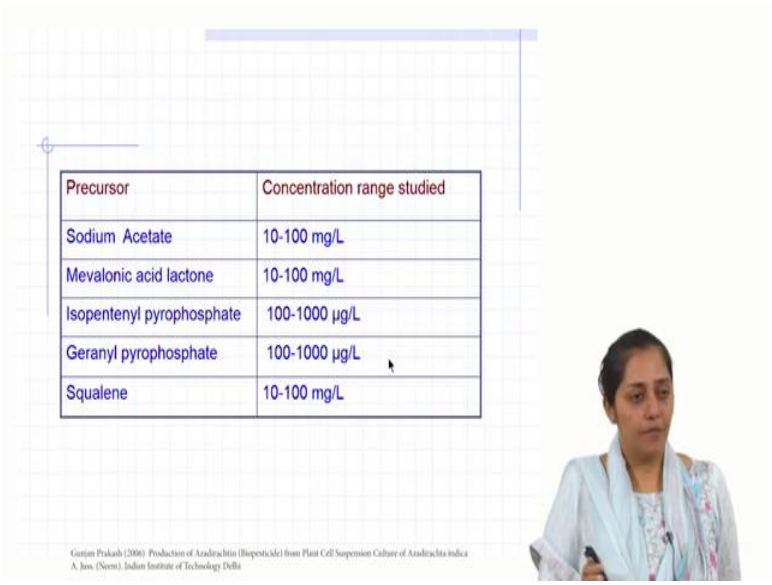

So, this was again a single factor study.

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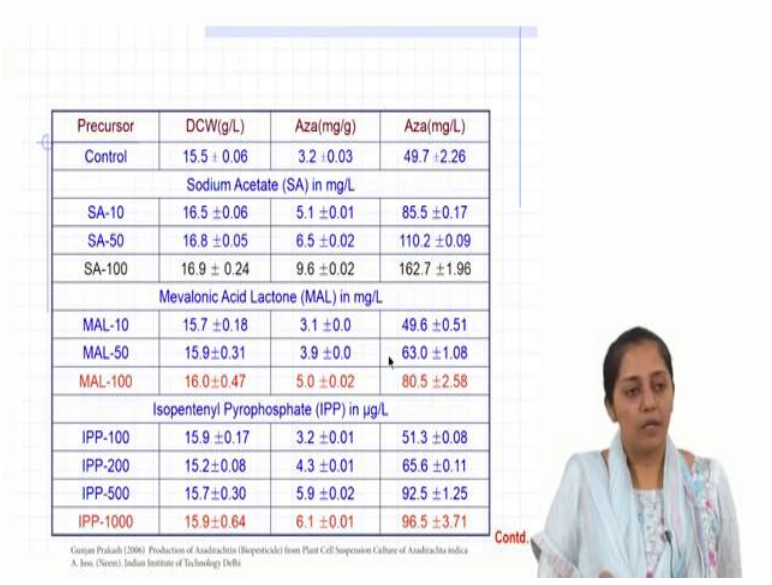

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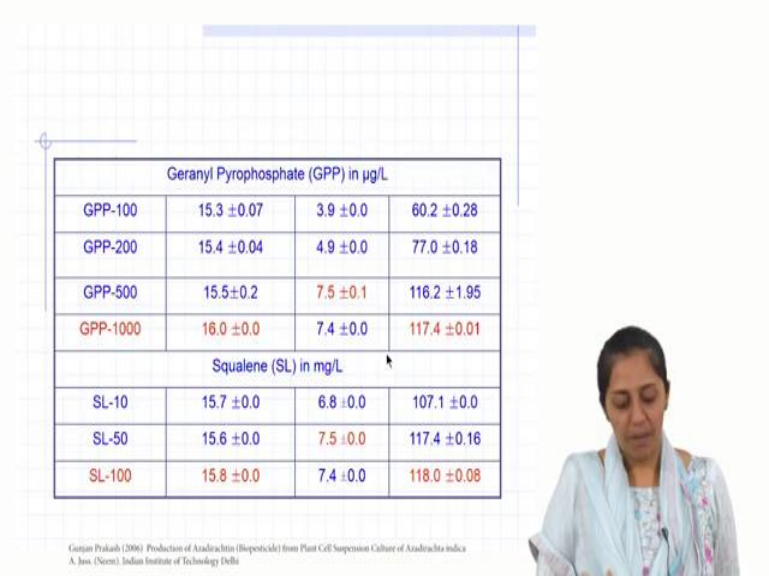

And they could chose IPP, then GPP geranyl pyrophosphate then squalene, mevalonic acid, lactone. And they could find that GPP at certain concentration level was giving more than 100 mg/l azadirachtin.

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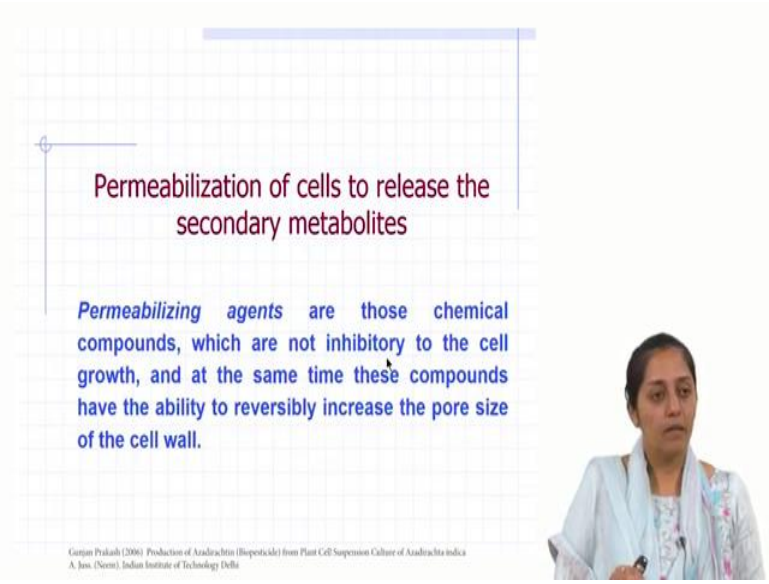

So, these are all independent experiments. So, there can be two ways of optimizing; one is you can run a relay system the other is you run a parallel system and then finally, integrate. So, generally you will observe people do independent studies and then finally,

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

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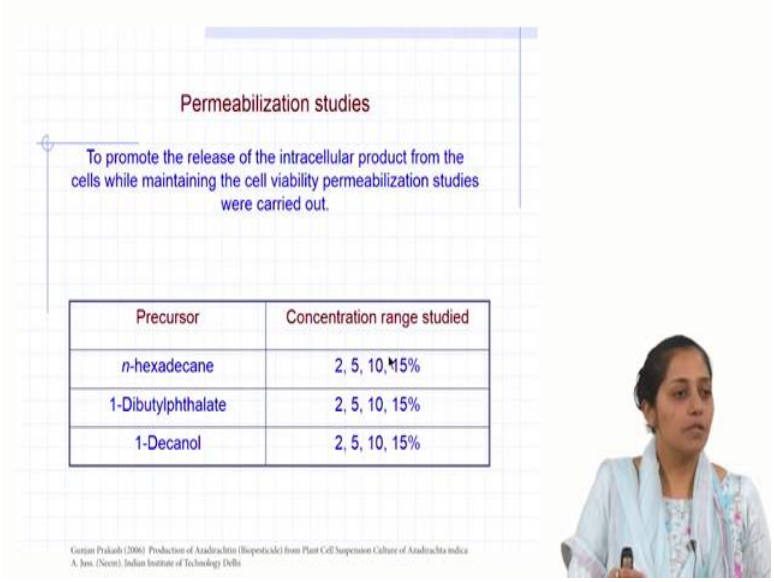

They used certain permeabilizing agents which are known in literature in concentration range which are well below that concentration which can affect the viability. But still viability was checked as the data from literature can vary among species.

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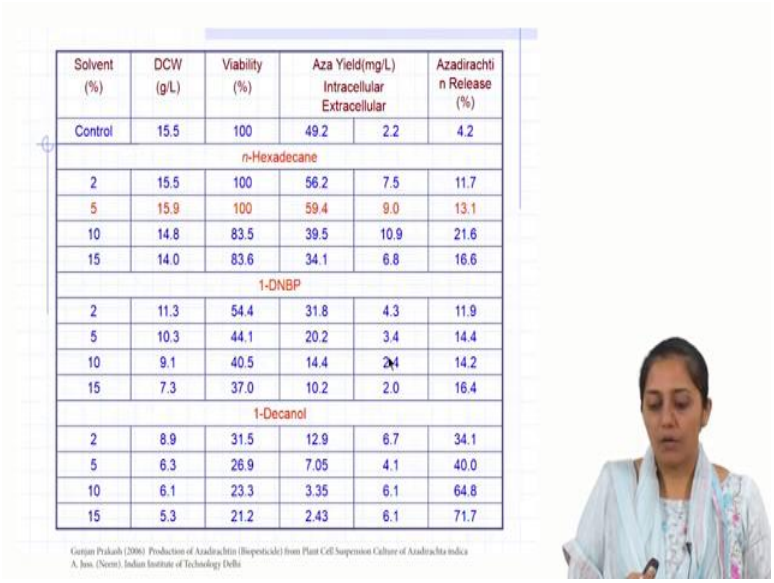

And they could find that n hexadecane at 5 percent volume could give you azadirachtin release up to 13 percent. Now, why do you think other permeability enhancers which are

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.

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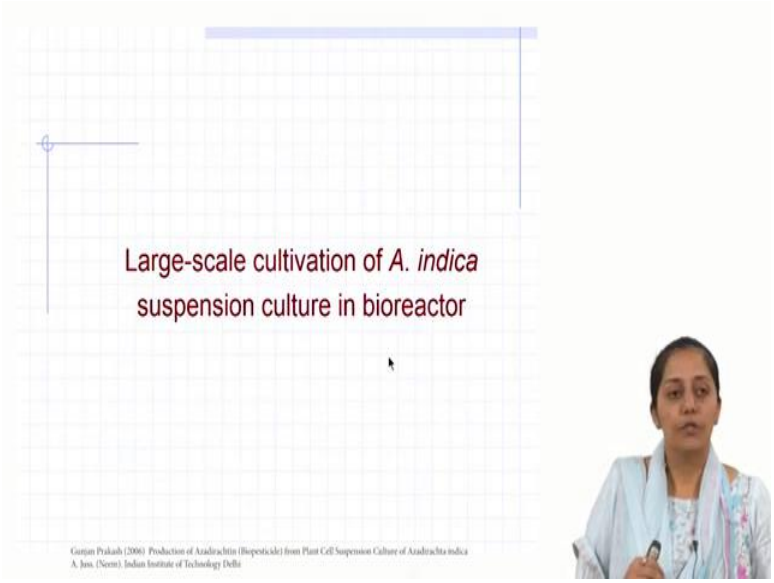

So, once we came to know that these are the strategies, which are leading to enhancement; then it is important to choose the right reactor for mass cultivation.

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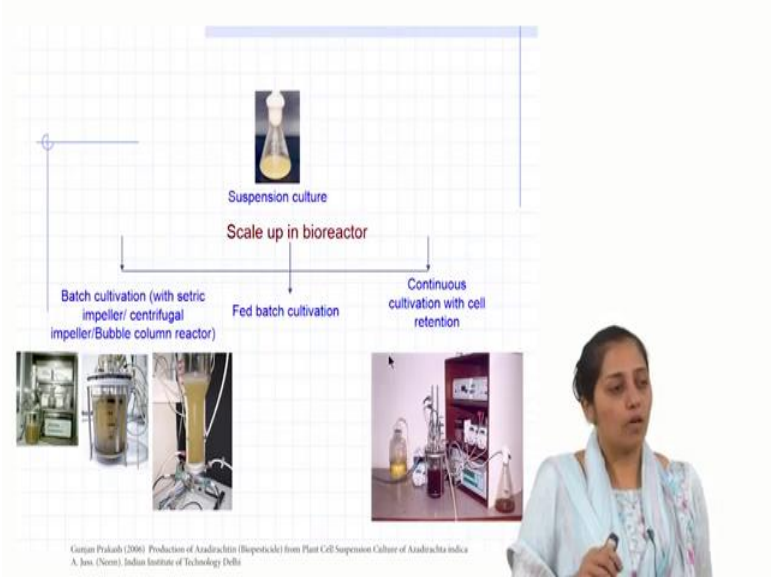

For that what was done? Batch cultivations were carried out. First even in the stirred tank reactor I mentioned that the shear forces can be varied by changing the impeller design. So, then they did a study in which in the same stirred tank reactor, there were different impellers which were used based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

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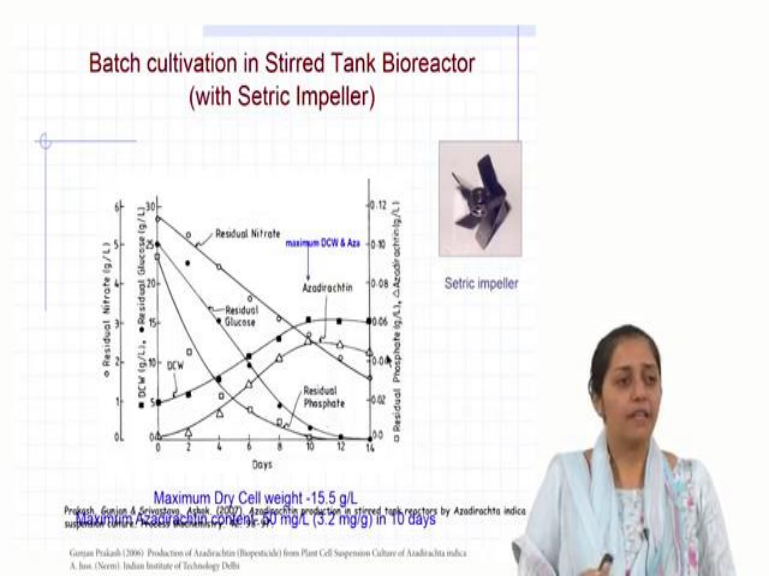

And for this then they drew the kinetics in each of these reactors. So, first is the simple reactor, which is easy to scale up which is your stirred tank configuration well known in industry. So, stirred tank configuration setric impeller was used and the kinetics was drawn. So, what could you see as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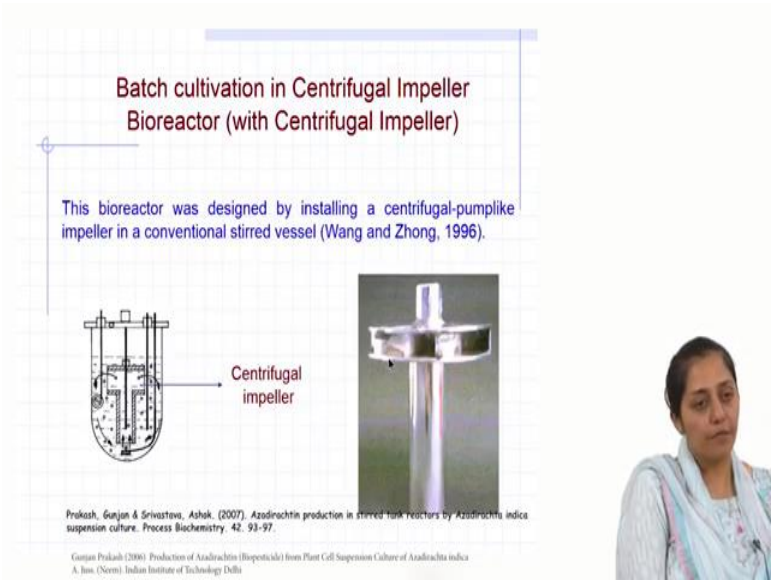

Then centrifugal impeller was used.

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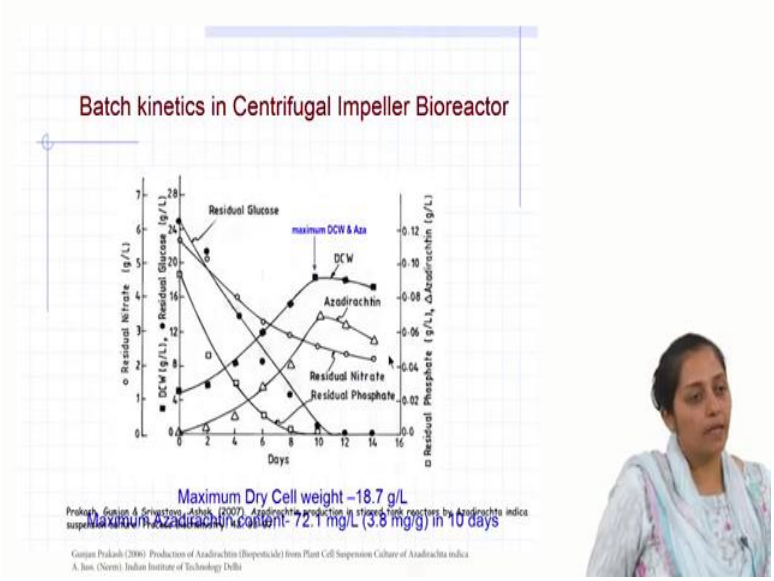

Now, again the kinetics was drawn. So, you can see that the biomass has been improved and the azadirachtin content also has improved in 10 days.

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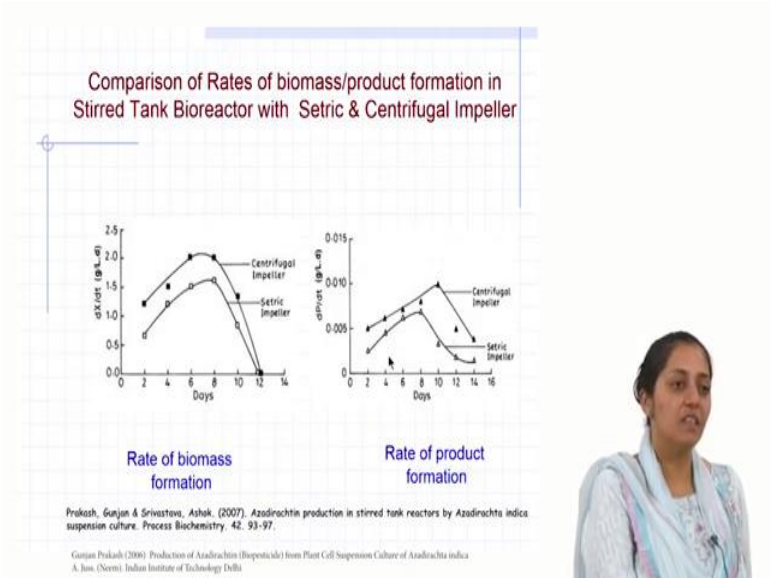

What could be the reason? They drew biomass formation rate and product formation rate. And they could see that you can see from the figures that centrifugal impellers leads to better biomass formation rates during the cultivation period and also better product formation rates. So, they wanted to justify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

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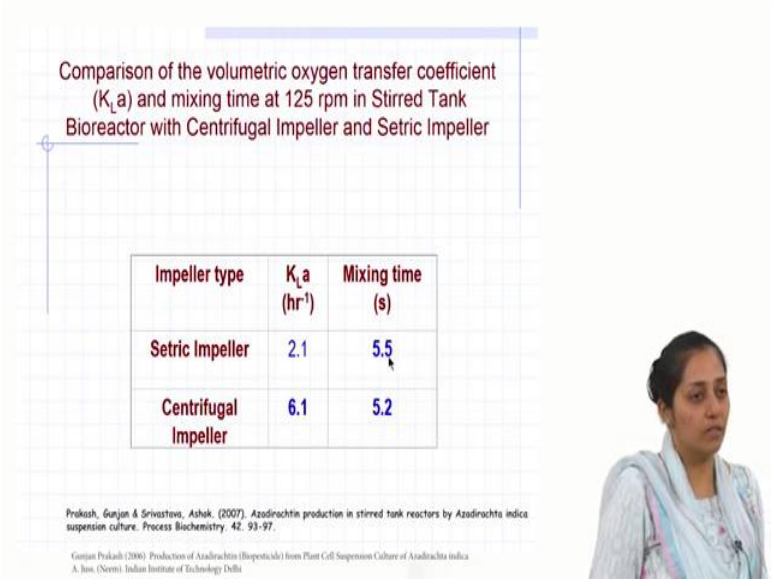

So, they calculated kLa under the given operating conditions in which the reactor was run, they calculated kLa and they also calculated the mixing time. After calculating can you see which one is better, which one is better?

Student: Centrifugal impeller

Why because kLa is better and mixing time is less

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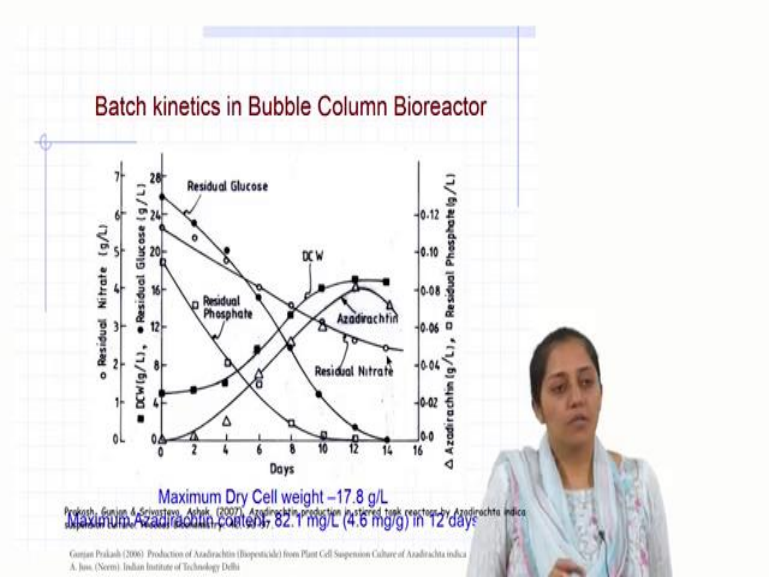

So, then pneumatically agitator reactor was used as no moving parts are there in the reactors. So, they could see that the dry cell weight was equivalent, but the growth rates have gone low. So, which means that, this may be because of better mixing because of the moving part inside the reactor in STR. 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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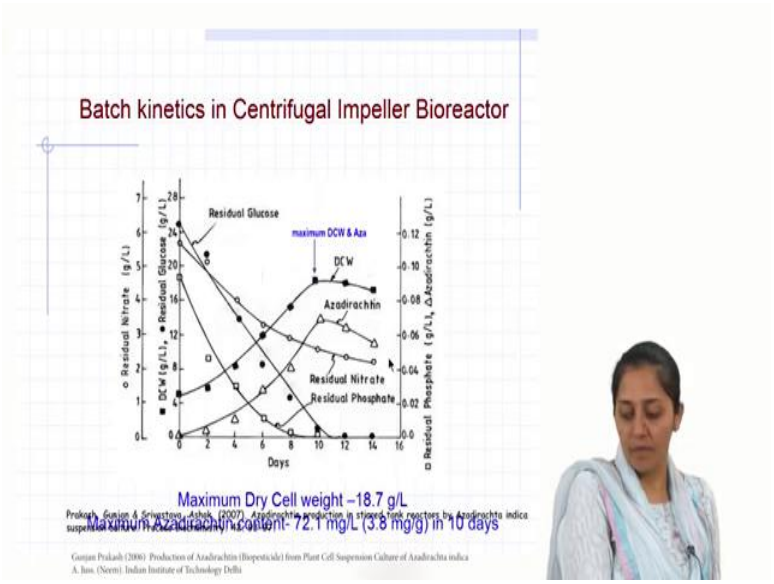

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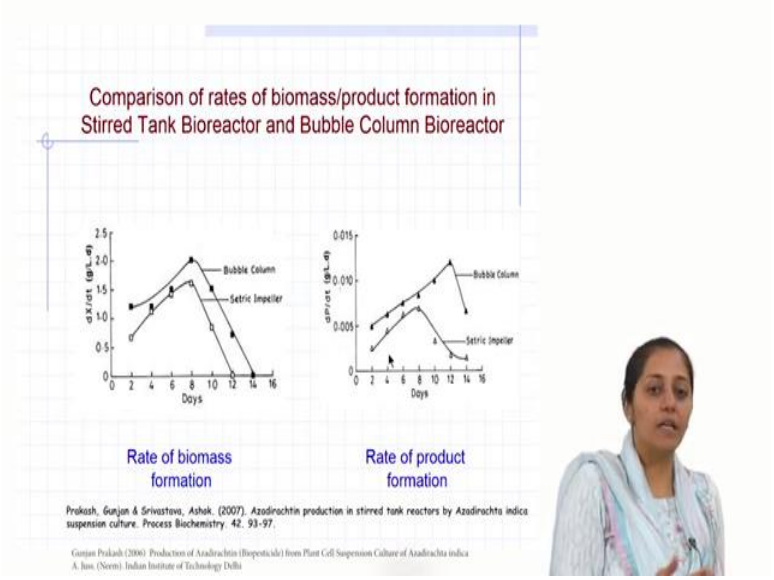

So, when they did a comparison in terms of setric impeller, they could find that the biomass formation rates and the product formation rates were better. But if you compare it with the centrifugal impeller, the bubble column was lower. So, finally, a stirred tank reactor configuration with centrifugal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