Analytical Technologies in Biotechnology

Prof. Dr. Ashwani K. Sharma
Department of Biotechnology
Indian Institute of Technology, Roorkee

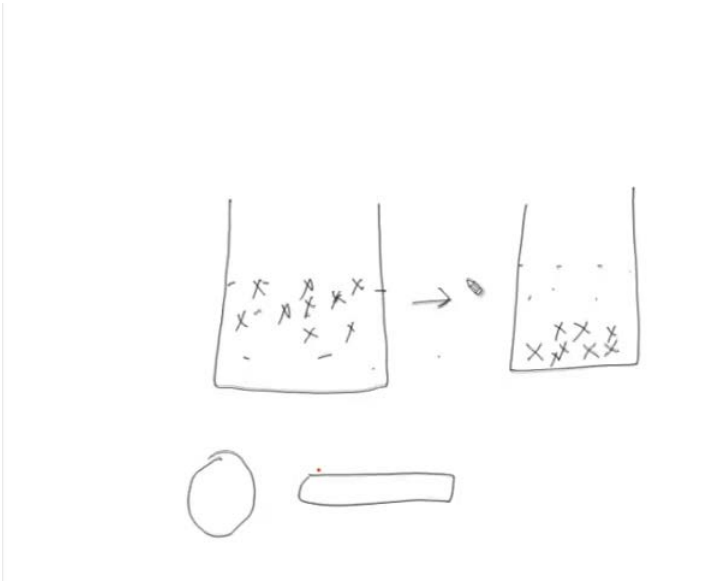
Module - 5
Centrifugation Techniques
Lecture - 1
Introduction and Basic Concepts 1

In previous lectures, we have covered topics including microscopy, radioisotope technique, chromatography techniques and electrophoresis. All of these techniques are widely used for different application in the various fields of biotechnology. In this lecture, we are going to start a new technique that is centrifugation technique. Now, centrifugation is one of the most important, and widely applied research technique in biochemistry and cell and molecular biology in medicine and in various other areas of biotechnology.

Now, current research and clinical applications rely on isolation of cells sub-cellular organelle or fraction membrane fractions macro molecules and lot of other constituents of cell in sometimes in high yield. Now, this separation technique exploits the inherent varied sedimentation property or sedimenting property of substances for their isolation by the application of centrifugation or centrifugal field. It is used as to separate or concentrate material suspended in a liquid medium and the resulting solution has two components namely the sediment and supernatant.

Now, before we go into the details let us let me give you an overview of this technique and let us understand little bit about what we are going to discuss in this particular lecture or in this particular section. So, but we were like first thing is let us understand this through a very simple experiment.

(Refer Slide Time: 02:26)



And if I take say certain particles of a particular size, which are suspended in a say liquid medium what is going to happen to these particle which are of a certain size. Like after sometime what is going to happen is after certain period of time these particles will settle down at the bottom. Now, these particles settle down they will certainly take more time to settle down, but these particles settle down because of gravitational pull or gravitational force.

Now, if we take an example here that if the size of the particles is larger than these particles then they will take less time that is gravitational pull will be more, and they will settle down faster. Supposing there are particles which are smaller than these particles then they are going to take more time to settle down. So, what we see is the size plays an important role where through gravity a particular particle is settling down. Now, the rate at which they are settling down it depends like we have seen size, but also the gravitational force acting or the pull or the gravitational pull.

Now, if you want to increase the rate of settling or sedimentation we can call it then you have to increase the force on these particles. So, that they can settle down faster and this force could be given by given in the centrifugation a particular centrifuge that is when you rotate a particular tube containing this material or this particle suspended in a liquid then they will experience a particular centrifugal field and they will settle down faster. Now, remember as the particle goes, now there are certain factors in which the whole

thing depends. One is like one thing is we are talking about size of the particle another thing could be shape of the particle.

Supposing you have a spherical a spherical material of a particular molecular mass and you have the same molecular mass your material is elongated or it is a flattened structure. Then certainly spherical structure or shape will settle down faster or will move faster under the influence of the centrifugal field rather than a flattened or elongated structure. The shape will also play a role and then density of the particle. Denser the particle more faster it will move in the liquid medium when during the centrifugation.

So, these factors that is size, shape and density will play an important role as far as particle is concerned when it is put under a centrifugal field. Now, other factors which are going to play are an important role is the medium in which they are suspended like density of medium, viscosity of the medium all these things will also play an important role and also the frictional force because if you remember in electrophoresis technique and other technique we have discussed about when a particle or an analyte move through certain medium.

And if it is a liquid medium there will be an upthrust and a frictional force will also be generated. So, what is happening here is that main thing is that through the centrifugation you are able to sediment a particular particle or particular say macro-molecule we can take an example of protein faster. Now, and as we are talking about particles type when we reach to cellular label these thing like cell or say sub-cellular organelle. And further if you go ribosome or protein materials lipoproteins or nucleic acids.

These are very small or as far as if we compare the sizes and for settling or for sedimentation they will require much higher centrifugation force. So, they can be only like they cannot be settled just on the basis of gravity. They need to be put under a particular centrifugal field for sedimentation. Now, these particles also have characteristic density they have characteristic shape like proteins could be spherical, they could be elongated depending on whether, they are particular function they have like say structural proteins are mostly elongated other proteins, which are functional proteins or which are like enzymes. They might have most of the time they will have a spherical or type of structure.

So, they will be accordingly they have a particular density and all these substances which could be say sub-cellular fractions of proteins which settled at characteristic centrifugal force. Now, centrifugation can be of two types one is like this technique could be both analytical as well as preparative. Now, when you have preparative centrifugation, then centrifugation is a done for a purpose of collecting a particular material after the centrifugation and for another biochemical investigation.

Now, when you are doing centrifugation I will show you that in a little while, but just to make you understand the centrifuge provides a means for centrifugation. And it has rotor actually these are different kinds of rotors, which we are going to discuss in detail, but just for now here there could be angled rotor or swinging bucket rotor or there could be other kinds of rotor, which these rotors could be placed on a drive shaft of the centrifuge and the material which needs to be centrifuged put in the tubes.

(Refer Slide Time: 09:21)

For example, there is a central drive shaft and if you are putting say there is a rotor here supposing this is your rotor which has an area to be kept in here. So, this rotor can be kept in here on a drive shaft and this contains areas where you can put your tubes actually. So, this will be circular fashion and this has an opening this could be closed in here when you open this then you can put your tubes in these slots, there could be a mostly these are even number slots. So, that you can balance them and rotor is balanced.

So, when you rotate this at high speed then there is a centrifugal force which is outward and that will lead to the settled settling of sedimentation of the particular material.

So, preparative centrifugation will result in certain material which could be recovered after the centrifugation and it could be used for the biochemical application or for the investigation. As far as like this could include like say you would like to purify or to get at then say you have microbial cells harvested and you want to get proteins in there you can lies the cells and then you can centrifuge and proteins will come in the supernatant. So, that can be done.

Next is that you can also separate say different sub-cellular fractions sub-cellular organelles like say mitochondria, nuclei or lysosomes and lot of like membrane fractions. Likewise you can also separate at higher speed protein fractions or ribosome's and other nucleic acids and other fractions. So, as the speed of this centrifugation or if we just call in terms of centrifugal force or revolutions per minute of the rotor, then as the speed increases the centrifugal force increases, and the particle which are smaller in size.

So, if you increase much more of the particles which are much smaller in size also can be sedimented. So, you go from you have centrifuges from low speed centrifuges which are very simple bench top centrifuges to a very high speed centrifuges like ultra centrifuges which can go up to like 600 or 700 1000 g force. We are going to discuss what is g force so in preparative that is what is that you recover the particular material. While in analytical centrifugation what is done it is mostly you are sedimenting or you are like a putting in mostly purified or partially purified material for analysis.

And analysis could involve this sedimentation behavior of a particular analyte or you want to calculate the sedimentation coefficient for interaction and lot of other studies could be done. Now, when you are doing it the rotor and there is a detaching system which is available in analytical centrifugation technique are analytical centrifuges where in real time you can monitor the whole process as it is going on.

So, analytical centrifugations are different than the preparative centrifugation. So, these are now if you consider different types of centrifuges likes I said it could be on basis of speed and capacity they could be differentiated. Like there could be like low speed and low capacity. Once there could be low speed and high capacity centrifuges. And they could go up to ultracentrifuges, which is like a very high speeds or very high centrifugal

forces available in there. There could be a like a the centrifuges could also be differentiated on basis of like a donor rotor.

There are simple rotors which are for small capacities there are rotors which are high speed and which can sustain high speed and which can have a high capacity as well. So, there are lot of differentiation which we are going to discuss about in terms of types of centrifugal types of rotors. And how do you maintain those rotors like taking care of those rotors for a like a they are increasing their age or for better performance.

Another important thing is what we are going to discuss about is certain method in centrifugation. Particularly ultracentrifugation like density gradient centrifugation, or differential centrifugation and other we will be discussing about certain zonal rotors also, where large amounts like there are one is bench type rotor another is continuous type rotor. Continuous type rotor is that you keep on applying the samples and the also the cells are sedimented, but rest of the material is taken out. And when this is utilized when large volumes are large volume of samples are available. Like I said there could be many liters of volume of sample, but containing very little number of cells where you'll be utilizing continuous flow centrifuge.

So, before little like an overview like what we are going to discuss in this section. So, as we were discussing theoretical basis of centrifugation technique is the effect of gravity on particles. So, like I told you like particles when they are just suspended in a liquid medium left then they will settle down after certain period of time depending on what is the size of the particle. And this could be like a when you are settling them two particles which are of different masses will settle in a tube at different rates in response to gravity.

So, if you want to settle them fast then centrifugal force is used to increase this setting rate and this centrifugal force in terms of multiple of gravity gravitation force. That is if you say like you can say that this many time of g force or gravity. Now, a centrifuge what it does is it utilizes the centrifugal force or g force to isolate suspended particle from their surrounding medium on either this could be a bench or like I was thinking continuous flow basis depending on your application.

Now, so this technique in particular is based upon behavior of particles in applied centrifugal field that is particles differ in shape size or density. And on this basis on their physical properties they can be separated as they sediment at different rates from each

other in the centrifugal field. Now, the rate is directly proportional to the applied centrifugal field. So, in very simple terms that if you have two particles if I say shape if you have two particles of the same size, but different shape they could be separated because they will settle down according to their shape also.

Likewise if the particles of same size but they have different kinds of like density then they could be separated, likewise if there are same density particles but they have different sizes then also they could be separated. So, these properties could be utilized to separate different kinds of substances or particles from each other. And in an centrifuge and the rate at which they will be sedimenting will be directly proportional to the applied centrifugal field.

Also one important thing that will come up is let centrifugal field of force is outward in direction. And it will also depend on the distance the particle present the distance between the drive shaft or the central drive shaft and the particle that is radius you can say more will be the radius of the particle position from the center more will be the centrifugal force. Alright so what is done essentially in this.

(Refer Slide Time: 18:58)

If you see this figure there is a centrifuge a very typical centrifuge it is a bench top centrifuge. And if you can see here there are like a essentially what I want like to show you if there is a rotor here inside it the tubes, which are put in to enlarge this here what is done is this rotor which is placed in on the central drive shaft of the centrifuge, which

contains some motor to run this. The material or the particle is suspended in a suitable liquid medium and then this particular medium with particle is kept in this centrifugation tubes. Now, these could be plastic tubes or they could be other glass tubes also, but glass tubes can crack when you are like a applying larger centrifugal force.

Now, if you see here these tubes are kept in this slots which are available in the rotor and this is the rotor is true here which can be closed here and this is center position where in the in the centrifuge it is placed. Now, remember these are centrifuged tubes has to be balanced there are even number of slots and even if you want to put only one sample, you have to balance it with the other tube of the same weight actually because otherwise, the balance of the centrifuge will be not proper and it will not run properly.

So, this is how centrifugation will be done in a centrifuge in a typical centrifuge. Now, this centrifuge could be a very small like micro-centrifuge where only micro they like very small amount of samples could be loaded, and could be as we big as floor models which will take many liters of the samples. So, but they will essentially many things are there they have all controls are there are refrigeration systems varied like ultra-centrifuges are vacuum systems, they have means to set up the speed a limit. And according to the rotor you can set the amount of centrifugal force to be applied remember these rotors.

Like they specify the kind of centrifugal force, which should be applied on them because the material which they are made of can only sustain certain amount of centrifugal force a certain amount of stress. So, these are all automated centrifuges and the function well and they are also refrigerated for a say biological molecules which can go bad as heat generation might take place. So, the centrifugation is utilized in many different fields like in the field of proteomics it has been quite utilized in the place a vital role and lot of other fields cell biology. Molecular biology plays a very important role.

It has come of like in proteomics where you require to isolate proteins in like I was telling you that a large amount of harvested cells are taken, where you are you have cloned and expressing a protein or from cell you are trying to read the proteome of a particular cell, so where it is necessary to disrupt or lice the cells. And this could be done by lot of different methods like or freezing or other methods. Once the cells have been

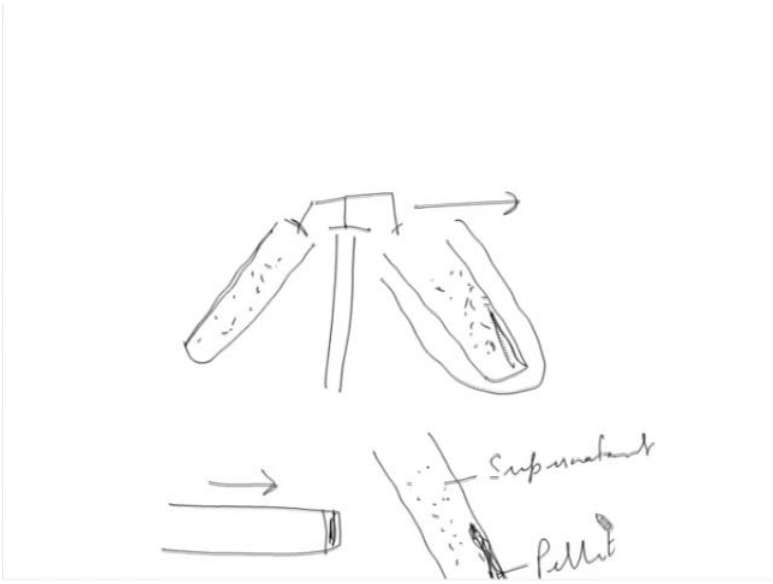
opened up. And all of their contents that is a cell membranes, RNA, DNA proteins, organelles will mix in the solvent with and the proteins as well.

And then you need to perform centrifugation so that it could be differential or density gradient as we are going to discuss in later part of this section. And the then what is done is they can be separated out from the non-protein material. Proteins could be separated out and this could be then a within the centrifuged samples are at very high speed. And the resulting force causes particles to separate settle down or palette actually you can say and like and based on their densities and other factors. And as per paletting is concerned like let me show you what does that mean actually.

(Refer Slide Time: 23:35)

You have say tubes here and say supposing this is your rotor. And now when these are centrifuged then you have material evenly distributed all over like cells or like say of you have the cells then both parts of cells like cell membranes inside content everything is in here. It is a hazy mixture or now when you centrifuge at a particular centrifugal field what you get is that a palette and a supernatant is the content. So, what does palette if I say here then after this centrifugation is complete.

(Refer Slide Time: 24:19)



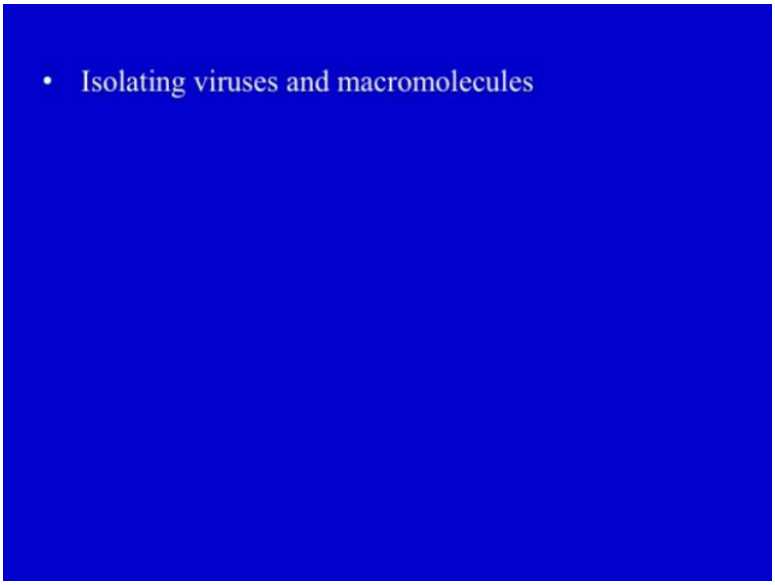
Then there will be certain material which will be settled on here on the wall of the of the of the tube and rest of the material which is liquid material clear liquid material that is supernatant. So, this is supernatant and this is palette. Now, remember depending in the kind of the rotor is palette like for in angled rotor this is angled rotor they will settle down at the side, if it is a swinging buckled rotor where these rotors are like when they when they is they are in the centrifuge is switched on. Then they will go out because they are free at least they are not constrained, like in here this rotor this tube is constrained the slot, but these are not constrained they are open.

So, they just fly in open and their palette will be at the bottom because centrifugal force is in this direction here centrifugal force is in this direction. Therefore, the paletting will take place on this wall. Likewise in vertical tube rotors it will happen. So, this is just to explain that a when you are doing proteomics and you are trying to read proteome. And you want to separate lot of proteins, and you have lice the cells then they could be separated out like protein and non-protein material could be separated on centrifugation.

So, there are lot of applications of this particular method or technique. And centrifugation is capable of doing lot of different things in various, and for various applications and all the laps routinely use centrifugation method. Centrifugation mostly like every lab will at least have a table top centrifuge with a decent capacity. And the most of the time they might be refrigerated as well. So, centrifugation is capable of like

for example, it is capable of removing cells or other suspended particles from the surrounding a milieu on either a bench, or a continuous flow basis like bench. We have seen just now like how cell lice cell material could be separated from protein solution.

(Refer Slide Time: 26:41)



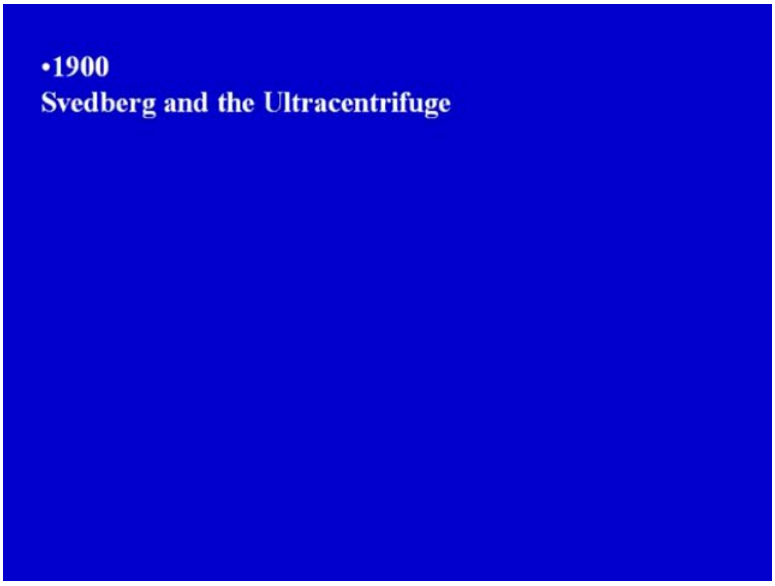
So, it could be utilized for a isolating viruses and various macromolecules including DNS, RNA, proteins, lipids, lipoproteins as well they could be also utilized for establishing physical parameters of these particle from their obscure behavior during that centrifugation. That is like sedimentation coefficient how fast they centrifuged in terms of Rhydberg unit we will be talking about that.

They could be utilized for separating dispersed tissues the various sub-cellular organelles sure for example, in nuclei mitochondria, chloroplast, golgi bodies, lysosomes, paroxysms, membranes, and the plasmic reticulum, ribosomal sub-units all these things could be separated by various methods like a density gradient centrifugation or differential centrifugation.

So, there are lot of different applications of the centrifugation technique actually. And like I said it could be both preparative as well as analytical. Now, development of centrifugation if you go in a bit of history it is started after 1850 and a few like important events which happened during the development of centrifugation. In 1864 invented the first dairy centrifuge in order to separate cream from milk.

So, that happened in eighteen sixty four then the potential of the centrifuge in the laboratory setting was first exploited by Fredrick Michel in 1869. And what he did was he used the crude centrifuge system to isolate a cell organelle. And this process led to the discovery of an important new class of biological constituents and these are macromolecules known as nucleic acids. In 1879 Gustaff d level demonstrated the first continuous centrifugal separation making it a commercial application feasible. So, the continuous centrifugal separator was invented.

(Refer Slide Time: 29:11)



Then in 1926 Theodar Svedberg, one of the person who has done a lot in the field of centrifugation and he received the Nobel also for mention of the analytical ultracentrifuge. And this was capable of achieving very high g force like 900 thousand g force. When we say g force it is a multiple of g x it is gravitational force. So, he was awarded Nobel for a this particular thing that is a invention of an analytical ultracentrifuge and his work on colloidal chemistry.

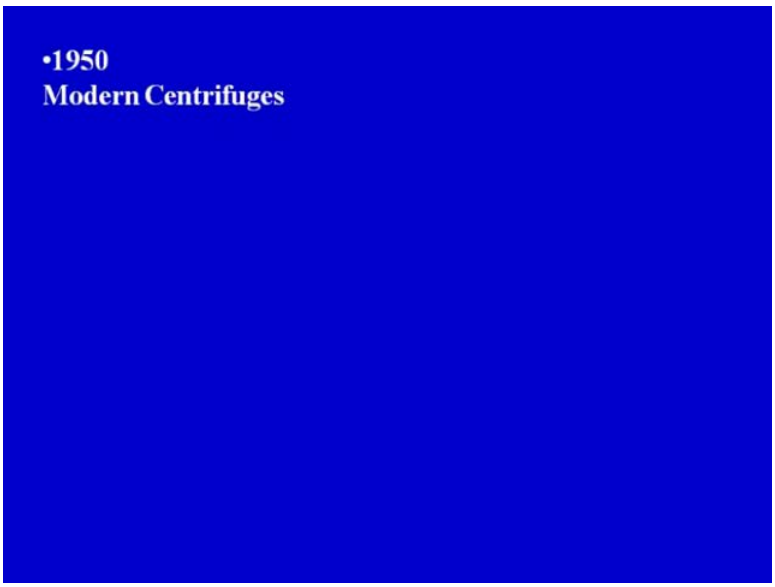
And this started a revolution in understanding of the structure of proteins, like you can analyze proteins with different properties of the proteins in terms of sedimentation coefficient others and here. Now, a friend physicist Emily Henroy, developed the first preparative ultracentrifuge, which was able to achieve very high rotational speed by means of a bearing less top driven and supported by compressed air. So, it was like a

very high rotational speed for preparative ultracentrifuge where and very spatial centrifuge.

Then interest in the isolation of viruses brought Edward Pickle and Johannes Bowl together to build the high speed vacuum centrifuge suitable for the study of filterable viruses. And later pickle on went on to develop the more conventional electrically driven ultracentrifuge. So, there are whole lot of development which took place in 1900 or 20th century. During the early 1930's Martin Behrens's developed improved centrifugation using density gradient of non-aqueous solvent for the separation of nuclei.

In 1942 Edward Cloud and James Porter published a landmark paper that is isolation of chromatin threads from the resting nucleus of leukemic cells. And this paper outlined a series of centrifugation steps in which either the supernatant, or the sediment was collected until chromatin threads were retrieved from the final sediment. In 1949 Espincorp introduced the model l first preparative ultracentrifuge to reach a maximum speed of forty thousand RPM, and this marked the dramatic change in the this particular company that is espincorp. And 50's like in early 50's they saw the introduction of density gradient centrifugation for tissue fractionation.

(Refer Slide Time: 32:12)

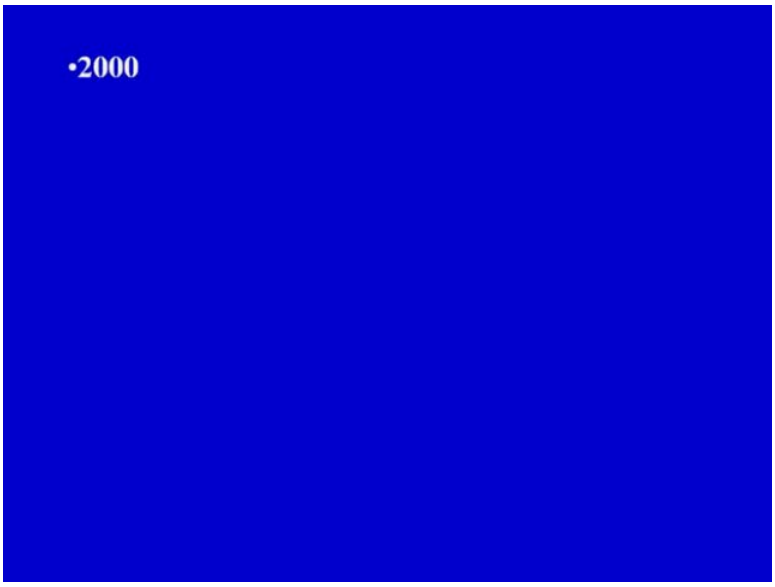


A process developed by a plant virologist Miron k Bragg working at the Brooklyn botanic garden. In 1954 Beckmen instrument now Beckmen actually started improving the design of centrifuges many of which are still used today. And the drive systems were

replaced with high speed motor and the material used for the rotor blade was upgraded, so Beckmen made very popular centrifuge. In 1962 there was like a company based in Hamberg, Germany and now it is known as the Eppendorf developed the first micro-centrifuge for a simple laboratory use. In 1976 the world's first microprocessor control centrifuge was launched by

So, during the 1980's Beckmen launched the first floor ultracentrifuges and during 1990 Beckmen launches the Avanti high performance centrifuge, which went to be one of the most popular centrifuges models in the history. Also during this decade the first centrifuge capable of robotic operation was developed by. So, this centrifuge also offered pc control and adjustable rotor positioning. There were lot of other developments where like in 1992, the PAC the pressure added centrifugation system was developed by Henkel.

(Refer Slide Time: 34:13)



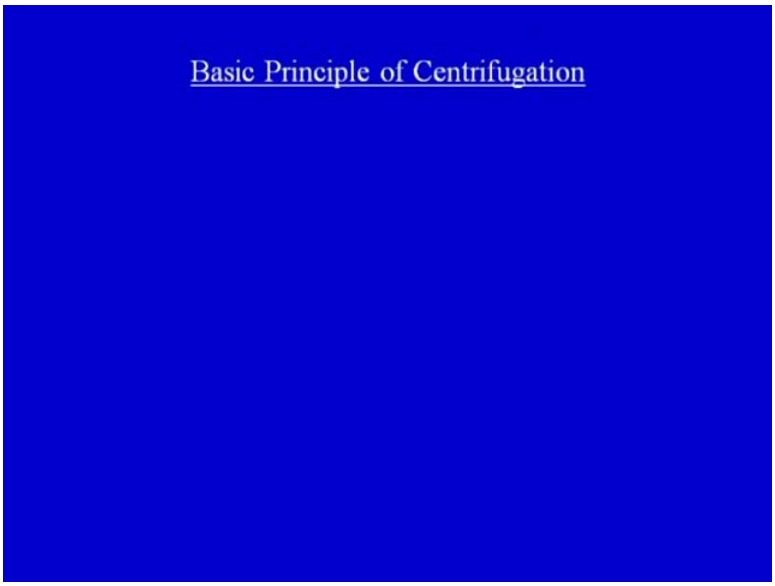
And lot of other developments took place like in 2000 Eppendorf invented his line of classic micro-centrifuges with the cooled, these are cooled centrifuges and smallest and quietest you can say centrifuges. And also Eppendorf also launched personal centrifuges like mini spin and mini spin plus. So, there was lot of development as the time passed. And now the modern centrifuges are very advanced and automated also, with lot of options. In future like now centrifuges have lot of different advanced technology like for example, in case of rotors.

Rotors have very high tensile strength the tensile string is like replaced by a material such as aluminum alloys, and particularly titanium in order resistant high centrifugal force. Most of the high speed rotors now rotors are made up from titanium or and they could withstand very high stress actually. There are standard features included in the centrifugal for say cooling for programming a particular run like automatic imbalanced action like. For example, you are putting in two tubes like I told you have to balance them if the balance is not there the centrifuge will not run.

So, those kinds of things are incorporated noise reduction help in a very important part of this development and changeable rotor systems. Like you can put many rotors of the same centrifuge you can put like say Eppendorf rotor or micro-centrifuge rotor or you can put 50 m l tubes rotor or swinging bucket rotor. Many different types of rotors are put so the centrifuges which are now operating.

And which are being developed are much more advanced and very high speed centrifuges like ultracentrifuges vacuum systems has been added in these modern centrifuges to reduce the friction and maintain the temperature control. So, lot of automation lot of advancement has taken place and it is still going on in the area of centrifugation and very good centrifuges are now available for various applications.

(Refer Slide Time: 36:50)



Till now I have given you an overview of the centrifugation technique. Now, let us discuss about the basic principle of centrifugation. Now, centrifugation method is based

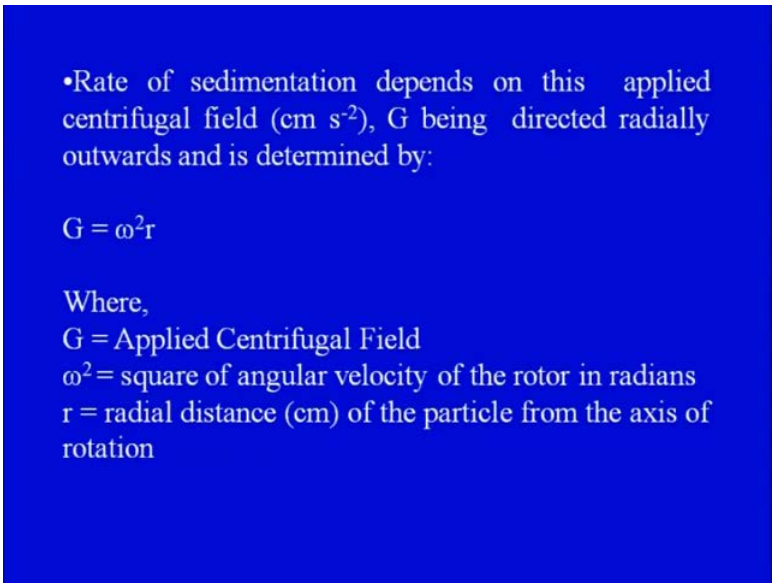
on the principle of sedimentation and when a particle sediments what it does is it displaces some of the solution in which it is suspended. So, that will result in an upthrust on the particle and that will be equal to the weight of the liquid displaced. Now, rate of separation in a suspension of particles by way of gravitational force mainly depends on the particles size and density.

So, size and density are the main and the intruding shape are the main factors which will determine the sedimentation behavior of a particular particle. Now, particles of higher density or the larger size typically travel at a faster rate. And at some point will be separated from a particles less dense, or smaller the smaller particles will travel at a smaller like they will take more time to settle down as compared to larger or higher density particles which will settle down faster.

Extremely small particles will not settle down or settle out of solution unless they are subjected to a very high centrifugal force like I was discussing in the very beginning that as the particle size becomes smaller then, the amount of centrifugal force which needs to be applied like becomes larger. So, that is how it will be smaller particles will not settle out easily and they require much higher force. For example, if you have to settle down say or sediment certain protein material then you have to apply much larger force, as compared to the if you want to settle or say sub-cellular fraction has to be sedimented where you require a less force.

So, when a suspension is rotated at the certain number of revolutions per minute now remember here there are two ways you can like understand the centrifugal force application. One is in terms of revolutions per minute and another is centrifugal force which is in real being experienced by a particle. So, when a suspension is rotated at a certain number of revolutions per minute, then the centrifugal force causes the particle to move rapidly away from the axis of rotation. And another thing is where the particle is present, whether it is very close to the center of rotation or it is very far from the center of rotation both of these particles are will experience different centrifugal force.

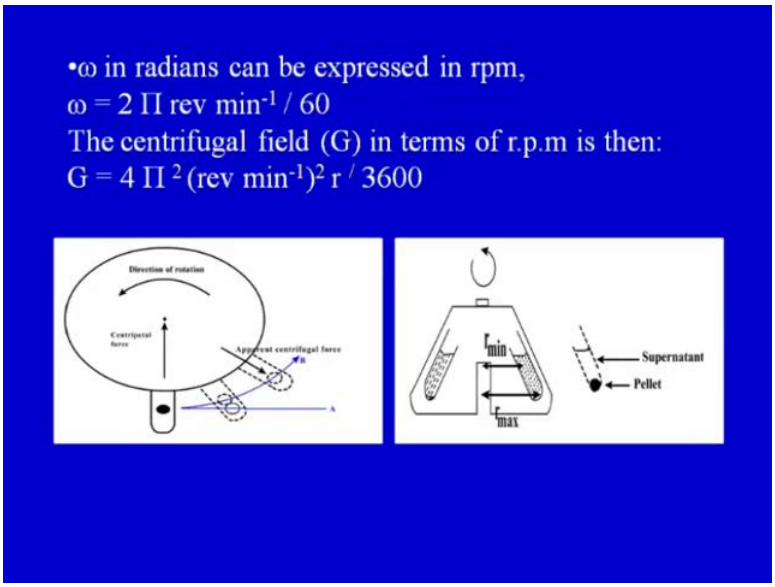
(Refer Slide Time: 40:01)



So, the rate of sedimentation depends on the applied centrifugal field. And that is represented if we say in capital G here which is being directed radially outwards actually. So, centrifugal force anything which moves in circular motion it will experience a outward centrifugal force. There is a centripetal force which is inward and there is a centrifugal force which is outward.

And this is given by omega square r which is it is the angular velocity and this is square of angular velocity and the r is the radial distance in centimeter and angular velocity is in radian per second it is in radians. So, r is the radial distance of the particle from the axis of rotation, so that is how particular centrifugal field is being experienced by a particle and rate of sedimentation will depend on that particular centrifugal field.

(Refer Slide Time: 41:13)

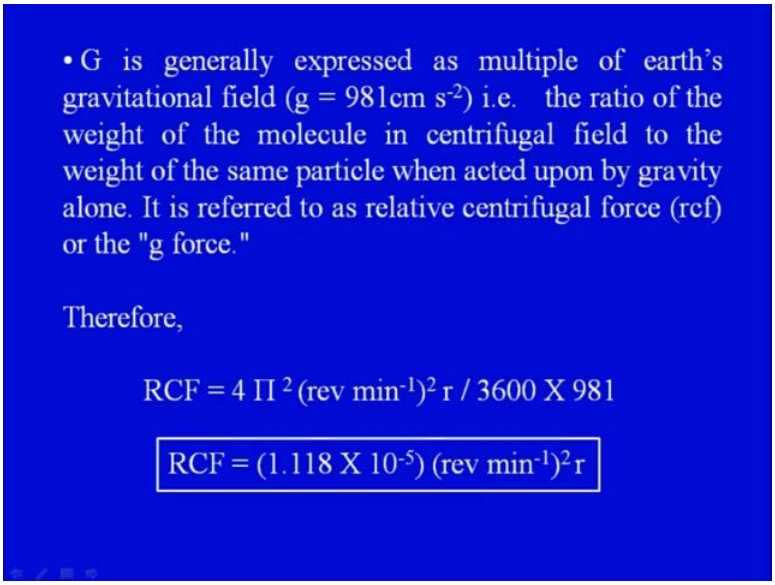


Now, this angular velocity can also be expressed in terms of the revolutions per minute. And you can write that angular velocity or omega equals 2 pi revolutions per minute divided by 60 or it could be written in terms of G for centrifugal field, which is 4 pi square revolutions per minute and to r divided by 3600 that factor 60 the square of the 60 here. So, if you see in this figure here this is typical like the direction of the rotation like when you switch on the centrifuge, the rotor will centrifuge in a particular direction and there will be an applied centrifugal force which will be in outward direction.

Like if it is the axis of rotation the outward force will be in perpendicular to that axis of rotation. And that will be outward centrifugal force will be applied depending upon what kind of rotor as I said, but the direction of applied centrifugal field will be the same here. As you were talking about the radial distance from the center, if you can see here if the is an angular rotor as we discussed in a coming time.

There will be tube like r minimum which is the meniscus here from the object tube from the center of the rotation, and this r maximum which is the bottom of the tube from the center of rotation. So, many times r average could be calculated or taken as we discussed later. So, this particular thing could be these are centrifugal effect you could say when these revolutions per minute are being taken here or you can take it in terms of angular velocity.

(Refer Slide Time: 43:11)



So, now the centrifugal field is generally expressed like I said in multiples of Earth's gravitational field. So, Earth's gravitational field is always present which is 981 centimeter per square second. And it is the ratio of the weight of the molecule in centrifugal field to the weight of the same particle when acted upon by the gravity alone. It is referred to as the relative centrifugal force or the g force. And the relative centrifugal force can be given by this particular equation which is like a you have divided it by 981 or gravitational force also to give the relative centrifugal force.

And in simplified terms it comes out to be 1.118 into 10 to the power minus 5 revolutions per minute square into the radial distance. So, this is like how you can calculate relative centrifugal force in terms of the gravitational force which is the most widely used term here. So, it could be either revolutions per minute it could be utilized or the relative centrifugal force could be utilized.

So, will stop here in this lecture and this lecture we have discussed about the basic features of centrifuge, an overview of the centrifugation techniques, where how a particular property that is size shape and density of a particle can be utilized to separate them in the centrifugal field. And for this purpose the particular purpose they are different kinds of centrifuges are used.

And as we have seen in the history of development of centrifuges they have developed from a very simple centrifuges to very complex and very advanced centrifuges. Now, there could be different types of centrifuges like from simple small capacity bench type centrifuges to ultracentrifuges. We will continue our discussion in the next lecture. And we will be discussing about the, we will extend our discussion on basic principles of centrifugation.

Thank you.