Experimental Biochemistry
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Lecture - 43
Basics of rDNA Technology Part-I

Welcome back. So, in today's lecture we will talk about the Basics of recombinant DNA Technology.

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Again in this first part, I will go through the basics of this process this technologies. So, I will talk about how do you clone a gene into bacteria even though you can use several systems. For this lecture we will keep ourselves limited to cloning into bacterial system; I will talk about all the different tools that we use for cloning experiments. And again I will talk about all the practical aspects that one has to take care of to do a successful cloning experiment.

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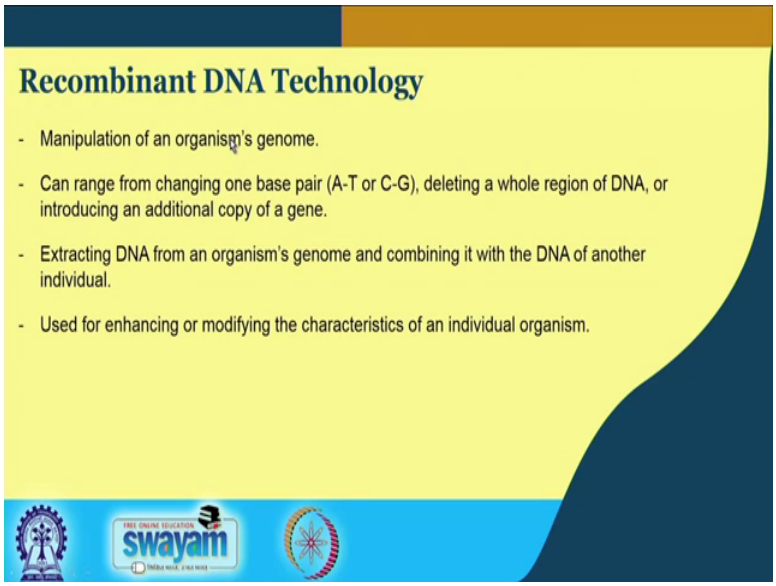

So, what is recombinant DNA technology or rDNA technology? So, it is manipulation of an organisms genome and such manipulation can range from a single base pair change. So, we can mutate one base pair to we can delete a big range of sequences from the genome.

We can add a big range of nucleotides into the genome or we can even introduce a completely new gene into the genome of an organism. So it also in first extracting of DNA from an organisms genome and combing it with the DNA of another individual; so, we do all of that in test tube and then again put back this newly engineered genome into the organism of our choice.

So, it can be used to enhance or modify the characteristic of an individual organism. So, in this case we will restrict ourselves to a bacterial system. So, what we will do is we will have a bacteria and we will introduce the gene of a protein that we want to express into that bacteria. So, the bacteria will not normally express that protein, but we want this modified bacteria to express our protein of interest.

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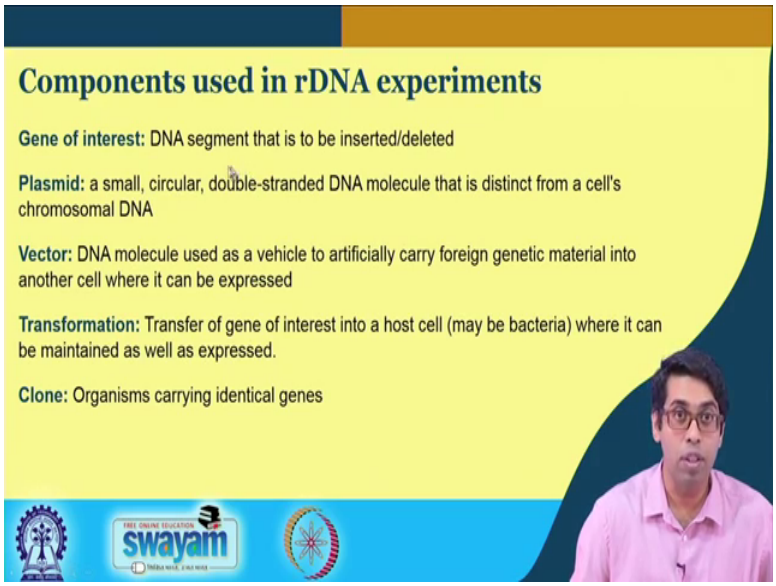

So, here are the components of that are used in our typical recombinant DNA experiment. So, the first one is the gene of interest; so this is the gene which encodes a particular protein that we are interested in. So, suppose you want to do characterize of particular protein as we talked in one of the previous lectures and you want to recombinantly explicit and purify it. So, what we do is; so that is the gene for that particular protein is the gene of interest.

Then there are two components one is plasmid and the other one is vector. So, these we use these two terms interchangeably. So, what is a plasmid? It is a small circular double stranded DNA molecule which is present inside the cell, but it is distinct from the organisms genome. So, plasmids they are typically of the size of several thousand base pairs, let us say 6000 to 10000 base pairs.

On the other hand if you think about the genome of a bacteria, then the bacterial chromosome will be in the order of hundreds of thousands to even millions of base pairs long. So, this plasmids are circular DNA, but they are much much smaller than chromosomal DNA. But they have all the properties of a chromosomal DNA so that they can self replicate. When these plasmid is used to express our gene of interest we call it a vector.

So, what we will do is we will take our gene of interest and insert it into a plasmid and then insert that circular plasmid into a bacteria. So, in that case we will refer to the

plasmid as a vector, because it carries our gene into the organism of our interest. Transformation is the process by which this plasmid or the vector is inserted into the host cell. In the example that we will see will be the host cell will be bacteria and then the bacteria will take care of all other activities; for example, expressing messenger RNA from the gene and making protein from the messenger RNA.

Cloning is the process by which we do all of this and the final organism that is now synthesize is a particular clones. So, if we have a colony of bacteria then all the bacteria are identical they have identical gene. So, then we will call those as a clone.

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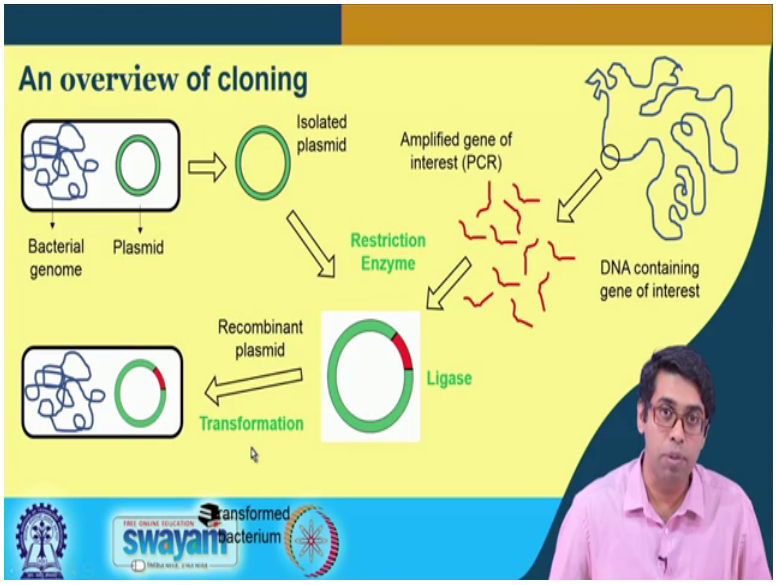

So, is a brief overview of this cloning process or the recombinant DNA technology that we are going to use to express a particular protein in a bacterial system. So, if you consider this as a bacteria so this is the bacterial genomes. So, this is its chromosome which is let us say 1 million base pair long and this one is the small circular plasmid which is roughly 6000 base pairs long. So, the first step is to extract out this plasmid from the bacteria. So, now what we have is the isolated plasmid in a solution inside our test tube.

On the other hand, these small gene or this particular small region which is a gene of our interest. So, it quotes for a particular protein that we want to characterize in our lab and these chromosome can belong to a completely different organism it can belong to a mouse or it can be even a human chromosome.

So, what we want to do is we want to extract out this small gene and we also want to make several copies of that. So, these two things are done in a single step by which is called a Polymerase Chain Reaction or PCR. So, I will go through in details about how you what is this technique and how we use it. So, using polymerase chain reaction we copy and amplify this gene of interest. Now this and these are both in isolated in two different test tubes.

So, now we combine them so that these particular genes are inserted into this plasmid. So, now this is the plasmid which carries our gene of interests and finally, we insert that into the bacteria to get our bacterial clone. And now this bacteria will make our protein and we can break up in this bacteria and purified using techniques that chromatographic techniques that I have already discussed before ok.

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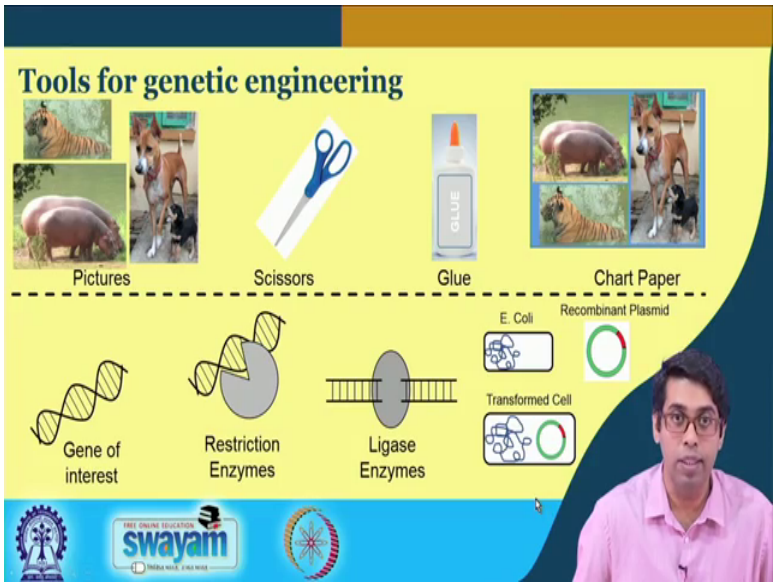

So, what are the tools for genetic engineering? So, as we have seen that it involves a lot of cutting and pasting right; so it seems it is a; it is really similar to making a collage that we often make.

So, suppose you have all these different pictures and you want to make a collage out of this picture. So, what you need, that you need are scissors so that you can cut this pictures according to you need. You want to tick them into a particular book or the chart paper, so you need glue for pasting them. And then this chart paper is the platform on

which you are going to put all this pictures. So, you cut them you paste them onto this and this is your final college right.

So, in a very similar manner for recombinant DNA technology; we have our gene of interest. So, this gene of interest is again coming from a particular chromosome, so we have to cut it out from that gene of interest. And I as I said before that we use polymerase chain reaction to do that, but even of once we have our PCR product; we need to trim them precisely so that we can insert them into the plasmid. So, such precise trimming are done by molecular scissors which in this case are a particular type of enzyme called restriction enzymes.

So, restriction enzymes are nothing, but our molecular scissors. So, DNA is very small which means that you cannot use anything mechanical to cut them in a very precise manner. So, we have to use a molecular scissor and they recognize particular sequences where they will cut. So, the sequences that you want to cut accordingly you will have to use a type of restriction enzyme. So, there are many many restriction enzymes you will you can choose which type of sequence you want to cut.

Once you have these different pieces of DNA, you will need to join them. So, that joining or pasting is done by a molecular glue which is another enzyme called ligase. So, ligase will take one double stranded DNA and another double stranded DNA and it will join them. So, it will join both the strands by forming a covalent linkage; so it will fill in the phosphodiester bond between these two strands so that you will have a complete DNA sequence that so there are no breaks between them.

And finally, once you have so that is what we do to get our plasmid the recombinant plasmid. And finally, you put that plasmid into your organism of choice in today's lecture we will keep ourselves limited to bacterial cell. So, E coli and that is your finished product and you can grow this E coli it will make your protein you can then purify protein using chromatographic techniques.

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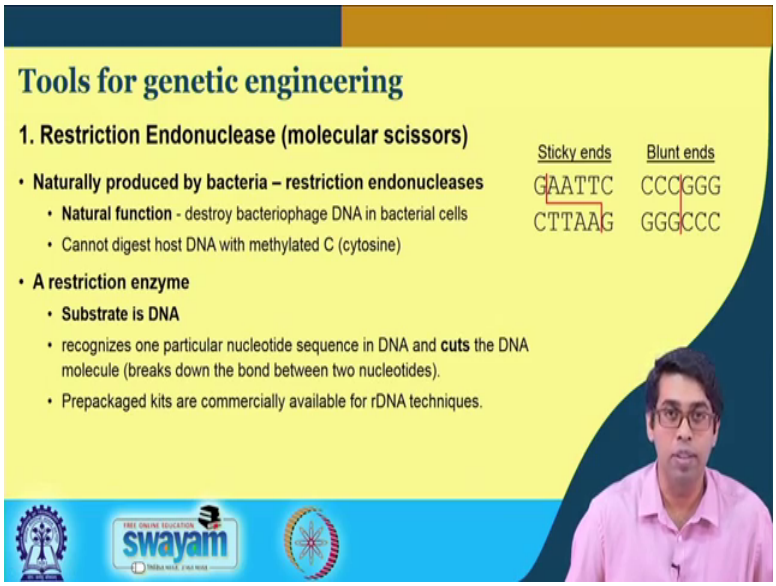

So, let us go through this tools that we are going to use for genetic engineering. So, in this lecture I am going to talk about these tools and then in the next lecture what I will do is I will go through a particular enzyme where we will use them specifically to build our clone.

So, the first one is the restriction in endonuclease which are basically the molecular scissors. So, naturally they are found in bacteria and they have a very specific function. So, their job is to destroy bacteriophage DNA; so bacteriophage is a type of virus which infects bacteria. So, when this virus inserts it DNA it is a foreign DNA and that DNA is quickly digested or chewed upon by this enzymes called restriction endonuclease.

Now, these enzymes do not act on the bacterial DNA because the host DNA has methylation on the C base, cytosine. So, the bacterial DNA is not destroyed, but the viral DNA will be destroyed. And we are going to use this particular enzyme to do our cutting job because as you will see that when you do PCR and other reactions, what you get is naked DNA which has no methylation or anything on it. So, it can be easily acted upon by these endonucleases.

So, for restriction enzyme the substrate is the DNA and it recognize the particular nucleotide sequence. So, here are two examples that I have shown here, the first one is GAATTC; if you notice that it is a palindromic sequence. So, whenever you write sequences for double stranded DNA, you should always know that this end is the 5 prime

end and this end is the 3 prime end. So this is the complementary strand which means that for C you have G, for T you have A and so on. So, for the complementary strand this is the 5 prime end and this is the 3 prime end.

So, now if you read the sequence GAATTC from this direction and in the complementary strand from 5 prime to 3 prime, it reads the same GAA TTC. So, it turns out that this endonucleases in most cases they recognize sequences which are palindromic in nature. This one is also very similar, it is also palindromic CCC then GGG and again if you read from this 5 prime to 3 prime, it is CCC GGG.

Now there are two types of endonucleases one which leaves sticky ends after cutting the DNA. And the other one which leaves blunt end; so what does that mean? Blunt end is actually very simple. So, it will cut exactly like this; so that there are no DNA overhangs left. But sticky ends they cut like this which means that these double stranded DNA will have this TTAA extra and this double stranded DNA, the other part will have these AATT extra; so those are the overhangs.

And they can easily go back and form the hydrogen bond, but there will be no phospodiaster linkage here which means that there will be no covalent bond. So, they can easily again come off, but this type of endonucleases will leave a sticky end and this type of endonuclease will not leave any sticky end.

So, there are many endonucleases which are now commercially available and depending on the type of sequence that you want to cut, you will choose them accordingly. So, in the example of cloning that we will see what we are going to do is we are going to use two endonucleases with both will have sticky end cuts, but they recognize different sequences.

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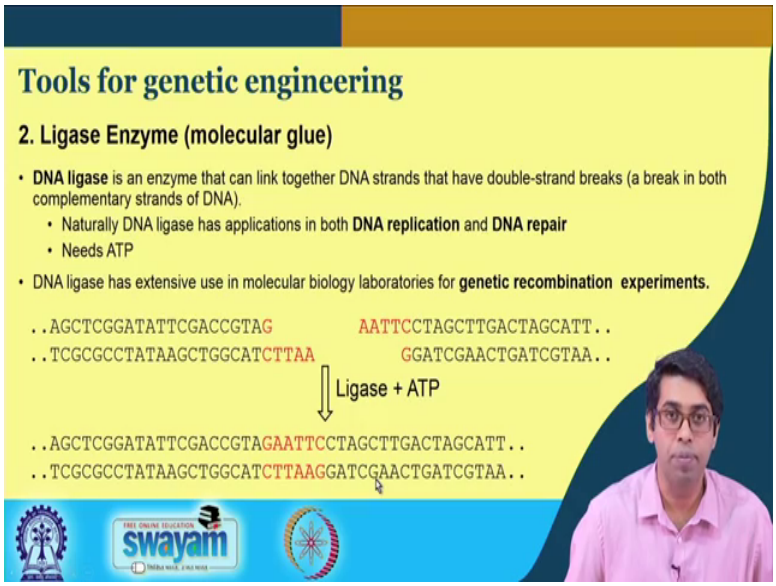

So, the next one is ligase enzyme; so this is the molecular glue . They are again present naturally in these organisms because they are involved in both DNA replication and in DNA repair, but we use them for joining different DNA fragments together. Because as you see that restriction enzymes can cut with sticky ends, but then there will be no covalent linkages between these ends; so we an use ligase to fill in that missing covalent linkage.

So, here is an example, let us this is one particular DNA that comes from some source and this is another particular DNA that comes from a different source. Both have been processed by the same restriction enzyme so that both have the exact same sticky end. So, you can see that these TTAA will easily base pair with this AATT, but they even if the form of base pair there will be no covalent linkage between these G and this A and this A and this G; so to make that happen we will use a ligase enzyme.

So, what you do is you simply add these two DNA fragments together add ligase and you need ATP because ATP is going to supply the phosphate that will form a linkage between these two basis. So, you have to wait for sometime all the enzymes have a have their prescribed temperature and how long you need to incubate them to get of complete reaction. So, once you do that you will have your full length DNA where these two fragments are now joined together.

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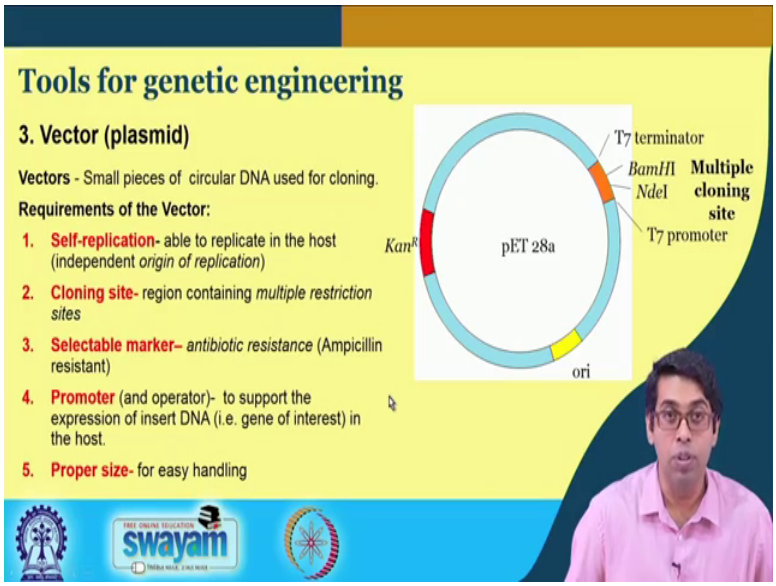

The third component that we are going to use is the plasmid and this plasmid is going to have it is going to carry our gene of interest; so we are going to call it our vector.

So, there are several important characteristics of this vector that are going to be useful for this purpose. The first one is self replication; so this vector is able to replicate on its own inside the bacteria. So, that is because this vector has its own origin of replication; it turns out that inside the bacterial cell these vectors are preset in multiple copies. So, the bacterial chromosome will be there only once, but then this vectors will have 50 to several hundred copies inside a bacteria; which means that if they are carrying your gene of interest somewhere here then there are multiple copies of here gene which results in over expression of your protein.

The second important characteristic is the cloning site. So, this is the cloning site it is also refer to as multiple cloning site. So, this cloning site is nothing, but it has these it codes for all these different restriction enzymes. So, only two restriction enzymes are shown here BamH1 and Nde1.

So, you can you can choose which clonings which restriction enzymes you will want to use and you can use both restriction enzymes simultaneously to cut opened this region of the DNA. So, the way this plasmids are designed is that those sequence is appeared only once in this multiple cloning site and never in this in the remaining of the plasmid.

Because if the same sequence appears here and here then the restriction enzyme will be cut in both places; you do not want to cut them in multiple places you want them to cut only in one place. So, that is why although sequences are encoded only in this small region which is referred to as the multiple cloning site.

So, what we are going to do is; we are going to choose BamH1 and Nde1 to digest these vectors. So, it will cut open this vector and what we will have is a linear DNA molecule. Then the third important feature that it has is the selectable marker, because when you are doing all these experiments; some of these vector will cut opened some will not. Some of this vector will have your gene of interest inserted into it and some vectors will not have the gene of interest inserted into it.

So, we need some way of selecting those bacterial cells which have these vector and from those bacterial cells which do not have this vector. So, these are the two types and also; so how do you do that? So, you want to select bacterial cells which carry this vector which ahs you gene of interest from bacterial cells which do not carry this vector. That is done by a selectable marker which is nothing, but a gene that codes for an antibiotic resistance. So, in this case it is written as ampicillin resistant; you can also have kanamycin resistant because that is what is shown here.

So, if you plate your bacteria on a agar plate which has kanamycin; the your then only those bacterial cells which carry this plasmid will survive because they have this gene. But those bacteria which do not carry this plasmid will automatically die out.

So, at the end of the day when you see colonies all of those colonies will have this plasmid. Then to expressive protein you need promoter; so those are also coded here and this size of this plasmid is also designed in such a way that it is easy for proper handling. Because if the DNA is too big then there is a chance of breaking it when your handling it; so, because of mechanical hesitation.

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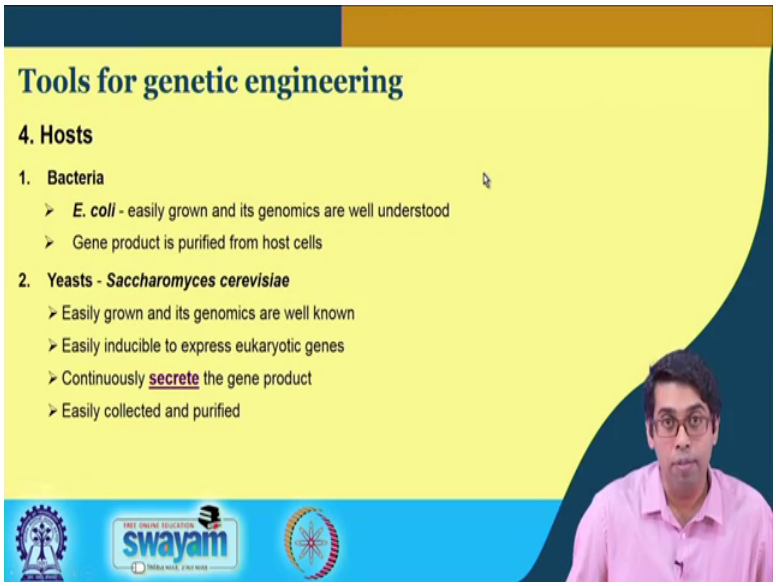

So, finally, once you have your vector ready with your gene of interest you need to insert it into an organism; we are going to limit ourselves to bacteria. And bacteria is a very useful organism because most proteins or many proteins can be easily cloned into vectors that can be over expressed into bacteria and purification of protein is also very easy. So, we can get large quantity of pure proteins from a bacterial cell.

Apart from bacteria is yeast can also be used and it is also widely used; so, for specially for eukaryotic proteins or eukaryotic genes which sometimes do not express well in a bacterial system; they are then expressed in yeast.

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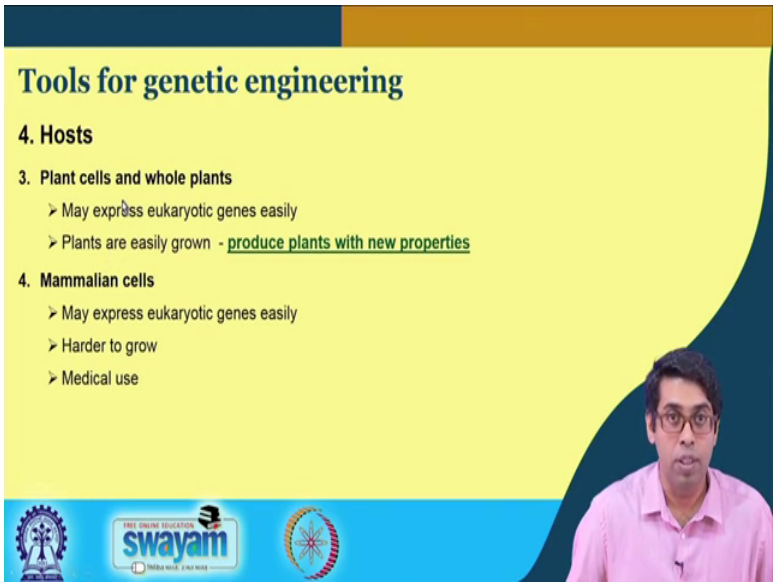

And the other two vectors which are higher organisms or also plants cells and mammalian cells. So, both are very good for expressing eukaryotic genes, but are more difficult to handle plants can be grown easily.

So, new plants with new properties can be generated by cloning new genes into them. And mammalian cells are mostly used for medical purposes where you want to test the effect of mutations. And if you want test the effect of a particular drug molecule on a on a on the expression or repression of enzymes and things like that ok.

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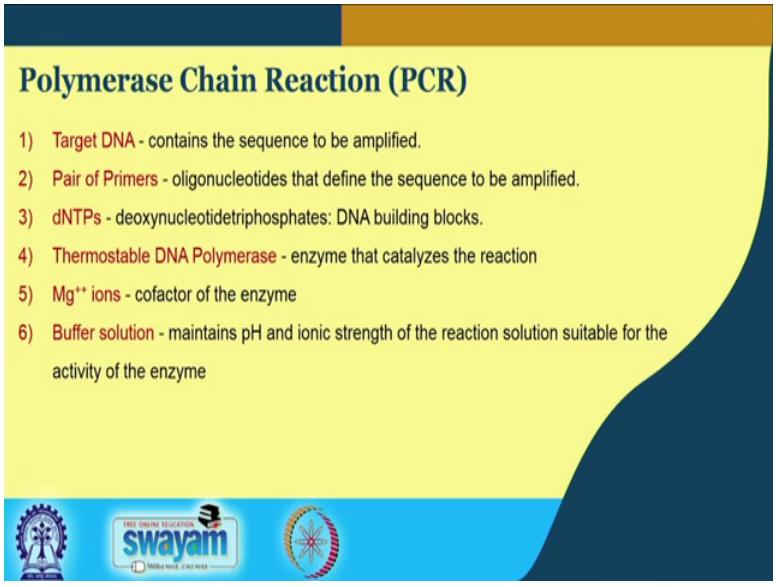

So, polymerase chain reaction; this technique is something that we are going to widely used for recombinant DNA experiments.

So, let me go through the basics and I will try to point out of you important aspects and in the next lecture we will design primers that we can use to extract out a gene and do our polymerase chain reaction. So, these are the components of polymerase chain reaction. So, the first one is the target DNA; it is the chromosome of the organism from which we want to extract out a gene; so that gene is our target.

Then we have a pair of primers, I will talk about the design of primers in the next lecture we need dNTPs because we are going to amplify the gene. So, the monomers of DNA are dNTPs; so you have all the four nucleotides. What we need is a DNA polymerase, but then this is special DNA polymerase because it is thermostable. Because you what you will see is that we are going to mix all of these together in a test tube and then heat them and cool them repeatedly.

So, we are going to heat them up to 95 degree centigrade and then cool them down to 50 or 55 degree centigrade and then we will keep on repeating this cycle. Magnesium ion is a cofactor which is needed by polymerase to perform its reaction and all of these components will be added in a buffer solution which maintains the particular pH and ionic strength that is required for the activity of the enzyme. So, how does this polymerase chain reaction works?

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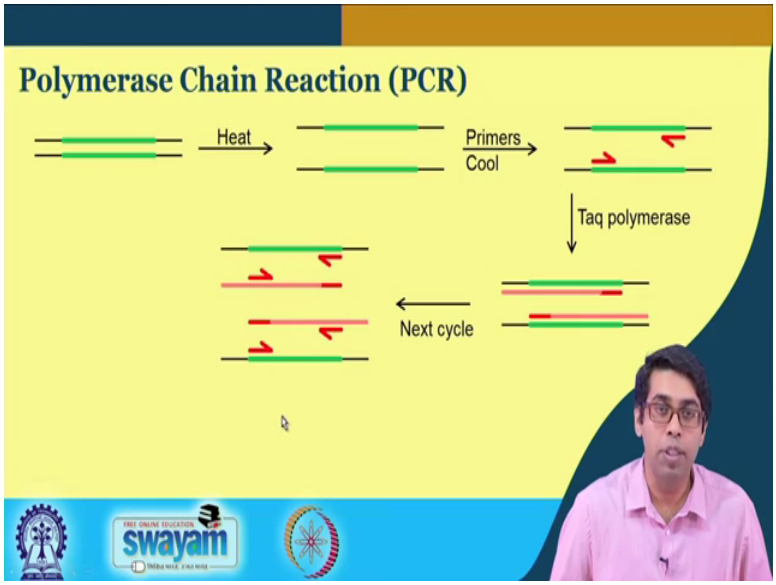

Let us say this is our double stranded DNA and this green part is the gene of interest. So, we want to amplify only this part and not the remaining. So, the first step that you do; so, you have mixed everything the first step you do is heat it you heat it up to 95 degree centigrades. So, that denatures the DNA which means that if breaks all the hydrogen bonds which are present here.

So, now, these two strands are separated out and then we cool it down to a much lower temperature let us say at around 55 degree centigrade. And in the reaction mixture we have added primers which are the small DNA sequences that are complementary to this part and this part ok.

So, again this is the 5 prime to 3 prime and this is 5 prime to 3 prime and again this is 5 prime to 3 prime; sorry this is 5 prime to 3 prime and this is 5 prime to 3 prime; so these primers are complementary to these two strands. And they typically have a length of 20 to 40 base pairs long. So, the primers are added in much more excess compared to the target DNA; so, they will bind there.

Now the polymerase comes into action and it will extend these two in then 5 prime to 3 prime directions. So, you see that when these extends it goes beyond the end point of our gene ok. So, then again you repeat this cycle; now in this cycle the primers will again stick here. And if you see this particular DNA; this primer will extend it from here to here and again this primer will extend it from here to here.

So, in this cycle what you are going to get is the right length of your gene. So, PCR does two things simultaneously one it amplify, so from one in first cycle you will have 2 and then in the second cycle you have 4; so it amplifies it in n exponential manner, so it goes as 2 to the power n. And it also amplifies only your gene of interest so that the amplified region will be only of gene; your gene of interest. The other thing that we do is we in built a restriction site in this primer. So, towards this end we have one restriction site and towards this end we have another restriction site. So, those are already encoded when we synthesize the primer so that we can easily digest the PCR product using our restriction endonucleases.

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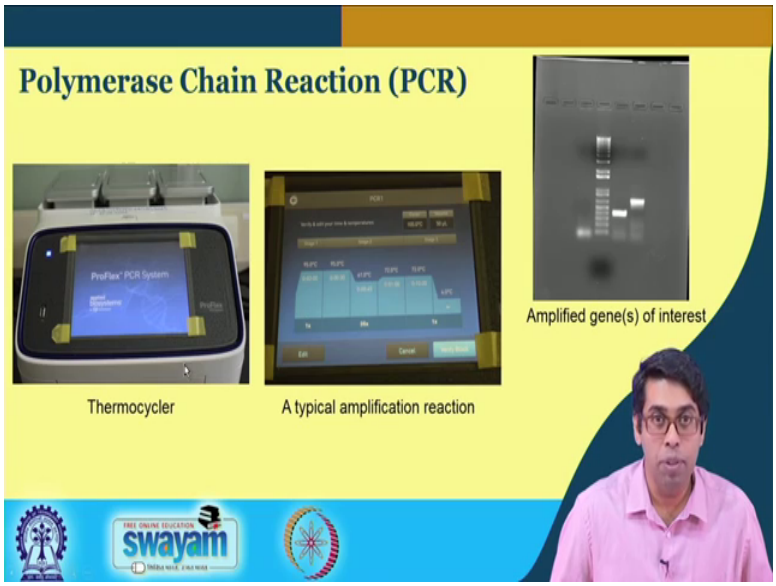

So, this is how a typical PCR arrangement looks like and the PCR cycle; one typical PCR cycle is shown here. So the initial one is where you heat it up to 95 degrees and then this particular steps are repeated several times; so here we repeat it 35 times and then we stop our reaction.

And if you run the PCR product on a agoras gel; then this is the molecular weight marker and you can see products from two PCR reactions;they are of different lengths, but you can clearly see that your PCR reaction has worked. The template vector template DNA that was used was of much longer length and it will show up somewhere here, but its concentration is so low that we do not see it in the agoras gel.

So, we can coming back to the overview slide; this is the overview slide. And when you have your isolated plasmid and your amplified gene from PCR; at this step we are going to use the restriction enzyme to digest them properly so that they will have this right sticky ends so that we can mix them together.

Once we mix them together there is a chance that it will combine like this and, but they are held together by hydrogen bonds only. So, we need a ligase to form the covalent linkage and then you have this complete circular DNA which will be transformed into the bacteria to get our bacterial clone. So, that is the step of transformation.

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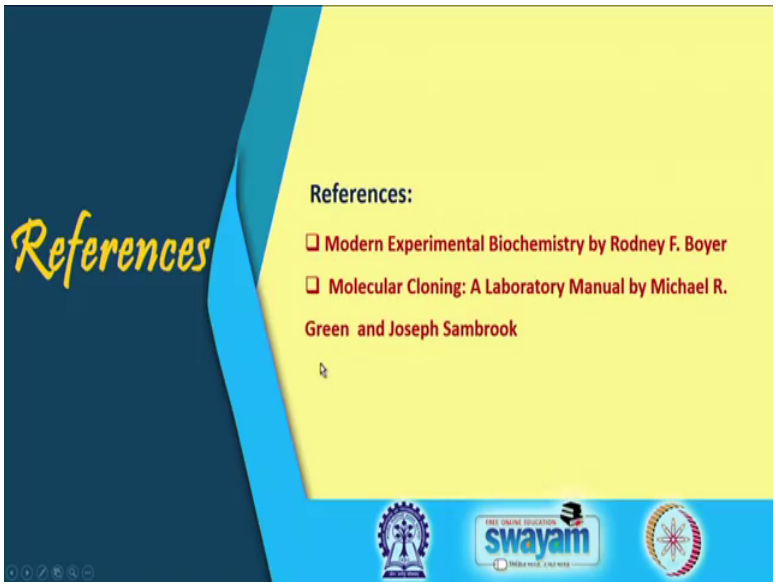

So, these are again he books that we are referring to.

Thank you.