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RESEARCH ARTICLE

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DEVELOPMENT OF DNA MARKER USING RESTRICTION DIGESTION ACTIVITY ON VARIOUS PLASMID VECTORS

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Abstract

This study is to report a simple method to prepare high molecular weight DNA ladders. Bacterial culture of DH5-alpha is transformed with pUC18, pYES2/NT A, pYES2/NT C and pYES2/CT vectors. The constructed plasmids are digested using restriction endonucleases to produce specific weights of the DNA fragments followed by purification and precipitation with ethanol. The obtained fragments are mixed proportionally as per the DNA construct, further the obtained fragments are observed on the agarose gel electrophoresis after restriction digestion. These fragments can be used for further studies or can be re-ligated. The DNA fragments obtained are re-ligated into specific plasmid and are transformed into bacteria which can be used for further ligation studies.

Introduction:-

Plasmid is the genetic material found apart from bacteria in other cellular organisms carry genes that are different from the genes present in the DNA of the organism, the genes present in the plasmid are helpful to the host organism[1]. Plasmids can serve alternate functions such as genes present in plasmid can enhance the survival of the host by completely killing a different organism or by synthesising toxins which act as defensive mechanisms[2]. An organism can contain multiple plasmids each with completely different functions[3].Restriction digestion is a method in which a specific region of the genetic material can be cut[4]. The final stage of restriction digestion includes incubation of the targeted sequence by the restriction enzyme that acknowledges and binds at specific DNA sequence and cleaves at specific nucleotides[5]. Restriction digestion ends with the generation of either blunt ends or sticky ends. Blunt ends usually contain nucleotides and sticky ends contain DNA molecule overhangs[6]. Restriction digestion allows for ligation of fragments of the genetic material which is required for biological research[7]. The end result of digestion is analysed by gel action in which the fragments obtained by digestion are separated by their molecular length[8]. NEBcutter accepts associate input sequence, which may be affixed or picked up from an area file or retrieved from NCBI as a GenBank file via its accession range. Numerous choices are on the market to pick out the set of restriction enzymes to be used and also the size of ORFs to be displayed[9]. The program determines

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the positions of all restriction endonuclease sites noting people who would possibly be blocked by overlapping methylation and finds the ORFs within the sequence[10]. When the method it displays a schematic diagram of the sequence, the long ORFs, supported the principles given within the strategies and every one restriction enzymes that cut it just the once[11]. The start show conjointly shows the enzymes are completely digestible to exercise every ORF that's displayed. If the initial desoxyribonucleic acid sequence is circular, then each linear kind and circular form displays are offered[12].

Materials & Methods:-

Selection of Plasmids:

The plasmids used in the study are pUC18, pYES2/NT A, pYES2/NT C and pYES2/CT, procured from Merck Biosciences (Mumbai, India) prepared and stored in Tris-Acetate EDTA buffer which is also used to separate the nucleic acids is mainly used in molecular biology[13].

Bacterial Transformation

Overnight culture were inoculated into 100ml LB medium and incubated at 37ºC. Grow until the OD A_{600} reaches 0.23-0.26. Chill the flask on ice for 10-20 minutes. Transfer 10ml of culture aseptically into sterile tubes and centrifuge at 6000rpm for 8 minutes at 4ºC. Decant the supernatant and add 15ml of cold 0.1M $CaCl_2$ solution aseptically. Place the tubes on ice for 30 minutes and centrifuge at 6000rpm for 8 minutes at 4ºC. Discard the supernatant and resuspend gently in 0.6ml of cold $CaCl_2$ solution. Aseptically aliquot 100µl of competent cells into pre chilled vials and place them in ice. Add 5µl of plasmid DNA to aliquots of 100µl of competent cells. Tap gently and incubate on ice for 20 minutes. Heat shock the cells by placing the vial in a 40ºC water bath for 2 minutes and then return the vials to ice to chill for 5 minutes. Add 1ml of LB broth aseptically to the vials and incubate at 37ºC for an hour. This allows the bacteria to recover and express the antibiotic resistance. Label two LB-Amp plates as 50µl, 100µl and pipette out the transformed cells. Mix well and spread gently with a spreader. Incubate the plates at 37ºC for overnight[14].

Agarose Gel Electrophoresis:

Pipette 1.5ml of culture to a labelled 1.5ml vial, and centrifuged at 14000rpm for 1 minute. Here, a tight creamy pellet may be seen. Decant the supernatant and place vial vertically for 5-10 seconds; remove liquid by aspiration with a pipette. Add 100µl of resuspension solution and resuspend the bacteria by shaking or vertexing to dissociate the bacterial pellet. Add 200 µl of lysis solution and mix by inverting the tube several times. The solution should quickly turn transparent and become more viscous indicating bacterial lysis has taken place. Allow at least – minute for lysis to take place and leave the vial to stand for a minute before opening. This will allow the liquid to return to the bottom of the tube. Add 150µl of neutralizing solution and invert the tube several times. At this point bacterial chromosomal DNA is usually seen as a white precipitate. Centrifuge the tubes for 10 minutes at 14000rpm. Remove the tubes from the centrifuge, being careful not to disturb the precipitation. Discard the supernatant, avoiding the white precipitate as much as possible. Transfer the liquid phase into the new tube containing 250µl of isopropanol. Vortex the tubes for 5-10 seconds and centrifuge the tubes in the microfuge for 1 minute at 14000rpm. The plasmid DNA precipitates as a white pellet. Decant the supernatant and wash the pellet by adding 750µl of 70% ethanol, vortex briefly and centrifuge at 14000rpm for 5 minutes. Discard the ethanol and centrifuge again for 1 minute to collect the remaining ethanol and leave the tube to dry on the bench for 5 minutes. Dispense 30µl of TE buffer into each tube and resuspend the pellet. Take 10µl of the resuspended pellet and analyse by agarose gel electrophoresis.

Restriction Digestion

The plasmids are subjected to double digestion with restriction enzymes. The restriction enzymes used are EcoRI, BamHI, HindIII and XhoI. Vials containing restriction enzymes on ice then thaw the vials containing substrate (specific plasmid DNA: pUC18, pYES2/NT A and C, pYES2/CT) and assay buffer. Prepare different reaction mixtures using the following constituents. Plasmid DNA - 8µl, Restriction Enzyme 1 - 1µl, Tango buffer of restriction enzyme 1- 5µl, Restriction Enzyme 2 - 1µl, Tango buffer of restriction enzyme 2 - 5µl,Assay buffer - 5µl. Incubate the vials at 37ºC for 1 hour and meanwhile, prepare a 1% agarose gel for electrophoresis. After an hour add 5µl of gel loading buffer to all the vials to arrest the reaction and load 10µl digested samples, control DNA, marker DNA and note down the order of loading. Electrophorese the samples at 50-100V for 1-2 hours then stain the agarose gel with ethidium bromide solution for 10-20 minutes on a gel rocker. Observe the bands on UV-Transilluminator and analyse the restriction pattern[15].

DNA Gel Extraction

Size fractionation of DNA is carried out on an agarose gel stained with ethidium bromide, along with a DNA molecular weight marker of known size and concentration and excise the DNA fragment of interest with a sterile scalpel and transfer the gel into a pre-weighed empty micro centrifuge tube, remove excess agarose using scalpel. Determine the weight of the agarose gel slice. Add 3 volumes of buffer G_A for each gram of the gel and then incubate at 50ºC for 10 min, inverting the tube intermittently every 2-3 minutes during incubation. The solution turns to pale yellow. The column is placed in 2ml collection tubes. The above suspension is transferred to the column and is centrifuged at 6500 rpm for 1 minute. Discard the flow through. Add 50µl of buffer G_A to column and centrifuge at 12000rpm for 1 minute and wash the column with 500µl of buffer G_B and centrifuge it again at 12000rpm for 1 minute. Repeat the before step. Spin the column with caps open at 12000rpm for 3 minutes. Leave the caps open at room temperature for 2 minutes. Place the column in a fresh 1.5ml microcentrifuge tube and add 30µl buffer G_C or water and incubate at room temperature for 10 minutes. Centrifuge at 12000rpm for 1 minute. The resulting eluate contains the expected DNA[16].

Results And Discussions:-

Plasmid Isolation

This approach is arduous, time-intensive and material and equipment-intensive, requiring the dissemination of the virus or cellular inclusion inside the host organism in question, as well as the purification and digestion of the host's nucleic acids from the genomic or plasmid polymer. Since the corresponding restriction nuclease sites of bacteriophages or cellular inclusion polymers aren't well balanced, the distribution of the ready polymer ladder is irregular and also the gaps of the DNA bands are variable in size and not convenient.

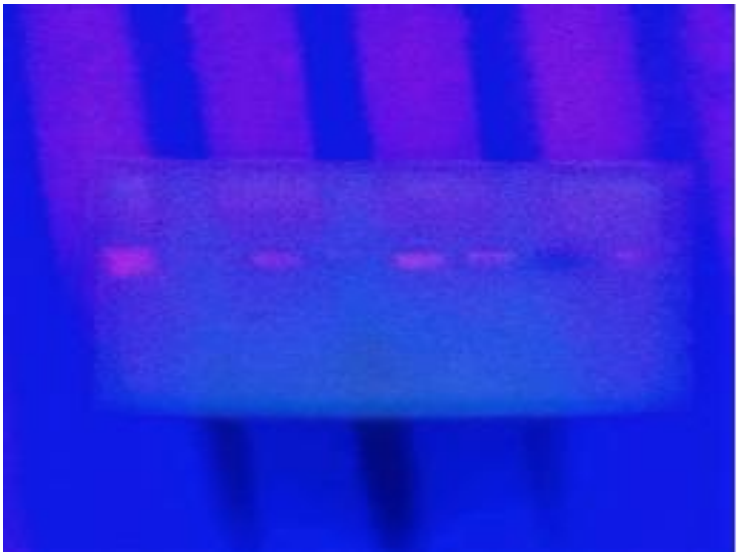

Fig 1:- Plasmids isolated from the transformed culture under UV-transilluminator. Lane 1: pUC18. Lane 2: pYES2/NT A. Lane 3:pYES2/NT C. Lane 4:pYES2/CT.

Restriction digestion

This technique was simple, practical and low value compared to business polymer ladders, and the polymer bands were also transparent. It will also be tailored to any molecular weight standard size in an extremely safe variance according to the experimental demand and will be used in molecular experiments as normal. In short, during this research, a brand-new technique was created to get high molecular weight polymer ladders ready.

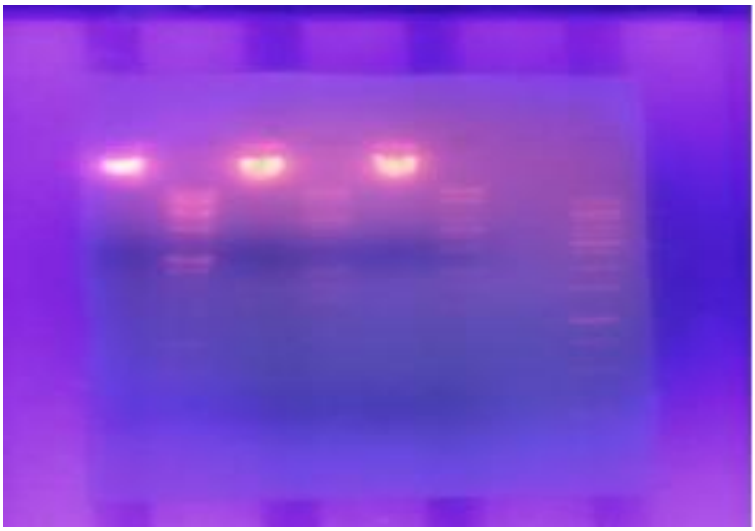

Fig 2:- Analysis of the digested sample under UV-Transilluminator. Lane 1: pYES2/NT A; Lane 2:pYES2/NT A double digested with EcoRI and XhoI; Lane 3: pYES2/NT C; Lane 4:pYES2/NT C double digested with HindIII and XhoI; Lane 5: pYES2/CT ; Lane 6: pYES2/CT double digested with EcoRI and HindIII; Lane 7: Nil; Lane 8:Marker DNA.

While PCR amplification is convenient to make the polymer ladder ready, standard PCR is intended to amplify one form of polymer fragment, one tube at a time, while the polymer ladder is an integration of several DNA fragments to the associated degree, which makes the technical method very advanced and bulky. In addition, the technology is hardly ideal for amplifying tiny polymer fragments, whereas the corresponding fragments of a polymer ladder of high molecular weight are difficult to obtain.

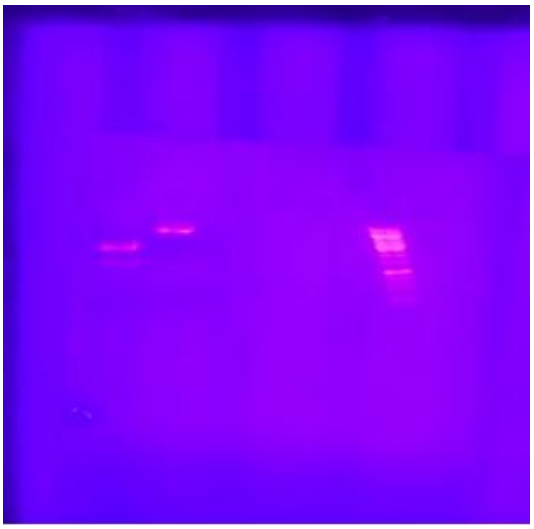

Fig 3:- pUC18 double digest analysis under UV-Transilluminator. Sequences of samples loading on Agarose gel electrophoresis. Lane 1: pUC18 double digested with BamHI and HindIII. Lane 2: pUC18.Last well: Marker DNA.

Gel elution

Despite the fact that giant fragments are amplified by precise polymer enzyme exploitation, the specificity is low, and the cost is high and cannot satisfy the experimental demand. The exploitation of bacteriophages or plasmids that are digestible by restriction of endonucleases is another routine technique for preparing a polymer ladder. This technique involves the formation of a series of vectors to prepare the compound ladder, any digestion of the pure polymer with some restriction endonucleases, and the acquisition of the combinations of necessary DNA fragments.

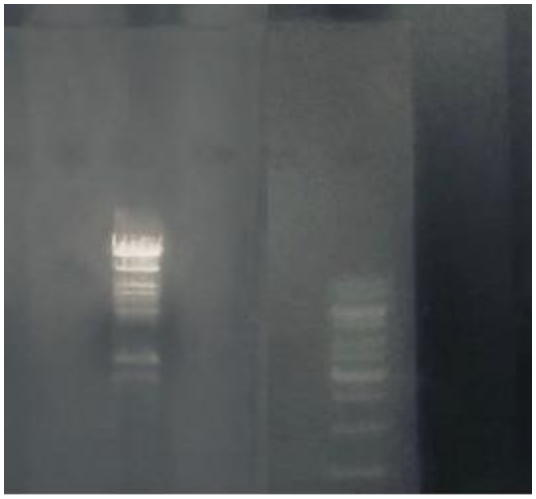

Fig 4:- Constructed DNA after elution of fragments after restriction digestion and its analysis under UV-Transilluminator. Lane 1: Constructed DNA marker; Lane 2: Commercial DNA marker.

Conclusion:-

The polymer ladder may be a widely used technique in action and is useful in experiments in biological science. In supermolecule action, it is customary to mark the molecular weight of unknown samples and to help judge the properties of polymer samples. Polymer ladders are commonly used in areas such as biotechnology, pharmaceuticals and agriculture, among others and consumer demand is therefore extremely high. As a selected polymer amplification technology, PCR has been initiated and has the advantages of easy activity, high sensitivity and specificity and intelligent property, and has been widely applied in the fields of biological science, genetics, organic chemistry, biotechnology and forensics.

References:-

1. Bagdasarian M, Lurz R, Rückert B, Franklin FC, Bagdasarian MM, Frey J, Timmis KN. Specific-purpose plasmid cloning vectors II. Broad host range, high copy number, RSF 1010-derived vectors, and a host-vector system for gene cloning in Pseudomonas. Gene. 1981;16(1-3):237-47.
2. Dietel AK, Kaltenpoth M, Kost C. Convergent evolution in intracellular elements: plasmids as model endosymbionts. Trends Microbiol. 2018;26(9):755-68.
3. Robicsek A, Jacoby GA, Hooper DC. The worldwide emergence of plasmid-mediated quinolone resistance. Lancet Infect Dis. 2006;6(10):629-40.
4. Botstein D, White RL, Skolnick M, Davis RW. Construction of a genetic linkage map in man using restriction fragment length polymorphisms. Am J Hum Genet.1980;32(3):314.
5. Moser HE, Dervan PB. Sequence-specific cleavage of double helical DNA by triple helix formation. Science. 1987;238(4827):645-50.
6. Walker A, Taylor J, Rowe D, Summers D. A method for generating sticky-end PCR products which facilitates unidirectional cloning and the one-step assembly of complex DNA constructs. Plasmid. 2008;59(3):155-62.
7. Kitagawa M, Ara T, Arifuzzaman M, Ioka-Nakamichi T, Inamoto E, Toyonaga H, Mori H. Complete set of ORF clones of Escherichia coli ASKA library.DNA Res. 2005;12(5):291-9.
8. Cleveland DW, Fischer SG, Kirschner MW, Laemmli UK. Peptide mapping by limited proteolysis in sodium dodecyl sulfate and analysis by gel electrophoresis. J Biol Chem.1977;252(3):1102-6.
9. Sheth RU. New Tools for Understanding and Engineering Complex Microbial Communities. Columbia University; 2019.
10. Vincze T, Posfai J, Roberts RJ. NEBcutter: a program to cleave DNA with restriction enzymes. Nucleic Acids Res.2003;31(13):3688-91.
11. Li MZ, Elledge SJ. MAGIC, an in vivo genetic method for the rapid construction of recombinant DNA molecules. Nat Genet.2005;37(3):311-9.
12. Bannister LJ. An ultrastructural and molecular analysis of neuronal degeneration in Huntington's disease. University of London, University College London (United Kingdom); 2004.

13. Parke D. Construction of mobilizable vectors derived from plasmids RP4, pUC18 and pUC19. Gene. 1990;93(1):135-7.
14. Johnston C, Martin B, Fichant G, Polard P, Claverys JP. Bacterial transformation: distribution, shared mechanisms and divergent control. Nat Rev Microbiol.2014;12(3):181-96.
15. Smith HO, Birnstiel ML. A simple method for DNA restriction site mapping. Nucleic Acids Res.1976;3(9):2387-98.
16. Zhou Y, Zhang Y, He W, Wang J, Peng F, Huang L, Zhao S, Deng W. Rapid regeneration and reuse of silica columns from PCR purification and gel extraction kits. Sci Rep. 2018;8(1):1-1.