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INDIGENOUS MOLECULAR SCISSORS FROM LABORATORY MICROBES - ISOLATION AND EVALUATION OF RESTRICTION DIGESTION EFFICACIES

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Abstract - Enzymes are important for the metabolic activities, self-protection and survival of the bacteria as well as are sources for the toxin production. Most of the bacterial enzymes are used in textile, food, pharmaceutical and in various different industries. Bacterial enzymes also play an important role in molecular biology techniques. Molecular scissors are available commercially, characterized with known activity. An attempt has been made to isolate, identify the bacteria and assess the potential of bacteria as an indigenous source of restriction enzymes. Bacterial enzymes from known laboratory microorganisms, capable of working as molecular scissors have been identified during the current study which can be compared to commercial kits. The indigenous procedure found to be easy, possible at laboratory levels with low production cost and efficient performance levels to obtain restriction enzymes.

Keywords: molecular scissors, microbes, enzymes, low production cost

I. INTRODUCTION

All living organisms irrespective of their unicellular or multicellular origin comprise a set of instructions stored in their DNA- deoxyribonucleic acid. Instructions for each metabolic activity are stored in each cell as blue prints in the genome in the form of codes (AGCT) otherwise known as bases. Nucleotides code the information into sets of nucleotide sequences called genes. Each gene represents a set of coding instructions to make a specific protein with a specific function. Each protein has been assigned a certain function. As per the requirement of the cell, and its

functions, many genes will be turned on or off. The functioning of the gene enables the cell, organ, organism to function in a desired manner.

A faulty gene can cause problems for the functional expression in an organisms' phenotypic expressions. Isolation of such a gene is necessary for further studies by scientists. Restriction digestion studies play a prominent role in which the gene will be cut and cloned or simply transfected into a replicable bacterial/ artificial plasmid. The digestion of gene can be carried out by a protein which behaves like a molecular scissors as it is capable of recognizing the sequence of DNA where to cut. The molecular scissors or restriction enzymes have prominent role in the discovery and manufacturing of artificial genes which play a significant role in pharmaceuticals industry. Similarly, sequencing of DNA also requires to cut it into smaller fragments. Enzymes of the pancreatic fluids comprise such DNA digesting enzymes. But most of these enzymes are of no use for sequence work as they cut each molecule randomly and produce a heterogeneous collection of fragments of varying sizes. The molecular scissors are used as tools in such instances, as they cleave the DNA molecule at a few precisely-located sites resulting in the formation of a small set of homogeneous fragments. The rarer the site it recognizes, the smaller the number of pieces produced by a given enzyme. Number of pieces produced by an enzyme depends on the rare site it recognizes.

Molecular scissors can be synthesized naturally or artificially. Naturally, all bacterial/viral cells have a unique mechanism to protect themselves from attack from viruses, fungi, and bacteria. Inside



each cell lies a group of special proteins called molecular scissors. These enzymes are just like scissors. They cut the DNA of a foreign organism into pieces before it has a chance to damage the cell. These enzymes don't just randomly attack foreign DNA. Instead, their attack is more targeted. However, they can be synthesized artificially as well by industrial processes.

Restriction enzymes cleave DNA at or near specific recognition sequences known as restriction sites by making an incision on each strand. This technique has a variety of uses in biotechnological applications i.e everyday medicine, including producing insulin, antibiotics, biofuel, transgenics, modern foods, synthesis of artificial and may help us make giant leaps in medicine and green fuel production in future. The production process has been commercialized. The kits are available widely which are expensive. The quantity produced by the established protocols is less, more production time, less longevity and require appropriate facilities for their storage. There is a need to find out an alternative cost-effective way to synthesize the restriction enzymes and develop a kit and to increase their longevity. Bacterial cells such as *E. coli* show a capability to protect themselves against foreign DNA or inhibit the activity of lambda phage through an enzymatically catalyzed genetic defense mechanism (Arber and Linn,1969; Seed,2015). Many restriction enzymes such as EcoB, EcoK and HindIII and KpnI were identified and isolated respectively from *E-coli*, *Haemophilus influenza* and *Klebsiella pneumonia* as well as the recognition sites (Kelly and Smith, 1970). The symmetry of this recognition sequence was found to be in the form of a palindrome, a nucleotide sequence in which the 5' to 3' sequence of one strand of a segment of DNA is the same as that of its complementary strand which was a basic feature of the action of the enzyme. Bacteria can be used as a natural source of restriction enzymes, as they produce these enzymes as a part of their regular metabolism.

Every restriction endonuclease named in such a way that represents both the genus and the species of the bacterium from which it was isolated, the strain number, and the order in series in which the enzyme was found. For example, EcoRI: Eco refers to the genus and species (1st letter of the genus; 1st two letters of species); R is the strain of *E. coli*; I (Roman numeral) indicates it was the first enzyme of that type isolated from *E. coli* RY13. Each enzyme has blade i.e. 4-6 teeth. It "scans" a DNA molecule for a particular sequence of four to six nucleotides and cuts the strands. This is known as enzyme digestion. On double-stranded DNA the recognition sequence is on both

strands but runs in opposite directions. The section of DNA that the scissors cut is called a 'recognition sequence' and is usually between 4-6 base pairs long. Host DNA (DNA from the animal or plant that is being attacked) can also contain these recognition sequences. Restriction enzymes catalyze the hydrolysis of the bond between the 3'-oxygen atom and the phosphorus atom in the phosphodiester backbone of DNA. The enzymes require Mg^{2+} or other divalent ions for their activity. Restriction enzymes typically recognize a symmetrical sequence of DNA – palindromic sequences

Recombinant DNA technology and certain of molecular biology techniques require restriction enzyme digestion to separate DNA fragments followed by electrophoresis. The use of restriction enzymes as a tool for recombining, or joining, different DNA fragments was first illustrated by Stanford University researchers (Mertz and Davis,1972). Simple and Cost-Effective Restriction Endonuclease Analysis methods will be great use in typing and characterizing Human Adenoviruses (Arun Kumaret.al.,2014) where the need for active quick work restriction enzymes are needed.

Similarly, the bacteria and some yeast comprise an extra chromosomal DNA of circular shape and, varying size and numbers called plasmids which can be modified and used as vectors (carrier) in rDNA technology. Genetic information contained in the plasmid of bacteria gives some advantage over other bacteria. Yeasts are small, lemon-shaped fungal cells with plasmids most widely used eukaryotic model organisms for genetic studies, cancer research, etc.

In the current study, an attempt has been made to identify the molecular scissors and assess their restriction digestion against plasmids that are also extracted and isolated from the laboratory bacterial species. This indigenous method key target is to help most of the educational institutions to set up and incorporate their own experiments at low costs. It enables short budget, more hands-on experience for the student and with reduced waiting time in the procurement.

II. MATERIALS AND METHODS

For the current study, the laboratory cultures were considered for the extraction of enzymes and plasmids. The bacterial and fungal cultures considered for the current study were laboratory bacterial strains of *Escherichia coli*, *Klebsiella pneumoniae* and *Staphylococcus aureus* and eukaryotic fungal sps. *Saccharomyces cerevisiae*. Bacterial strains were cultured in sterile Nutrient Broth at 37°C for 24 hours.



Yeast was cultured in sterile Sabouraud's broth at room temperature for 24 hours. All the chemicals used were analytical grade.

A. Isolation and extraction of Plasmid DNA:

Plasmid isolation methods are of various types based on the extraction and rapidity that one can apply (Kado and Liu,1981). In the current study, the bacterial plasmid was isolated by a modified Alkaline Lysis Method (Bimboim and Doly.1979). Broth containing culture was transferred to centrifuge tubes and centrifuged at 12000 rpm for 2 minutes. Supernatant was discarded and pellet was re-suspended in Solution I (1ml), solution II (1ml) and incubated for 10 minutes on ice followed by addition of chilled solution III (1 ml). The mix was centrifuged at 12000 rpm for 10 minutes. Pellet was discarded and to the supernatant, twice the volume of chilled ethanol was added, mixed and kept on ice for 15 minutes. After centrifuging at 12000 rpm for 10 minutes, the supernatant was discarded. The pellet was re-suspended in 100 μ l Tris-EDTA buffer and stored at 4°C.

Yeast plasmid was isolated by Phenol – Chloroform Method (Sobanski and Dickinson ,1995). The culture broth was transferred in centrifuge tubes which were centrifuged at 10000 rpm for 10 minutes. The pellet was suspended in 1 ml Solution I. Acid washed glass beads were added until just below the level of liquid & vortexed at maximum speed for 2 minutes. 1ml ice cold Solution II was added. To this, 1ml ice cold Solution III was added, mixed and incubated on ice for 5 minutes. The sample was treated with glass beads with an equal volume of phenol-chloroform – Iso-amyl alcohol (25:24:1), vortexed and centrifuged for 2 minutes. The upper aqueous phase was transferred to a second tube and the phenol-chloroform extraction repeated. The aqueous upper layer was transferred to another tube and plasmid DNA was precipitated with 2 volumes of chilled ethanol (-20°C) plus 0.1 volume of Sodium acetate, pH 6.0. After incubation for 30 minutes on ice, plasmid DNA was pelleted by centrifugation at room temperature for 10 minutes. Pellet was washed with 1ml 70% ethanol and air dried. Plasmid DNA was suspended in Tris – EDTA buffer, pH 8.0 and stored at 4°C. The TAE based AGE method was used for isolation of the plasmid as per the standard protocol (Armstrong,1983).

B. Isolation and processing of Bacterial Proteins

The bacterial cells were lysed and precipitated by centrifugation. The supernatant was collected for the

isolation of bacterial proteins. The supernatant broth was supersaturated with Ammonium Sulphate under cold conditions and stored at 4°C overnight. The broth was centrifuged at 10000 rpm for 10 minutes and the supernatant was discarded. The pellet was suspended in Phosphate buffered saline (pH 7.0) (Rosenberg,2005) The protein pellet was dialyzed against Dialysis buffer. The proteins thus obtained were assessed quantitatively by Biuret Method. The biuret method depends on the presence of peptide bonds in proteins. Biuret reagent is an alkaline copper sulfate solution (Copper Sulfate, NaOH). When a solution of proteins is treated with cupric ions (Cu^{2+}) in a moderately alkaline medium, a purple colored Cu^{2+} - peptide complex (purple chelates) is formed. The intensity of the color produced is proportional to the number of peptide bonds that are reacting, and therefore to the number of protein molecules present in the reaction system. The intensity can be measured quantitatively by spectrophotometer in the visible region at 560 nm.

C. Separation of Proteins

Partially purified proteins were separated by Polyacrylamide Gel Electrophoresis. The 6% polyacrylamide gel was prepared by using 30% Polyacrylamide, 10% Ammonium persulfate, TEMED, Tris – HCl (pH 8.3 – 8.6). The gel was set in gel casting set up of vertical electrophoretic apparatus (Genei, India) as per the standard protocols (Rosenberg,2005). The sample was diluted with gel loading dye comprising Bromophenol blue for tracking purpose. 20 μ l of the sample was loaded in each well. At an electric current of 100 V, considering Tris- Glycien buffer (pH8.6) as tank buffer the gel electrophoresis was run for 2-3 hours. The gel was stained with Staining Solution (Coomassie brilliant blue R250, Methanol, Distilled water, Glacial Acetic Acid) for an hour. Destaining of the gel was carried out till sharp bands started appearing (Walker ,2002).

Assessment of Restriction digestion capability of proteins extracted from *E.coli*, *K. pneumoniae* & *S. aureus* from the supernatant of culture media was carried out using the plasmids extracted from bacteria (*E.coli*, *K.pneumoniae*,*S. aureus*) and yeast (*S.cereveseae*) as substrates. . A common restriction buffer was used while assessing the activity which contained Tris – HCl, Magnesium chloride, Potassium chloride, Dithiothreitol.

D. Standard protocol for Restriction digestion:

The requirements for restriction digestion were mixed as per the standard protocol table (Table-1) The mixture was spun at 8000 rpm for 1 minute and incubated at 37 °C for 2 hours.. Mixture spun at 8000 rpm for 1 minute. AGE was performed to check restriction fragments.

Agarose Gel Electrophoresis: The plasmid extracts and restriction digests of DNA and plasmid were observed on agarose gel prepared in TAE buffer after Agarose gel electrophoresis as mentioned in the standard protocols (Rosenberg,2005). 0.8% agarose gel was prepared comprising 20 µl Ethidium Bromide. The sample was diluted with tracking dye in the ratio of 5:1. 20 µl of the sample was loaded in different wells. An electric current of 100 V was applied. DNA bands were visualized in the gel using U.V. trans-illuminator at 265 nm (Short wavelength) and 365 nm (long wavelength).

Reagents	Test	Sample
DNA pellets	4 µl	4 µl
Restriction buffer (10X)	2 µl	2 µl
Enzyme (Crude protein extract)	10 µl	-
Distilled water	13 µl	23 µl
Loading dye	5 µl	5 µl

Table-1: Standard protocol for restriction digestion

III. RESULTS

A. Plasmid Extraction:

The standard protocol of alkaline Lysis method was followed to extract the plasmid DNAs of laboratory microbes such as *S. aureus*, *E. coli*, *K. pneumoniae*, and *S. cerevisiae* were obtained in the form of white pellets. The pellets were suspended in TE buffer (pH 8.0) prior to the use of restriction digestion by the proteins extracted from the same bacterial species.

B. Protein Concentration:

Proteins from the nutrient broth were separated by salting and desalting procedures. The obtained protein pellet was dissolved in PBS and their concentration was estimated by Biuret Method. The concentration of protein in the sample varied from 8 ± 0.2 mg/ml. However, the protein extracted from the broth concentration was more for *Klebsiella pneumoniae* bacteria than the others.

C. Identification of bands:

The extracted protein samples of different bacteria were isolated on native PAGE. Four different bands for each protein sample were detected on the gel stained with CBB R250 whereas five bands were detected for the marker. The Rf values of the protein band number 1, 2 and 4 of the sample matched with the Rf values of marker protein band number 1, 4 and 6 and thus their molecular weights were known. The Rf values of protein band number 3 of the sample did not match with any of the protein marker bands. The molecular weights of these bands were calculated (Rosenberg,2005). The log 10 of the molecular weights were plotted against their Rf values. The Rf values of the unknown were extrapolated on the standard graph. Antilog of the same gives the molecular weight of the band. All the bacterial proteins showed a similar banding pattern and approximately equal Rf values. The molecular weights of the bands were found to be 14 ± 0.1 kDa; 15 ± 0.1 kDa. 29 ± 0.1 kDa, and 66 ± 0.1 kDa in case of *S. aureus*, *E. coli*, and *K. pneumoniae*. (Fig.1)

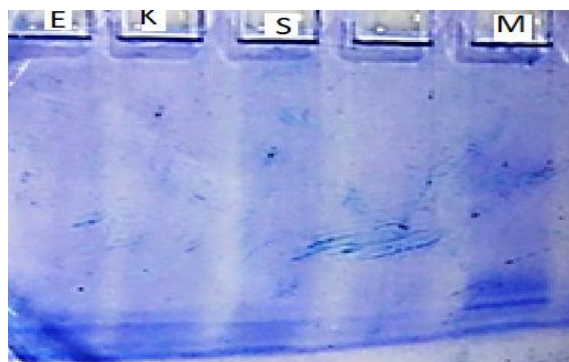


Fig 1 : Protein bands of sample and marker after PAGE (E-*Escherichia coli*.; K-*Klebsiella pneumoniae*.;S- *Staphylococcus aureus*, M-*Marker*)

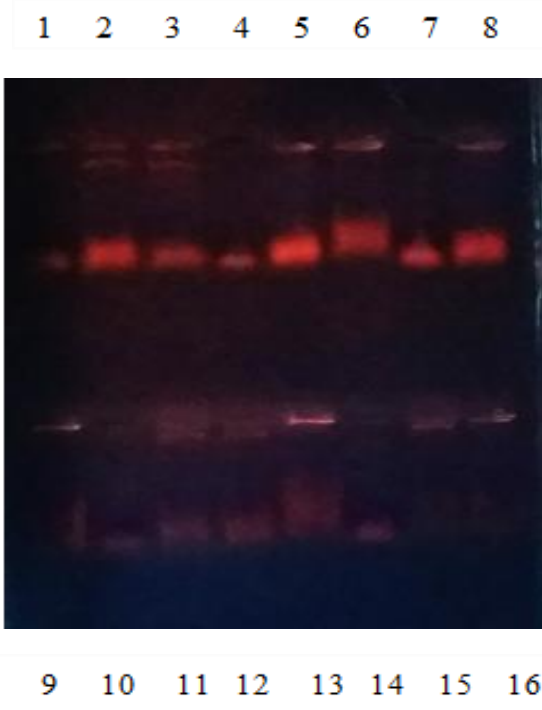
The Agarose gel electrophoresis indicated that the plasmids of yeast were effectively digested by the proteins of the three bacterial strains indicating that they represent the restriction enzymes. Similarly, the extracted proteins of the three bacterial strains effectively broken the plasmids of each other indicating that they can be effectively used for digestion of DNA (Fig.2). The overall study indicated that restriction enzymes extracted from the laboratory strains of

bacteria can be effective and can be used for academic purposes

IV. DISCUSSION

A number of restriction enzymes are isolated from bacteria where they are thought to play a role in host defense by cutting and destroying foreign DNA, for example, of invading viruses. The methods such as alternating sonication with cooling on the ice were used to disrupt cells, protein purification and extraction by His Trap, Exclusion, and DEAE anion exchange chromatography followed SDS PAGE for the isolation (Loenen et.al., 2013) In the current method, a simple method of using glass beads to disrupt cells and to extract proteins/restriction enzymes.

Type I enzyme (Roberts et.al., 2013) Type II enzymes, purification procedures comprised a high-speed supernatant of a cell lysate, removal of nucleic acids by streptomycin or polyethyleneimine and several column chromatography techniques (Phospho-cellulose, DEAE-cellulose, hydroxyapatite, and gel filtration). Preparations were purified to reduce the interfering activities, but usually not to homogeneity (Pingoud et.al., 2014).



1 – Yeast Plasmid Control ; **2, 3, 4, 5, 6** – Action of *E. coli* 1, *E. coli* 2, kit, *S. aureus*, and *K. pneumoniae*

proteins on yeast plasmid resp.; **7**- *E. coli* plasmid control; **8, 9** - Action of *S. aureus*, and *K. pneumoniae* proteins on *E. coli* plasmid resp.; **10** - *S. aureus* plasmid control; **11, 12, 13** - Action of *E. coli* 1, *E. coli* 2, and *K. pneumoniae* proteins on *S. aureus* plasmid resp.; **14** - *K. pneumoniae* plasmid control; **15, 16** - Action of *E. coli* and *S. aureus*, proteins on *K. pneumoniae* plasmid respectively.

Fig. 2: Visualization of Restriction fragments after AGE

Crude lysates of *E. coli* K(P1) exhibited no apparent REase activity. But fractionation gave an enzymatic activity, which specifically cleaved the phage DNA (Kasarjian, 2005) Hinf III REase was purified from restricting strains using phospho-cellulose and DNA-agarose chromatography (Rao et.al., 2013). Similarly, sequence of restriction endonuclease KpnI from *Klebsiella pneumoniae* was extracted and identified whose specific activity 50,000 units/mg (Tomassini et.al., 1978). Class II Restriction Endonuclease from *Staphylococcus aureus* was obtained by suspending cells in 8 ml of (Tris)-hydrochloride and B-mercapto ethanol buffer (pH 7.4), cell disruption by sonic treatment at 0°C followed by centrifugation. The supernatant was collected, and nucleic acids were precipitated by streptomycin sulfate (2 ml of a 10% streptomycin sulfate solution in 0.01 M Tris-hydrochloride-0.01 M β-mercaptoethanol, pH 7.4) followed by centrifugation. The supernatant was dialyzed against 0.01 M Tris-hydrochloride to 0.01 M β-mercaptoethanol, pH 7.4. The crude extract (about 10 ml) was tested for the presence of endo-nucleolytic activity (Stobberingh ,et.al.,1977; Xu et.al.,2011).

In the current study, an attempt was made to isolate restriction endonucleases indigenously from common laboratory bacteria with basic laboratory techniques during the current study. The enzymes were isolated by a general cell lysis method (Bimboim and Doly,1979; Sobanski and Dickinson1995). The lysate was subjected to partial purification by precipitation with ammonium sulfate (salting) followed by desalting. The crude extracts were also electrophoresed using polyacrylamide gel. Four different bands for each protein sample were detected whereas five bands were detected for the marker. Out of four bands, the Rf values of three matched with that of the marker. The molecular weights of these proteins were calculated to be 14 ± 0.1 kDa, 15 ± 0.1 kDa, 29 ± 0.1 kDa, and 66 ±



0.1kDa in case of *S. aureus*, *E. coli*, and *K. pneumoniae*.

The crude extract was assessed for digestion activity by incubating with the plasmids as per the standard procedure as mentioned earlier. Restriction fragments were detected by Agarose gel electrophoresis using Ethidium Bromide as an intercalating substance and fluoresce wherever the DNA fragments were found. In this study, after AGE, test samples showed more bands as compared to the control. This indicates the crude extracts possess restriction digestion activity. Also, with respect to the commercial kit, the crude extracts showed comparable activity.

The isolated proteins from the selected bacterial species of laboratory were checked for effective restriction digestion against the other bacterial and yeast plasmids. For example, the proteins isolated from *S.aureus* were checked against the plasmids of *E.coli*, *K pneumoniae* and *S cerevisiae*. The restriction digestion activity found to be effective. The proteins extracted from broth of *S. aureus*, *E. coli*, and *K. pneumoniae* cultures can be considered as restriction enzymes. The crude extract thus obtained was showed significant digestion activity. Also, the plasmids against which the digestion activity was tested were obtained from common laboratory bacteria by a standard cell lysis method. However, no further purification steps were carried out.

Each restriction modification system has a different mechanism of action as well as physical and chemical requirements. There are several important factors to consider when using restriction enzymes such as buffer composition, incubation temperature, DNA methylation, and star activity. Buffers vary with respect to the type of enzyme and mode of action as they have different preferences with respect to ionic strength and cations such as Sodium and potassium. There is no universal common digestion buffer for restriction enzymes. There are 3–4 commonly used buffers that are generally used for most enzyme conditions. Most enzymes work around pH 8.0, however, some enzymes are more particular and have specific buffers. Buffer systems may vary with respect to components such as Bis-Tris-Propane-HCl/ Tris HCl, MgCl₂, NaCl and Dithiothreitol, and their concentrations, pH and temperature conditions depending on the type of restriction enzymes origin. However, in this study, a single universal digestion buffer was developed and used for assessment of digestion efficiency of all restriction endonucleases. Minimal facilities were required for the storage of these enzymes. The commercially available enzymes are

costly; require high maintenance and waiting time to procure them is long. An attempt was made to develop a cost-effective method to procure restriction enzymes

V. CONCLUSION

The use of restriction enzymes has proved invaluable in molecular biology, cloning, genetic engineering, immunology, forensic analysis and a number of other scientific disciplines such as the creation of gene libraries (Durmaz et al. 2015, Heather and Chain 2016). However, the cost of these enzymes limits their use and applications. Thus, there is an absolute need to devise an alternative way to obtain the restriction enzymes. An attempt was made to develop a cost-effective method to procure restriction enzymes indigenously with minimal facilities for production, maintenance, and storage during the study. The cost is further reduced by the use of a single universal buffer for all enzymes. This also reduces the waiting time for procuring the enzymes. Also, the enzymes obtained show a broad spectrum of activity, as they work on eukaryotic as well as prokaryotic plasmid DNA. As of now, these enzymes can be used for academic purposes, to teach students the concepts and give them hands-on training. However, after further analysis, these can be commercialized as well.

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