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To study the extraction of DNA from *Polyporusmonticola* and *Trichodermaviride* and amplify it using PCR to ascertain its purity.

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ABSTRACT

In the present study the extraction of DNA from Polyporusmonticola and Trichodermaviride and amplify it using PCR to ascertain its purity and it was carried out using two wood decay fungiPolyporusmonticola and Trichodermaviride. It was concluded that the powerful fermation effect was attributed to the greater amount of DNA obtained from various extracts of Polyporusmonticola and Trichodermaviride.

Keywords:Polyporusmonticola, Trichodermaviride, DNA, polymerase chain reaction.

1. INTRODUCTION

*Trichoderma*is a filamentous, asexual spore producing ascomycetic fungi belongs to class Deuteromycetes. T*richodermaviride*is one of many species of mold which is almost found in all types of soils and is most prevalent and culturable fungi [9, 33, 5]. *Trichoderma*is a very useful fungus for industry and as a biocontrol; it has shown little host specificity, colonizing most plants and it is widely studied fungi and most commonly used as biological control agents in agriculture and their products as alternatives to synthetic agro-chemicals [34].*Trichoderma*species have shown biocontrol potential against many plantpathogens including diseases caused by *Sclerotinia minor* (Jones and Stewart,1997; Dolatabadi*et al*., 2011).

Identification and characterization of fungi using molecular methodologies -DNA- based methodologies have been used to examine the relationships within and between two genera of fungi for and they assist a great deal in their identification and characterization. The polymerase chain reaction (PCR) is a rapid procedure for in vitro enzymatic amplification of a specific segment of DNA.

The number of applications of PCR seems infinite-and is still growing. They include direct cloning from genomic DNA or cDNA, *in vitro* mutagenesis and engineering of DNA, genetic fingerprinting of forensic samples, assays for the presence of infectious agents, prenatal diagnosis of genetic diseases, analysis of allelic sequence variations, analysis of RNA transcript structure, genomic foot printing, and direct nucleotide sequencing of genomic DNA and cDNA. There are three nucleic acid segments: the segment of double-stranded DNA to be amplified and

two single-stranded oligonucleotide primers flanking it. Additionally, there is a protein component (a DNA polymerase), appropriate deoxyribonucleoside triphosphates (dNTPs), a buffer, and salts.

2. MATERIALS AND METHODS

Extraction of DNA from partially purified Trichodermaviride and *Polyporusmonticola was carried out using hexadeclytrimethyl ammonium bromide (CTAB) (Brook man et al., 2000) as described below.*

Ground sample as fine as possible in liquid nitrogen using a pestle and mortar.Collected the ground sample in a 1.5 ml eppendorf tube to about the 0.1ml mark.Added 800microliter CTAB isolation buffer (100mMTris-HCl,pH8;1.4MNaCl;20mMEDTA (sodium salt);2%hexadeeyltrimethylammonium bromide [CTAB]) and mixed thoroughly by vortexing.Incubate at 70ºC for 30 minutes and vortex again at 15 minutes.Added 500µl chloroform and vortexed to form a white emulsion.Spinned at top speed (1300rpm) in a microfuge for 10 minutes.Taken off 500µl of the upper aqueous layer and dispensed into another eppendorf tube.Added 300 µl isopropanol and mixed gently inverting the tube several times.Incubated at room temperature for 5 to 10 minutes to allow the DNA to precipitate then spinned in a microfuge at top speed for 15 minutes.Carefully poured off the supernatant.

The DNA was visible as a grey pellet.The pellet was washed in 1ml 70% ethonol. The pellet was freed from the base of the tube by flicking the tube.Incubated at 70°c for 10 minutes and during the incubation agitate the pellet by flicking the tube. The pellet should become whiter and more clearly visible.Spinned at top speed for 10 minutes.Carefully taken off the ethanol and dry the pellet (either at room temperature or at 70°c for 5 minutes being careful not to over dry).The pellet was resuspended in appropriate volume of TE (approximately 50 to 100µl depending on the size of the pellet) warmed if necessary to help dissolve the pellet.

I. Quantification of DNA using absorption spectroscopy:

Absorption of DNA sample is measured at several different wavelengths to assess purity and concentration of nucleic acids. A_{260}measurement are quantitative for relatively pure nucleic acid preparation in microgram quantities .Absorbance readings cannot discriminate between DNA and RNA ; however ,the ratio of *A* at 260 and280 nm can be used as an indicator of nucleic acid purity .Proteins ,for example , have a peak absorption at 280 nm that will reduce the A_{260} / A_{280} ratio .Absorbance at 325 nm indicates particulates in the solution or dirty cuvettes; contaminants containing peptide bonds or aromatic moieties such as protein and phenol absorb at 230nm.DNA extracted from *Polyporusmonticola* and *Trichodermaviridec* were quantified.

II. Polymerase chain reaction (PCR)

The polymerase chain reaction (PCR) is a rapid procedure for in vitro enzymatic amplification of a specific segment of DNA. Like molecular cloning, PCR has spawned a

multitude of experiments that were previously impossible. The number of applications of PCR seems infinite-and is still growing. They include direct cloning from genomic DNA or cDNA, in vitro mutagenesis and engineering of DNA, genetic fingerprinting of forensic samples, assays for the presence of infectious agents, prenatal diagnosis of genetic diseases, analysis of allelic sequence variations, analysis of RNA transcript structure, genomic foot printing, and direct nucleotide sequencing of genomic DNA and cDNA. There are three nucleic acid segments: the segment of double-stranded DNA to be amplified and two single-stranded oligonucleotide primers flanking it. Additionally, there is a protein component (a DNA polymerase), appropriate deoxyribonucleoside triphosphates (dNTPs), a buffer, and salts.

The primers are added in vast excess compared to the DNA to be amplified. They hybridize to opposite strands of the DNA and are oriented with their 3' ends facing each other so that synthesis by DNA polymerase (which catalyzes growth of new strands 5'→3') extends across the segment of DNA between them. One round of synthesis results in new strands of indeterminate length which, like the parental strands, can hybridize to the primers upon denaturation and annealing. These products accumulate only arithmetically with each subsequent cycle of denaturation, annealing to primers, and synthesis.

However, the second cycle of denaturation, annealing, and synthesis produces two singlestranded products that together compose a discrete double-stranded product, which is exactly the lemgth between the primer ends. Each strand of this discrete product is complementarey to one of the two primers and can therefore participate as a template in subsequent cycles. The amount of this product doubles with every subsequent cycle of synthesis, denaturation, and annealing, accumulationg exponentially so that 30 cycles should result in a 228-fold(270million-fold) amplification of the discrete product.PCR was carried out using the method of sambrook et al (1989).

Reagents and equipment:-

- 10 X PCR Reaction buffer.10mM deoxynucleosidetriphosphates(dNTPs), 2.5 mM each of dATP,dCTP,dGTP and dTTP. TaqDNA polymerase, a heat-stable polymerase. Many different polymerases are available, the most common being Taq polymerase isolated from the organism Thermophilusaquaticus.
- Primers, short single stranded DNA fragments (20-30bp), which allow the DNA polymerase to bind to the DNA template and initiate the synthesis of DNA.
- Template, and DNA containing ssmple.Thermal Cycler(PCR machine).0.2mL PCR tubes

Method :-

1. Setup the PCR master mix excluding DNA template.

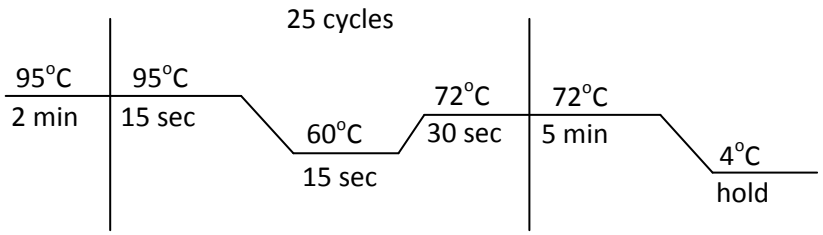
Component	For 1 reaction	for n reaction*
10x PCR buffer	5µl	
dNTPs	1µl	
$MgCl_2$	3µl	

Forward primer (10μm)		1.25μl
Reverse primer (10μm)		1.25μl
Template DNA	1 μl	
Taq polymerase (5U/μl)		0.2
ddH_2O		37.3μl
Final Volume		50μl

*n reactions=reaction required+1

2. Pipette 49μl of PCR master mix into 0.2 ml micro centrifuge tubes.Add 1μl of template, seal tubes with lids and gently mix by flicking .Briefly centrifuge to collect content at bottom of tubes (Fig. 2).

Fig. 2: Programme used for PCR thermal cycle



III. AGAROSE GEL ELECTROPHORESIS:-

Agarose gel electrophoresis is a simple and highly effective method for separating, identifying, and purifying 0.5- to 25-kb DNA fragments. The protocol can be divided into three stages: (1) a gel is prepared with an agarose concentration appropriate for the size of DNA fragments to be separated; (2) the DNA samples are loaded into the sample wells and the gel is run at a voltage and for a time period that will achieve optimal separation; and (3) the gel is stained or, if ethidium bromide has been incorporated into the gel and electrophoresis buffer, visualized directly upon illumination with UV light.

Preparing the gel:-

1. Prepare an adequate volume of electrophoresis buffer (TAE or TBE) to fill the electrophoresis tank and prepare the gel.

2. Add the desired amount of electrophoresis-grade agarose to a volume of electrophoresis buffer sufficient for constructing the gel. Melt the agarose in a microwave oven or autoclave and swirl to ensure even mixing. Gels typically contain 0.8 to 1.5% agarose .Melted agarose should be cooled to 55°C in a water bath before pouring onto the gel platform. This prevents warping of the gel apparatus. Gels are typically poured between 0.5 and 1 cm thick. Remember to keep in mind that the volume of the sample wells will be determined by both the thickness of the gel and the size of the gel comb. To facilitate visualization of DNA fragments during the run, ethidium bromide solution can be added to the electrophoresis buffer or agarose gel to a final concentration of 0.5 ug/ml. Gel should not be added until it has cooled to 55°C.Ethidium

bromide is a mutagen and potential carcinogen. Gloves should be worn and care should be taken when handling ethidium bromide solutions.

3. Seal the gel casting platform if it is open at the ends. Pour in the melted agarose and insert the gel comb, making sure that no bubbles are trapped underneath the combs and all bubbles on the surface of the agarose are removed before the gel sets. Most gel platforms are sealed by taping the open ends with adhesive tape. As an added measure to prevent leakage, hot agarose can be applied with a Pasteur pipet to the joints and edges of the gel platform and allowed to harden. Alternatively a gel casting apparatus can be employed.

Loading and running the gel :-

4. After the gel has hardened, remove the tape from the open ends of the gel platform and withdraw the gel comb, taking care not to tear the sample wells. Most gel platforms are designed so that 0.5 to 1 mm of agarose remains between the bottom of the comb and the base of the gel platform. This is usually sufficient to ensure that the sample wells are completely sealed and to prevent tearing of the agarose upon removal of the comb. Low percentage gels and gels made from low gelling/melting temperature agarose should be cooled at 4°C to gain extra rigidity and prevent tearing.

5. Place the gel casting platform containing the set gel in the electrophoresis tank. Add sufficient electrophoresis buffer to cover the gel to a depth of about 1 mm (or just until the tops of the wells are submerged). Make sure no air pockets are trapped within the wells.

6. DNA samples should be prepared in a volume that will not overflow the gel wells by addition of the appropriate amount of 10 X loading buffer. Samples are typically loaded into the wells with a pipettor or micropipette. Care should be taken to prevent mixing of the samples between wells. Be sure to include appropriate DNA molecular weight markers .

7. be sure that the leads are attached so that the DNA will migrate into the gel toward the anode or positive lead. Set the voltage to the desired level, typically 1 to 10 V/cm of gel, to begin electrophoresis. The progress of the separation can be monitored by the migration of the dyes in the loading buffer.

8. Turn off the power supply when the bromophenol blue dye from the loading buffer has migrated a distance judged sufficient for separation of the DNA fragments. If ethidium bromide has been incorporated into the gel, the DNA can be visualized by placing on a UV light source and can be photographed directly.Gels that have been run in the absence of ethidium bromide can be stained by covering the gel in a dilute solution of ethidium bromide (0.5 ug/ml in water) and gently agitating for 10 to 30 min. If necessary, gels can be destained by shaking in water for an additional 30 min. This serves to remove excess ethidium bromide which causes background fluorescence and makes visualization of small quantities of DNA difficult.

IV. Photography of DNA in agarose gels:-

DNA can be photographed in agarose gels stained with ethidium bromide by illumination with UV light (>2500 uW/cm2). A UV transilluminator is typically used for this purpose, and commercial models are available designed specifically for DNA visualization and photography. The traditional means for photographing agarose gels uses a bellows-type camera equipped with a Polaroid film holder. An orange filter is required to achieve a desirable image of light transmitted by fluorescing DNA. Polaroid type 667 film (ASA 3000) offers ideal sensitivity, allowing as little as several nanograms of DNA to be detected on film after making adjustments of exposure time. Gel photography systems can be set up by purchasing and assembling the individual components (e.g., camera, film holder, filters, and UV transilluminator). Alternatively, complete photography systems are commercially available.

A major disadvantage of traditional photography is the high cost of film. This can have a significant impact on a laboratory budget, particularly if large numbers of gels are processed on a routine basis. Because of this, traditional photography systems have been largely replaced with video imaging systems. Such systems use a CCD camera to capture the gel image and a video monitor to adjust focus and exposure time. A thermal printer generates inexpensive prints suitable for data documentation. A second advantage of imaging systems over traditional photography is that many systems come with video-processing computers and analysis software. This enables the degree of DNA fluorescence to be quantified (and thereby the relative amount of nucleic acid), and images can be stored for documentation and publication. Although the commercially available imaging systems are convenient, they can also be assembled on a less costly basis by purchasing the individual components and using public domain or commercially available software.

3. RESULTS

The internal transcribed spacer region for *Trichodermaviride*corresponded to a single band of 600 base pairs while for *Polyporusmonticola*it corresponded to 550 base pairs table 1. Plate I,II,III.

Table 1: Results of the spectro photometric measurements of Purified DNA samples of *Trichodermaviride*and *Polyporusmonticola*

Sample*	Absorbance 260 mm	Absorbance 280 nm	260nm / 280 nm	Concentrate of DNA (ng)
TV	0.163	0.147	1.00	0.343
PM	0.045	0.152	0.1124	93.33

* Values are averages of two readings

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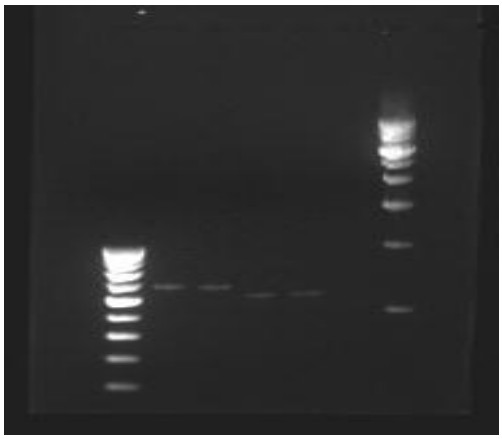
Plate I:*Polyporusmonticola*cultured in agar slopesPlate II: *Trichodermaviride*cultured in agar slopes

Plate III: Agarose gel electrophoresis (1%) of amplified product of Internal Transcribed Spacer regions of *Trichodermaviride*and *Polyporusmonticola*
M1 L1 L2 L3 L4 L5 M2



M1=100 bp DNA Marker (Bangalore Genei Pvt. Ltd. Bangalore)
L1= Amplified Internal Transcribed Spacer region ofTrichodermaviride (~600 bp)
L2= Amplified Internal Transcribed Spacer region of Trichodermaviride (~600 bp)
L3= Amplified Internal Transcribed Spacer region ofPolyporusmonticola (~550 bp)
L4= Amplified Internal Transcribed Spacer region of Polyporusmonticola (~550 bp)
L5=Negative Control (template DNA absent)
M2=500 bp DNA Marker (Bangalore Genei Pvt. Ltd., Bangalore)

CONCLUSION

*Trichodermaviride*and *Polyporusmonticola*proved them very effective in breakdown of ragi straw employing Solid State Fermentation and could be utilized for the transformation of lignocelluloses as well as enzyme production. The polymerase chain reaction (PCR) is a rapid procedure for in vitro enzymatic amplification of a specific segment of DNA.

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