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Rapid DNA Extraction for Screening Soil Filamentous Fungi Using PCR Amplification

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Abstract

A simple and rapid procedure for efficiently isolating fungi DNA suitable for use as a template for PCR amplification and other molecular assays is described. The main advantages of the method are: (1) the mycelium is directly recovered from Petri-dish cultures; (2) the technique is rapid and relatively easy to perform , and (3) it allows for processing of around 50 samples during a single day; (4) it is inexpensive; (5) the quality and quantity of DNA obtained are suitable for molecular assays; (6) it can be applied to filamentous fungi from soil as well as from a fungi from other environmental sources; and (7) it does not require the use of expensive and specialized equipment or hazardous reagents.

Keywords: DNA isolation, soil fungi, PCR amplification

Introduction

Molecular methods are useful analytical tools for evaluating the microbial communities structure and function, including both the cultivated and non-cultivated parts. These techniques can be applied to both pure and mixed cultures. DNA analysis have been applied at different resolution levels for whole communities, bacterial, fungal, yeast isolates and clones of specific genes [1]. Therefore, in all these various samples DNA extraction procedures are important parts of the investigations. These procedures must provide DNA in sufficient quantity and purity for molecular analyses. The DNA extraction techniques can potentially remove inhibitory materials, i.e. polysaccharides, proteins, mineral salts, etc., which limit the sensitivity of the different reactions in which isolated DNA is applied [1].

Filamentous fungi have sturdy cell walls which are resistant to standard DNA extraction procedures for yeast and bacteria [2]. To reduce the cost, equipment, and time involved in molecular experiments, it is also desirable to isolate fungal DNA from a plate rather than using conidia and hyphal fragments from plate cultures to seed larger volume broth cultures [2].

Nucleic acid detection methods such as PCR have become a common tool for identification and characterization of microbial communities. The polymerase chain reaction (PCR) is increasingly being used as an alternative to culture-based methods for the detection and, in some cases, quantification of microorganisms in various environmental samples including air, soils, landfills, waters, etc. [3, 4]. Although PCR amplification can be performed directly on various microbial cultures, for filamentous fungi and yeast, prior isolation of DNA is often preferred. The DNA extraction procedure eliminates many interfering substances like mineral salts, proteins, polysaccharides, and plays an important role in ensuring consistent results. Considerable efforts have been made to improve the preparation of DNA from fungi [5-10]. This is a significant challenge, especially when dealing with a large number of samples. However, many of the methods are often laborious, expensive and time consuming. These methods often rely on using a grinder (with or without liquid nitrogen) for initial breakage of the mycelia or require

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special hydroxyapatite columns, specific instrumentation and matrices [2, 5].

The objective of the study was to develop a rapid and simple method for the direct extraction and purification of DNA from filamentous soil fungi.

Experimental

Fungal Cultures

Pure cultures of 50 filamentous fungi were obtained from soil contaminated by petroleum hydrocarbons and now under bioremediation treatment for environmental restoration [11]. The isolated fungal cultures were grown on MEA (Malt Extract Agar – DIFCO) supplemented with 100µg/ml chloramphenicol (Sigma) for 7 days at room temperature prior to DNA extraction.

DNA Extraction for Fungal Strains

The DNA extraction procedure was adopted with some modifications from the methods of Tsai and Olson [12] and Furlong *et al*. [13]. Fungal mycelium were directly collected from culture plates and 200-500 mg of mycelium material (wet weight) was added to 1.5 mL microcentrifuge tubes. Mycelium material from the fungi were suspended in 500 µl of a bead beating solution containing: 0.1M NaCl, 0.5M Tris-HCl, (pH8.0), and 5% sodium dodecyl sulfate. Approximately 0.2 g of mixed diameter (1.0mm/0.5mm/0.1mm) glass beads for crushing of cell walls were also added. The tubes were then placed into a TurboMix™ adapter (Scientific Industries, INC.) attachment for a Vortex Genie 2 (Fisher Bioblock Scientific) and homogenized for 10 min at maximum speed. Then, the tubes were centrifuged for 10 min at 11,000 g. After centrifugation, the supernatants were decanted into new tubes and the extraction procedure was repeated. An equal volume of phenol: chloroform:isoamyl alcohol (25:24:1) (Amresco) was added to each sample; the samples were then vortexed briefly, and centrifuged for 5 min in a microcentrifuge. The aqueous layer was transferred to a new tube and extracted again with an equal volume of chloroform: isoamyl alcohol (24:1). The tubes were mixed vigorously and centrifuged for 5 min at 10,000 g. The supernatant was transferred to the new Eppendorf tubes, and 2.5 volumes of isopropanol was added for precipitation of DNA. The tubes were incubated in a refrigerator for 1 hour, and centrifuged at 4°C for 10 min at 14,000 g. The pellets were washed twice with cold 70% ethanol, air-dried, and then resuspended in sterile double deionised water. The samples were treated with RNase (DNase free) (Roche Molecular Systems, INC.). The subsequent DNA yields and quality were assessed by standard electrophoresis through a 1% (w/v) ethidium bromide-stained agarose gel.

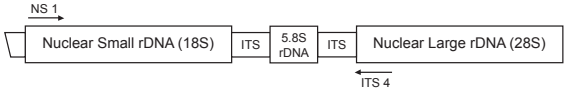

Fig.1. Schematic representation of primers position on rDNA (source: http://plantbio.berkeley.edu/-bruns/picts/results/map).

PCR Amplification of rDNA

The PCR reaction for isolated fungal DNAs was performed with puReTaq™ Ready-To-Go™ Polymerase Chain Reaction (PCR) beads (Amersham Biosciences) as previously described [4]. Beads are premixed and predispensed complete reactions for performing PCR amplifications. With the exception of primers and template, the ambient temperature-stable beads provide all the necessary reagents to perform 25 µl polymerase chain reactions, e.g. stabilizers, BSA, dATP, dCTP, dGTP, dTTP, ~2.5 units of puReTaq DNA polymerase and reaction buffer. When a bead was reconstituted to a 25µl final volume, the concentration of each dNTP was 200 mM in 10 mM Tris-HCl (pH 9.0), 50 mM KCl and 1.5 mM $MgCl_2$. A typical PCR contains < 1µg of template DNA and primers at a concentration of 0.2-1 µM. The samples were subjected to 30 cycles of 95°C for 1 min, 56°C for 3 min and 72°C for 2 min for denaturation, annealing and elongation steps, respectively. An initial denaturation step (95°C, 1 min) was used to ensure complete denaturation of the template DNA. PCR amplification was performed in a Mastercycler® gradient machine (Eppendorf). The primers for the reactions were as follows: forward primer: NS1 (GTAGTCATATGCTTGTCTC) and reverse primer: ITS4 (TCCTCCGCTTATTGATATGC) [14]. Localization of the primers to fungal rDNA is presented in Fig.1. PCR products were first analyzed

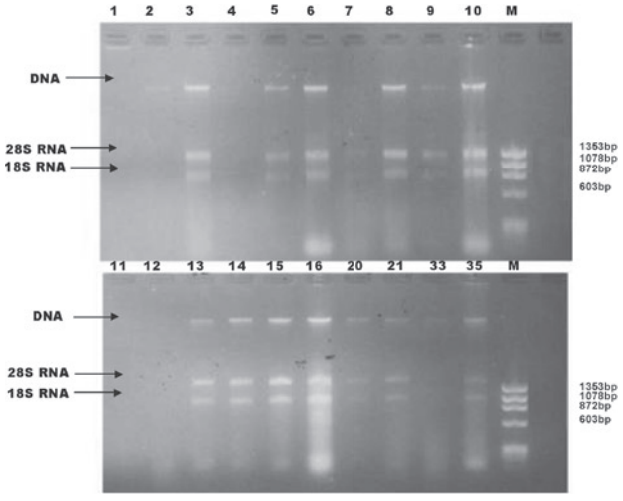

Fig.2. Electrophoresis of DNAs isolated from filamentous soil fungi. DNA were seperated on a 1% agarose gel in 1xTAE buffer. Lanes 1-10 and lanes 11-16, 20, 21, 33, 35 - number of fungal cultures from which DNAs were isolated. M - X174 DNA/HaeIII ladder; bp - base pairs.

by electrophoresis in 1% (wt/v) agarose gels and ethidium bromide staining. PCR products were purified using a High Pure PCR Product Purification Kit (Roche Molecular Systems, INC.) and then sequenced.

RFLP

Restriction enzyme digestions of PCR amplification products were performed according to the manufacturer specifications. Approximately 2-5 µl of PCR products were digested with 1 U of Hae III (Boehringer Mannheim GmbH) in the M buffer supplied by the manufacturer. Total volume of digestion reaction was 10 µl. RE reactions were performed in 0.5 mL microfuge tubes and incubated for 2 hours at 37°C, DNA samples were electrophoresed in 2% (wt/v) agarose gel for 2 hours in 1xTAE buffer (40mM Tris-acetate, 1mM EDTA). Fungal DNAs were viewed on a UV transilluminator box and photographed. As DNA molecular weight markers ΦX174 DNA/HaeIII (Promega, UK) and 1Kb DNA (Invitrogen™ Carlsbad, CA) ladders were used.

Results and Discussion

The method presented in this paper eliminates much of the laborious and time-consuming steps of most other protocols [2, 5, 6]. DNA was isolated immediately from mycelium. In this procedure cell walls are broken by a bead beating solution with a mixture of glass beads with different diameters. The amount and quality of the DNA obtained by this procedure were suitable for PCR amplification and other molecular assays. Results of DNA electrophoresis in 1% agarose gel are demonstrated in Fig. 2. PCR products before the purification step are presented in Fig. 3. One of the advantages of this procedure is that many samples can be simultaneously processed. In our experiment, approximately 50 fungal strains isolated from soil contaminated by petroleum hydrocarbons and under bioremediation were examined. Of particular interest is the recovery of 28S RNA and 18S RNA as visualised by electrophoresis in ethidium bromide-stained gel (Fig. 2). The recovery of these single stranded RNAs indicated that the procedure was relatively gentle. The procedure minimizing contamination risks between samples can be completed within 2 hours. It is applicable to various filamentous fungi isolated from diverse environment samples. The DNA yields were reasonably high and compared with the literature data [2, 5, 7]. Nevertheless, genomic DNA extracted by the procedure has been readily amplified by PCR (Fig.3), and PCR products have been digestible with restriction enzymes, e.g. HaeIII (Fig.4).

It is likely that this procedure could be applied to the examination of many other fungal cultures. It provides a rapid, reliable, and low-cost alternative to the existing DNA purification protocols used in research and clinical laboratories [2, 5, 9, 15]. Use of this procedure will allow researchers to obtained DNA from fungi quickly and inexpensively for molecular assays and replaces expensive and consuming procedures [5, 8, 16].

Conclusions

The protocol is effective, easy, fast, and does not require the use of expensive equipment and reagents. DNA extracted using the method was used to successfully amplify regions of interest. The amplicons were suitable for further applications such as sequencing and PCR-RFLPs.

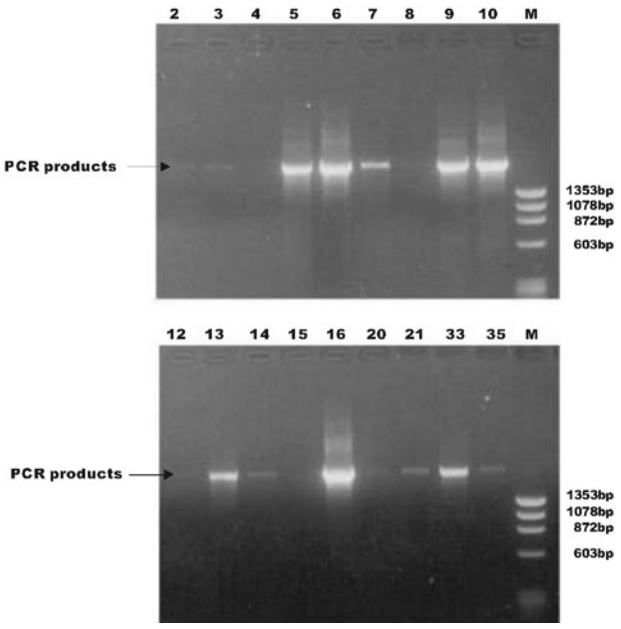

Fig.3. Agarose gel electrophoresis of PCR-amplified products.
5 uL PCR reactions were run on 1% agarose gel.
Lanes 2-10 and lanes 12-16, 20, 21, 33, 35 - number of fungal cultures, Lane M - DNA marker.

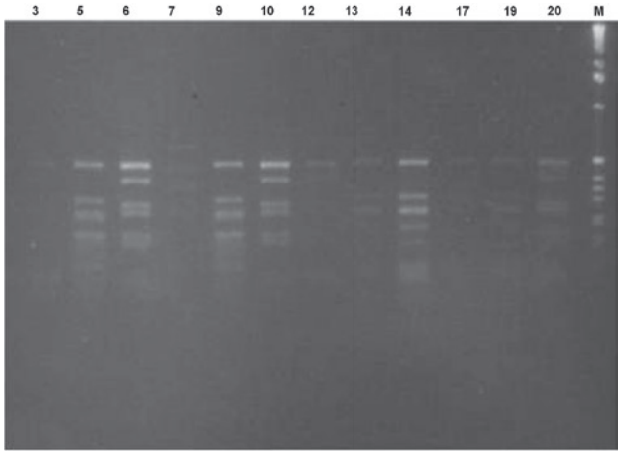

Fig.4. HaeIII restriction patterns of PCR products.
PCR products were digested by HaeIII and run on 2% agarose gel.
Lanes 2, 3, 5, 6, 7, 9, 10, 12, 13, 14, 17, 19, 20 - number of fungal cultures, Lane M - DNA molecular weight marker - 1Kb DNA.

SIGNIFICANCE AND IMPACT OF THE STUDY: The described method can be applied in environmental microbiological studies for sensitive screening of filamentous fungi isolated from different environments. This technique could be used for rapid screening of different species, activity, and/or products for biotechnology.

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