A simple and rapid DNA extraction protocol for filamentous fungi efficient for molecular studies

Iiti Gontia-Mishra, Niraj Tripathi and Sharad Tiwari*
Biotechnology Centre, Jawaharlal Nehru Agricultural University
Jabalpur 482 004, India

Received 4 January 2013; revised 22 May 2013; accepted 16 July 2013

A simple and rapid protocol for extracting high-quality DNA from filamentous fungi was studied. The method involved disruption of fungal cells by employing glass bead method, followed by inactivation of proteins using CTAB/protease K. The DNA yield from fungal isolates varied from 310-1879 µg g⁻¹ dry mycelium and a clear intact DNA band was observed upon agarose gel electrophoresis. Absorbency ratios (A₂₆₀/A₂₈₀) for DNA ranged 1.7-1.9, which indicated minimal presence of contaminating metabolites. PCR analysis like 18S rRNA gene amplification, random amplified polymorphic DNA (RAPD) and PCR-restriction fragment length polymorphism (PCR-RFLP) showed that DNA was compatible for downstream applications. This method can be applied to extract genomic DNA of filamentous fungi from different environmental sources.

Keywords: DNA isolation, filamentous fungi, PCR-RFLP, RAPD, 18S rRNA

PCR-based methods have become a common tool for fungal identification and diagnosis. Although PCR amplification can be performed directly on various microbial cultures, prior isolation of DNA is often required for fungi and yeast. DNA extraction process eliminates many unknown interfering substances, such as, salts, proteins, polysaccharides etc., and plays a significant function in ensuring consistent test results. Hence, for any PCR based analysis, the rapid isolation of relatively pure genomic DNA of high mol wt is a prerequisite. Many fungal DNA isolation kits are available in the market, which are quite expensive and cannot be used by laboratories extensively involved with DNA isolation in developing countries. Far-reaching efforts have been made to improve DNA preparation from fungi. However, the fact remains that filamentous fungi have a strong cell wall, which is resistant to standard DNA extraction procedures of yeast and bacteria. The methods often depend on using a grinder (with or without liquid nitrogen) for the initial breaking up of mycelia or require special hydroxyapatite columns and matrices. Protocols for extraction of DNA of fungal cells are either very time-consuming or show poor release of fungal DNA. Therefore, the objective of present study was to develop a rapid, simple, low-cost and reliable method for DNA extraction from filamentous fungi.

The filamentous fungi, viz., Aspergillus niger, A. flavus, A. awamori, A. fumigatus, A. ficuum and A. terreus were isolated from soil samples. These fungal isolates were cultured on potato dextrose agar medium (Difco). The plates were incubated at 28°C for 72 h. The mycelium from pure fungal colonies was used for DNA isolation.

Fresh fungal mycelium (~200 mg) was transferred to a sterilized 1.5 mL eppendorf microcentrifuge tube with the help of sterilized scalpel and 800 µL of extraction buffer (0.1 M Tris-HCl pH 8, 10 mM EDTA pH 8, 2.5 M NaCl, 3.5% CTAB, 150 µL of 20 mg/mL protease K) was added with mixed sterilized 0.5-1 mm glass beads. The mixture was vortexed at high speed on a homogenizer (Spinix, Tarsons, India) for 5 min. The samples were placed in a water bath at 65°C for 30 min. The samples were then centrifuged at 10,000 rpm for 10 min at room temperature. Supernatant was collected and equal volume of phenol-chloroform-isoamylalcohol (25:24:1) was mixed. The samples were again centrifuged at 10,000 rpm for 10 min at room temperature. Supernatant was again collected and equal volume of chloroform-iso-amyl-alcohol (24:1) was mixed. Samples were again centrifuged under the conditions mentioned above. Supernatant was collected and equal volume of ice-cold isopropanol was added. Supernatant were mixed and incubated at −20°C for 1-2 h. The samples were centrifuged for 15 min at 13,000 rpm to pellet the DNA. Supernatant was decanted and DNA pellet was washed with 800 µL of 70% ethanol. DNA pellet was air-dried and dissolved in 200 µL TE buffer (10 mM Tris-HCl pH 8, 1 mM EDTA). 5 µL RNase A (20 mg/mL) was added to DNA samples, mixed and incubated at 37°C for 1 h. DNA was recovered and air-dried as described above. DNA was reconstituted in TE buffer for further use for PCR amplification.
Quality of the extracted DNA was obtained by means of electrophoresis in 0.8% agarose gels, followed by staining with ethidium bromide. The purity of the DNA was estimated from the $A_{260}/A_{280}$ ratio, whereas the yield was obtained by measuring absorbance at 260 nm with a spectrophotometer. A variety of PCR-based assays were done to check the suitability of extracted DNA for downstream analysis.

PCR amplification of fungal small-subunit rDNA (18S rRNA gene) was carried out using the primer set EF4/EF3, which amplified a 1.5 kb section of the 18S rDNA and had the following sequences: EF4 (5′-GGAAGGG [G/A] TGTATTTATTAG-3′) and EF3 (5′-TCCTCTAAATGACCAAGTTTG-3′). PCR amplification was performed in 25 µL reaction mixture containing: 2.5 U Taq DNA polymerase (Sigma) along with 10× manufacturer’s buffer (Sigma), 200 µM each deoxynucleoside triphosphate (dNTPs), 20 pM primers EF4 and EF3 and 50 ng genomic DNA. The reaction conditions were as follows: initial denaturation step at 94°C for 4 min, 40 amplification cycle of denaturation at 94°C for 1 min, annealing at 50°C for 1 min and primer extension at 72°C for 3 min; followed by a final extension at 72°C for 10 min. PCR amplifications were carried out using a Thermo-Hybad PCR thermal cycler (Thermo Fisher Scientific, USA). Aliquots of the PCR products (5 µL) were analyzed in 1% (w/v) agarose gels (Sigma, USA) by horizontal gel electrophoresis. DNAs were visualized by UV excitation after staining with ethidium bromide (0.5 mg/L).

Random amplification of polymorphic DNA (RAPD) was performed using 3 random primers, i.e., OPB-07, OPC-06 and OPC-07 (B-07: 5′-GGGTACGCCAG-3′; C-06: 5′-GAACGGACTCTC-3′; C-07: 5′-GTCCCGAGCGA-3′). PCR amplification was carried out in 25 µL reaction mixture containing: 1 U Taq DNA polymerase (Sigma) along with a 10× manufacturer’s buffer (Sigma), 200 µM each deoxynucleoside triphosphate (dNTPs), 20 pM primers OPB-07, OPC-06 and OPC-07 (used separately); and 50 ng genomic DNA. PCR conditions were as follows: initial denaturation at 94°C for 4 min, 39 cycles of 1 min at 94°C, 1 min at 37°C, 2 min at 72°C and followed by final extension at 72°C for 5 min in Thermo-Hybad PCR thermal cycler (Thermo Fisher Scientific, USA). Gel electrophoresis (1.5% agarose) of amplified DNA was done under standard electrophoresis procedure.

For the study of PCR-restriction fragment length polymorphism (PCR-RFLP), 18S rRNA amplified PCR products were purified using Qiagen PCR purification kit. The PCR product was digested using restriction enzymes TaqI, AseI and VspI (Fermentas). The restriction digestion reaction consisted of 3 µL purified PCR product, 1 U restriction enzymes, 1 µL buffer supplied by manufacturer and volume was adjusted to 10 µL with milliQ water. The reaction mixture was incubated for 3 h at 37°C. The restriction digested products were then electrophoresed on 2% agarose gel. DNAs were visualized by UV excitation after staining with ethidium bromide (0.5 mg/L).

Genomic DNA was isolated from all the fungal cultures. DNA purity and quality were tested by means of electrophoresis in 0.8% agarose gels, followed by staining with ethidium bromide. DNA obtained was an intact band of good quality as shown in Fig. 1. $A_{260}/A_{280}$ ratio of DNA ranged 1.68-1.9, which confirms that it is free from protein impurities. The amount and quality of DNAs obtained by the present protocol were suitable for PCR amplification and other molecular assays. The DNAs were then utilized for various PCR based analyses. PCR amplification of 18S rRNA gene using specific primers was performed and a desired band size of 1.5 kb was obtained from fungal isolates as shown in Fig. 2. RAPD was also performed with 3 primers, namely, OPB-07, OPC-06 and OPC-07, which produced amplification of products ranging from 0.25 -2 kb as shown in Fig. 3. Furthermore, PCR-RFLP of 18S rRNA was also performed using TaqI, AseI and VspI restriction enzymes. These enzymes can digest the PCR products and produced bands of different sizes as shown in Fig. 4.
The method for DNA isolation from filamentous fungi presented in the present study eliminated the laborious and time consuming steps involved in previous protocols. The DNA yields were higher as compared with the available data. Some of the important features of the present protocol were: small amount of sample (mycelium) was sufficient for DNA extraction; large numbers of samples could be processed parallely; and it did not require liquid nitrogen for grinding of mycelium, and thus the method was simple. The extractions could be carried out in 1.5 mL micro centrifuge tubes, which minimized the chances of contamination and loss of DNA. Lysis buffer used in this method was composed of 3.5% of detergent (CTAB), whereas much higher concentration of detergents (5% SDS) has been used in other DNA extraction process. This method needs a single extraction step with lysis buffer, whereas the other methods require twice extraction with lysis buffer for efficient recovery of DNA. Further, the present procedure was successfully extended to recover DNA from *Fusarium oxysporum* and *Macrophomina phaseolina* (data not shown). Thus the present method of DNA isolation from fungi could be efficiently used for various molecular assays including PCR, and may replace the other described procedures for DNA isolation.

**Acknowledgement**

IG-M is grateful to Department of Biotechnology, Ministry of Science and Technology, Government of India, New Delhi for financial assistance.

**References**


9 Stewart C N Jr & Via V E, A rapid CTAB DNA isolation technique useful for RAPD fingerprinting and other PCR applications, Biotechniques, 14 (1993) 748-751.


