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Optimization of protocol for DNA isolation from filamentous fungi and its identification

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Abstract

A study was carried out to isolate the DNA from fungi of filamentous nature using sonication method. As sonication method is a very efficient and reliable tool that allows a complete control over different parameters and ultrasonic waves can easily destroy cell wall. But in contrast, other studies carried out to investigate the most effective method for DNA isolation from filamentous fungi holds sonication method as low DNA yielding method. In contrast, our study involves the optimization of sonication method for DNA isolation from filamentous fungi; yields high quality DNA, absorbency ratio (A_{260}/A_{280}) for DNA ranged 1.8, which indicated minimal presence of contaminating metabolites. Moreover, the method is cost effective, efficient and reliable. The filamentous fungi isolated from wastewater of Bagru region, Jaipur, Rajasthan, India were further sequenced for their identification and identified as *Aspergillus niger* isolate AN6 and *Aspergillus niger* isolate CBS 120.49.

Keywords: Sonication method, DNA isolation, Filamentous fungi, Bagru

Introduction

Microorganisms represent half of the biomass of our planet, yet we know as little as 5% of the microbial diversity of the biosphere **[1].** Considering filamentous fungi and its identification, filamentous fungi have a sturdy cell wall which is resistant to standard DNA extraction procedures for yeast and bacteria **[2].** Moreover, fungal nucleases and high polysaccharide content (mannan, β-glucans and chitin) in rigid cell wall add to the difficulties in isolating DNA from filamentous fungi **[3].** A number of protocols have been designed for fungal DNA extraction include use of liquid nitrogen, combined with extraction buffer **[4]**, bead vortexing with SDS lysis **[5]** and glass bead/magnetic bead-vortexing **[6].** Moreover, In recent years, commercial kits have been used more frequently **[7]** but disadvantages associated with these kits involves their high cost and number of samples can be extracted. Other factors for modification often adopted to minimize the time and to avoid the use of expensive and hazardous chemicals. This may be achieved by agitating the fungal samples using

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sonication (ultrasound disruption: >20-30 kHz) **[8].** Sonication is a quite versatile method, suitable to all cell types and easily applicable in small and large scale **[9]** but is less used for extraction of DNA as earlier studies shows it to be less yielding method.

Protocols for fungi DNA extraction involves number of steps, either less efficient or involve the use of highly expensive chemicals. To combat such limitations in our study, fungal sample were agitated using sonication method. Optimized procedure is modification of the (CTAB and Sonication) method of **[2]**. The findings indicate protocol is precise, proved highly efficient and cost effective as well.

Materials and Method

Fungal cultures

Fungal isolates isolated from water samples of Bagru region (Rajasthan) were named as SRF2 and SRF6. These fungal isolates were cultured on potato dextrose agar medium. The plates were incubated at 28^{O}C for 72 h. The mycelium from pure and fresh fungal colonies was used for DNA isolation.

DNA Extraction from Fungal Strains

Reagents

The following solution and reagents were used: extraction buffer solution (100mM Tris Buffer; 25mM EDTA; 1.5M NaCl; 2% CTAB); 10X Phosphate Buffer Saline (PBS) (137mM NaCl; 8mM Na_2HPO_4; 2.7mM KCl; 2mM KH_2PO_4); Phenol:CCl_3:Isoamyl alcohol (PCI) (25:25:1); CIA (24:1) and cold absolute ethanol.

The DNA extraction procedure consisted of the following steps.

1. Purified colonies of fungal isolates were grown on Potato Dextrose Agar slants for 5 days at 28^{O}C.

2. Fungal mycelium along with spores was then transferred to eppendrof (1.5 ml) containing 500µl PBS solution.

3. The reaction mixture was centrifuged at 5000 rpm for 5 mins and supernatant was discarded.

4. To the centrifuge tube pre-warmed 500µl extraction buffer (65^{O}C) and 10µl β-mercaptoethanol were added and the reaction mixture was vortexed for 1 min vigorously.

5. The reaction mixture was incubated at 65^{O}C for 1 hour and then in ultrasonicator (Phoenix ultrasonic cleaner) at frequency of 40 KHz at 50^{O}C for 30 mins respectively.

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6. To the reaction mixture 500µl of PCI was added and was centrifuged at 13,000 rpm for 10 mins.

7. The supernatant was transferred to fresh centrifuge tube and 500µl of chilled absolute ethanol was added and as centrifuged at 13,000 rpm for 10 mins.

8. The supernatant was discarded and pellet was air dried for 15 mins.

9. Finally, the isolated DNA was resuspended in 50µl of nuclease free water and stored at -20^{O}C for further use.

10. Extracted DNA samples were run on 1 % agarose gel (w/v) in 1.0% TBE buffer at 90 V for 1 hr to detect the presence of DNA. Gel was visualized using UV Transilluminator.

PCR amplification was performed in a reaction mixture (reaction mixture kit was purchased from Kappa) of 50µl, containing Taq master mix (20µl), $MgCl_2$ (2.0µl), 2µl of each ITS-1 and ITS-2 primers, genomic DNA (5.0µl) and nuclease free waster (19.0µl). Fungal specific primers ITS-1 (5'-TCC GTA GGT GTA CCT GCG G-3') and ITS-2 (5'-GCT GCG TTC TTC ATC GAT GC-3') were used (purchased from GCC Biotech).PCR conditions were as follows: 35 cycles each of 94^{O}C for 3 mins and 95^{O}C for 30 sec for denaturation, 55^{O}C for 30 sec and 72^{O}C for annealing and final elongation at 72^{O}C for 5 mins. The reaction was carried out in the MWG AG Biotech Primus 96 Plus PCR system.The presence and yield of PCR amplified amplicons was ascertained on 2.0% agarose gel (w/v) prepared in 1% TBE. Gel electrophoresis was carried out at 90 V for 45 mins in 1X TBE Buffer, Staining was done with ethidium bromide and gel was visualized using UV transilluminator.

PCR product cleaning up, sequencing, annotation and blasting

The PCR product obtained were of high quality and were got sequenced from 1st BASE DNA Sequencing Division. The received sequences were in FASTA format was cleaned up to remove the misleading sequences and was improved upon using Chromas software . The improved consensus sequences were blasted using BLASTn **[10]** of NCBI (http://blast.ncbi.nlm.nih.gov/Blast). Both the forward and reverse sequences were aligned using BLAST tool, aligned sequence were downloaded and then search in 18S rDNA database, 10 sequence with highest query coverage, E value, percentage identity were downloaded for phylogenetic tree construction using Mega 7.0 software (Allele Life Sciences Pvt Ltd).

Result and Discussion

Filamentous fungi cell wall disruption is crucial and should be achieved without the disruption of genomic DNA. Our study includes the fungal DNA extraction using CTAB and sonication method. The optimized protocol yields high DNA (Fig 1) with absorbance ratio of 1.8.

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Few studies have been carried out on DNA extraction using CTAB and sonication method. An investigation carried out by Van Burik et al 1998 comprising of comparison of six DNA extraction techniques from filamentous fungi states low amount of DNA extraction using soniaction and CTAB method. Similarly one more investigation carried out by Motkova and Vytrasova 2011 states low yield of DNA obtained using ultrasound method.

Contrast results of our study maybe due to following factors varied in our research from others: (a) Composition of extraction buffer (Table 1); (b) Use of PBS solution for maintenance of pH throughout the process; (c) Temperature, frequency and time period for which ultrasonicator was used.

Our findings indicate sonication is a very efficient and reliable tool that allows a complete control over different parameters and ultrasonic waves can easily destroy cell wall. Significance of above described optimized protocol for fungal DNA extraction involves: (1) Exclusion of costly chemicals like urea and lysozyme and other enzymes generally used DNA isolation. (2) Less number of procedural steps. (3) The method is efficient because DNA obtained is of high quality can be used for various downstream processes, moreover unlike **[11]** only 0.5g of mycelium gives large amount of pure, intact DNA. (4) It exempts the need of breaking of fungal mycelia by grinding with glass rod or mortar pestle in presence of liquid nitrogen as achieved by **[12].**

Identification of fungal isolates

Fungal isolates were named as SRF 2 and SRF6. Identification of fungal isolates SRF2 and SRF6 were done on the basis of 18S rRNA gene sequence. The sequence of SRF2 and SRF6 showed maximum similarity 98% and 96% respectively with *Aspergillus niger* during similarity search using BLAST tool and identified as *Aspergillus niger* isolate *AN6* and *Aspergillus niger* isolate *CBS 120.49* (Table 2). The 18S rDNA partial sequence of *Aspergillus niger* isolate *AN6* (SRF2) and *Aspergillus niger* isolate *CBS 120.49* (SRF6) has been deposited in GenBank under the accession number LCO 62385.1 and MF 152936 respectively. In phylogenetic analysis (Fig 2 and Fig 3), SRF2 and SRF6 strains fall in cluster of *Aspergillus* sp. The evolutionary history was inferred using the neighbor joining. The evolutionary distances were computed using the Maximum Composite Likelihood method and are in the units of the number of base substitution per site.

In our study, the fungal specific primers used, Internal transcribed spacer (ITS1) region I (ITSI) and ITSII are more variable than the rest of the ribosomal gene subunits and are found between SSU rRNA and 5.8 rRNA and between 5.8S rRNA and LSU rRNA, respectively **[13]**.

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Table 1.Composition and comparison of Extraction buffer used in different studies

Our study	Van Burik et al 1998	Motkova and Vytrasova 2011
100mM Tris Buffer	100mM Tris Buffer	200mM Tris Buffer
25mM EDTA	20mM EDTA	25mM EDTA
1.5M NaCl	1.4M NaCl	250Mm NaCl
2% CTAB	1% CTAB	0.5% SDS
pH 8.0	pH 8.0	

Table 2 Identification of bacterial isolate inferred from 18S rRNA gene sequences by BLAST

Name of Isolates	Maximum Score	Maximum Identity	Query Coverage	Description Name	Gene Bank Accession no
SRF2	313	98%	98%	*Aspergillus niger* isolate *AN6*	LC062385.1
SRF6	309	100%	100%	*Aspergillus niger* isolate *CBS 120.49*	MF 152936

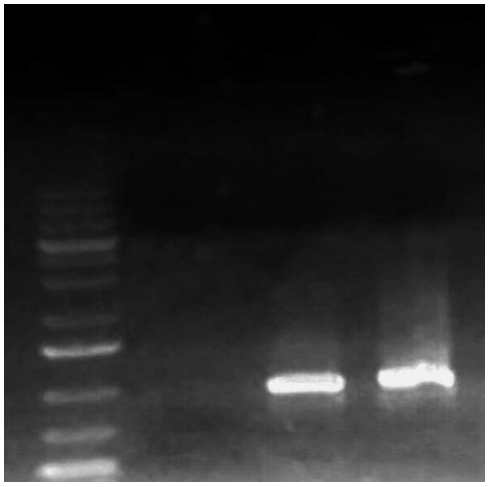

Fig 1. Products of PCR amplification of DNA obtained. Lane 1, Ladder (size standard): 1000 bp, 700 bp, 500bp, 400 bp, 300 bp, 200 bp, 75 bp. Lane 3-4 rDNA amplified from *Aspergillus niger* isolate *AN6* and *Aspergillus niger* isolate *CBS 120.49* respectively.

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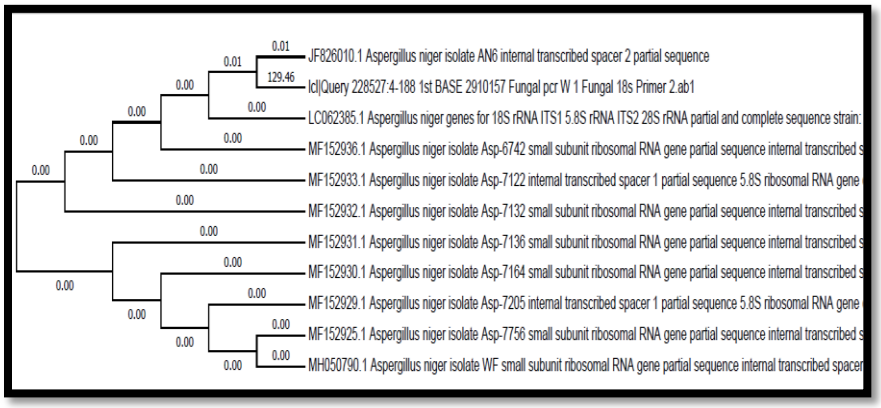

Fig 2 BLAST tree view of potential identified fungal isolate *Aspergillus niger* AN6

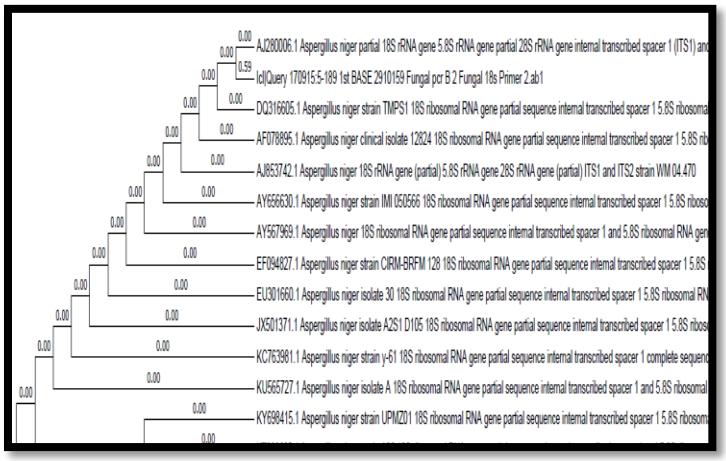

Fig 3 BLAST tree view of potential identified fungal isolate *Aspergillus niger* CBS 120.49

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