Global Journal of Molecular Sciences 5 (1): 37-42, 2010
ISSN 1990-9241

Modified Simple Protocol for Efficient Fungal DNA Extraction Highly Suitable for PCR Based Molecular Methods

[1]*V. Karthikeyan,* [2]*S. Patharajan,* [3]*P. Palani,* [1]*D. Spadaro,*
[1]*M.L. Gullino and* [1]*A. Garibaldi*

[1]Centre of Competence for the Innovation in the Agro-environmental Sector, Agroinnova, University of Torino, Via Leonardo da Vinci 44, 10095 Grugliasco-(Torino) -Italy
[2]Centre for Research and Development, PRIST University, Thanjavur, Tamil Nadu, India
[3]Centre for Advanced Studies in Botany, University of Madras, Guindy Campus, Chennai, Tamil Nadu, India

Abstract: Using only 5 mg of three to four days old fungal mycelium of *Fusarium*, *Verticillium*, *Bipolaris oryzae*, and *Rizoctonia solani*, up to 9091ng/ul of genomic DNA was extracted by combined lysis buffer-microwave treatment. The obtained DNA was proved to be enough for PCR assay with translation elongation factor based sequence matching coefficient with NCBI species identification of *Fusarium* and *Verticillium* species and efficient to specific gene tag analysis of fumonisin producing PKS domine gne called *fum5* similarly for *p450-4* fragment of GA3 biosynthetic gene cluster in *Gibberella fujikuroi* sps. We developed a simple method for fungal DNA extraction which is quite rapid and highly cost-effective by means of minimizing the usage of costly chemicals like CTAB, SDS etc., The amount of DNA was competent compared with classical and Kit based DNA extraction methods. Furthermore this exaction is quite suitable for DNA fingerprinting, transform ants analysis and Southern blot hybridization.

Key words: *Gibberella fujikuroi* · Verticillium · PKS · CTAB

INTRODUCTION

A good amount of DNA is a primary need for all kind of DNA based molecular biology works. In modern research environment, dozens of new protocols have been developed in order to reduce time constraints [1] like CTAB based extraction described by O'Donnell and Sreenivasa *et al.* [2, 3] mitochondrial DNA extraction by Laday *et al.* [4] and total DNA extraction by Leach *et al.* [5] and from different filamentous fungi was elaborated by many researchers [6-9]. For *Fusarium* DNA extraction elaborated by Moller *et al.* [10]. Though the amount and quality of obtained DNA was satisfactory, these techniques are time consuming, involve the usage of toxigenic chemicals like Phenol, chloroform and â-mercapto-ethanol etc. and too labor intensive also. Despite the range of techniques available for the preparation of fungal DNA, some fungal mycelia and most fungal spore samples remain intractable to extraction by these procedures.

Recently, the use of microwave for extraction of plasmid [11] as well as total DNA from *E.coli* and other Gram negative bacteria was described by Fujikawa *et al.* [12] and Tendulkar *et al.* [13] who reported about isolation of fungal DNA of *Magnaporthe grisea* by microwave radiation method. Though this method is simple but lack of reproducibility and consistency of this method with other fungus like *Fusarium, Bipolaris oryzae, Rizoctonia oryzae* was observed. Though this microwave treatment was proven to be effective in obtaining DNA from plant, animal cells and fungi such as *Xyleria hypoxylon, Heterotextus alpinus, Crucibulum leave, Lycoperdon* spps, but the use of phenol/chloroform prior to PCR amplification [14] is the major drawback of this experiment. This strategy tents to develop new simple procedure of extraction excluding the usage of above chemicals. The present study aimed to obtain fungal DNA with simple and efficient procedure from most widely used fungal pathogens. We modified the microwave treatment along

Corresponding Authnor: V. Karthikeyan, Centre of Competence for the Innovation in the Agro-environmental Sector, Agroinnova, University of Torino, Via Leonardo da Vinci 44, 10095 Grugliasco-(Torino)-Italy.
E-mail: karthickeyanv@yahoo.com.

with addition of lysis buffer treatments yielded very good amount of DNA with the above mentioned fungus.

MATERIALS AND METHODS

Fungal Cultures: The fungal cultures was grown and maintained in PDA medium. And for liquid culture medium all fungal cultures were sub cultured in PDA kept in controlled environment growth chambers under continuous florescent light for 6 to 7 days at temperature of 24 to 26°C. On the 7th day fungal cultures were examined for CFU using hemocytometer and the spore suspension of 1.10^4 concentration were inoculated in potato dextrose broth medium.

The fungal strains isolated from infected samples of rice collected from rice growing regions of North-Western Italy, Two representative (ITEM504 (*F.verticillioides*), ITEM1720 (*F.proliferatum*), strains of the species were obtained from Culture collection of Institute of Sciences of Food Production, Bari, Italy (http://www.ispa.cnr.it/Col-lection).

DNA Extraction by Modified CTAB Extraction Method According to Moller *et al.* [10]: 50mg of fungal mycelia was scraped from 10d old PDA cultures, manually ground in 1.5ml of microfuge tubes with micro pestle adding 500ul of pre-warmed (60°C) TES lysis buffer (100mM Tris pH 8.0; 10mM EDTA pH 8.0; 2% SDS). 50ug of proteinase K were added to the ground material, incubated in 60°C for 60 min. 140ul of 5M Nacl and 64ul of 10% (w/v) of CTAB were added to the suspension incubated at 65°C for 10 min. DNAs were extracted by adding equal vol. of chloroform:isoamylalcohol (24:1) centrifuged at 14000xg/10min. DNA was precipitated by adding 0.6vol of cold isopropanol and 0.1 vol of 3M sodium acetate pH 5.2 and maintained at -20°C, centrifuged and washed twice with 70% ethanol suspended in 100ul of TE (10mM Tris pH 8.0; 1mM EDTA pH 8.0). RNA was digested by adding 10mg/ml of RNAse A and incubating at 37°C for 45min and stored -20°C for further use.

DNA Extraction by Kit Method: All fungal DNA was extracted using Nucleo-Spin Plant DNA extraction kit (i.e., NucleoSpin, Macherey-Nagel,) according to the user manual instructions.

DNA Extraction by Lysis Buffer-microwave Treatment: 5-10mg of fungal mycelia was scraped from 4-7d old PDA cultures, liquid cultures were filtered through cheese cloth and manually ground in 1.5ml of microfuge tubes with micro pestle by adding 500ul of TES lysis buffer (100mM Tris pH 8.0; 10mM EDTA pH 8.0; 2% SDS) followed by microwave treatment at 800W frequency in microwave oven of 230 V output (Severin and Co, Turin, Italy) at 28°C for 15s. incubated in 60°C for 30min. centrifuged at 1000xg/5min. DNA was precipitated by adding 0.6vol of ice cold isopropanol and suspended in 100ul of DNAs free sterile water and stored-20°C for further use. The optical density of DNA measured by 260/280nm using eppendorf spectrophotometer, quantification expressed in amount of nano grams per micro-litters.

Polymerase Chain Reaction: A standard polymerase chain reaction (PCR) protocol is used to amplify the TEF gene region. ef1 (forward primer; 5-ATGGGTAAGGA(A/G)GACAAGAC-3) and ef2 (reverse primer;5-GGA(G/A)GTACCAGT(G/C)ATCATGTT-3 *(15,2)* primers are used in a PCR reaction, for fum 5 gene Fum5 (5-GTC CTA CGC GAT ACA TCC CAC AAT -3) and fum 6 (5-GAT CAA GCT CGG GGC CGT CGT TCA TAG-3 *(16)* for GA3 P450-4-GD1 5'-TTT CTC GGT CCA GAG CAC TGC CGC-3'; P450-4-GD2 5'-CGT GGT CTT CCT TTC CCA TCT GGC-3' and for *Aspergillus* sps. ITS 4 and 5 with Reactions were performed in volumes of 50 µl and contained 20 ng of fungal DNA for each reaction. The reaction mixture consisted of a 10X buffer (Taq Polymerase Kit - Quagen, Italy) solution consisting of 50 mM KCl and 10 mM Tris-HCl (pH 9), 0.2 mM of each dATP, dCTP, dGTP and dTTP, 0.5 mM of each forward and reverse primer pairs and 1.5 mM of MgCl2. Amplification was performed using the Biomerta gradient thermo cycler. The PCR program included the following temperature regime: 95°C for 3 min, The remaining steps in the program were repeated 32 times and consisted of 95°C for 1 min, 60°C for 1 min, 72°C for 3 min and the final extension was at 72°C for 5 min. The PCR products (15 µl) were separated by gel electrophoresis in 2.0 % agarose at 1 X TAE gel and stained with Cyber safe (Invitrogen-Italy) for photography. An ~700 bp product is amplified. The PCR conditions and reactions mixtures were same to all primers except the annealing temperature for Fum 5 -58°C for 30s; GA3-60°C for 1min; ITS 4and5-56°C for 45s. were maintained.

DNA Sequencing: The PCR product generated using the ef1 and ef2 and ITS 4and5 primers is used as a template for DNA sequencing, send to BMR Genomics-Padova-Italy. Sequencing data were BLAST with FUSARIUM-ID developed by Geiser *et al.* [17] and for *Aspergillus* species confirmation the sequences were BLAST in Entez website at the US National Centre for Biotechnology Information (NCBI): http://www.ncbi.nlm.nih.gov/Entrez/.

RESULTS

The present method of combined lysis buffer-microwave treatment which is rapid, cost-effective and less time consuming. The whole procedures required approximately 1hr of time and was not only specific for *Fusarium*, as it also allowed rapid isolation of genomic DNA from *Aspergillus*, *R. solani* and *Verticillium*. In all cases, we obtained good yields of high-quality genomic DNA (Fig.1a,b).

Totally five Fusarium two *Bipolaris oryzae*, three *R. solani,* three *Giberella sps.* obtained from rice and two *Aspergillus sps.* of grapes, collected from North-Western-Italy three *Verticillium* sps. obtained from nematodes, collected from Tunisia were used for this study (Table1). Five mg of fungal mycelium was scraped directly from 5 days old PDA plate containing *Fusarium*, *Bipolaris, Aspergillus, Verticillium sps* and *R.solani* and used for microwave treatment with lysis buffer, addition of lysis buffer facilitate the faster extraction with little amount of mycelium and DNA was released by treating less than 30s in microwave oven. When the amount of DNA yield from Fusarium varies from 1120-2210ng/ul in CTAB extraction method 901-1811ng/ul, but its varies from 1602-2610ng/ul in the present method, similar strategy was observed in *Bipolaris oryzae* 1213-2610ng/ul, in *R.solani* 2310-6128ng/ul, in *Verticillium* sps.1716-719ng/ul and 2718-7192ng/ul in *Apergillus.* Levels of fungal DNA recovered with the three extraction methods are displayed in (Table 2). Another major advantage of this procedure is it reduces the time for growing the mycelium on liquid medium for more than 10 days 4 to 5 days grown mycelium on petri-dish is more than sufficient for extraction its speeds up the extraction procedure.

Table 1: Isolates used for this study

S.No	Name of fungal cultures	Host plant	Origen
1	*Fusarium verticillioises*	Rice	Italy
2	*Fusarium proliferatum*	Rice	Italy
3	*Fusarium graminearum*	Rice	Italy
4	*Fusarium equseti*	Rice	Italy
5	*Fusarium oxysporum*	Rice	Italy
6	*Bipolaris oryzae*	Rice	Italy
7	*Bipolaris oryzae*	Rice	Italy
8	*Rizctonia solani*	Rice	Italy
9	*Rizctonia solani*	Rice	Italy
10	*Rizctonia solani*	Rice	Italy
11	*Verticillium leptobactrum*	Nematode*Meloidogyme*	Tunisia
12	*Verticillium leptobactrum*	Nematode*Meloidogyme*	Tunisia
13	*Verticillium chlamydosporium*	Nematode*Meloidogyme*	Tunisia
14	*Gibberella moniliformis*	Rice	Italy
15	*Gibberella fujikuroi*	Rice	Italy
16	*Gibberella fujikuroi*	Rice	Italy
17	*Aspergillus niger*	Grapes	Italy
18	*Aspergillus niger*	Grapes	Italy

Table 2: *Comparative DNA quantification with other extraction methods*

S.No	Name of fungal cultures	CTAB Methodng/ul	Kit extractionng/ul	Lysisbuffer-Microwave methodng/ul
1	*Fusarium verticillioises*	2380	1310	2490
2	*Fusarium proliferatum*	1050	904	1902
3	*Fusarium graminearum*	2210	1811	2610
4	*Fusarium equseti*	2345	1345	1953
5	*Fusarium oxysporum*	1120	901	1602
6	*Bipolaris oryzae*	665	765	1213
7	*Bipolaris oryzae*	1715	1671	2610
8	*R.solani*	2380	3281	6128
9	*R.solani*	1820	810	2310
10	*R. solani*	3045	2032	2918
11	*Verticillium leptobactrum*	1505	1215	1716
12	*Verticillium leptobactrum*	6996	5986	7119
13	*Verticillium chlamydosporium*	3045	2124	3218
14	*Gibberella moniliformis*	1050	951	1710
15	*Gibberella fujikuroi*	1050	820	2189
16	*Gibberella fujikuroi*	6996	4982	9091
17	*Aspergillus niger*	6996	4985	7192
18	*Aspergillus niger*	2200	1145	2718

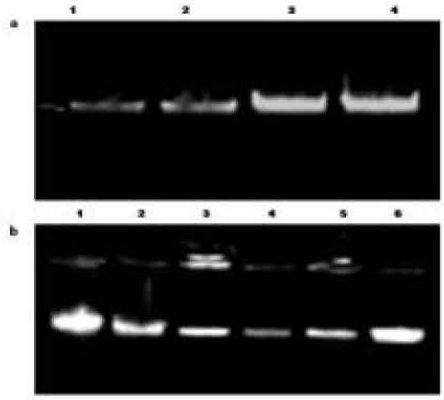

Fig. 1a,b: Representative DNA gels
1a. Shows DNA of lane 1 - *R.solani*,2- *Bipolaris oryzae*,3-*Verticillium sps*, 4-*Aspergillus sps*
1b. Shows DNA of 1, *F. verticillioides*, 2, *F. proliferatum*, 3, *F. graminearum*, 4. *F. equseti*, 5. *G. moniliformis*, 6. *G. fujikuroi*

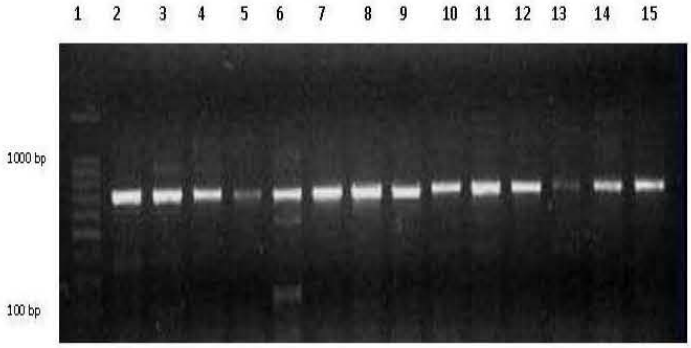

Fig. 2: PCR products of TEF of *Fusarium sps.*

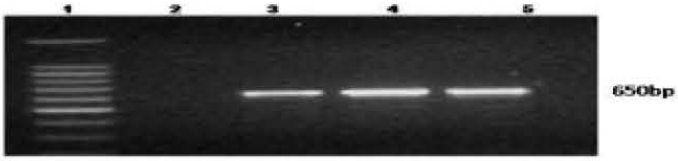

Fig. 3: PCR amplification of the ITS region from *Aspergillus sps*. Species
Lane 1-1kb ladder, lane 2,positive control; Lane 3,4,5-ITS amplification region

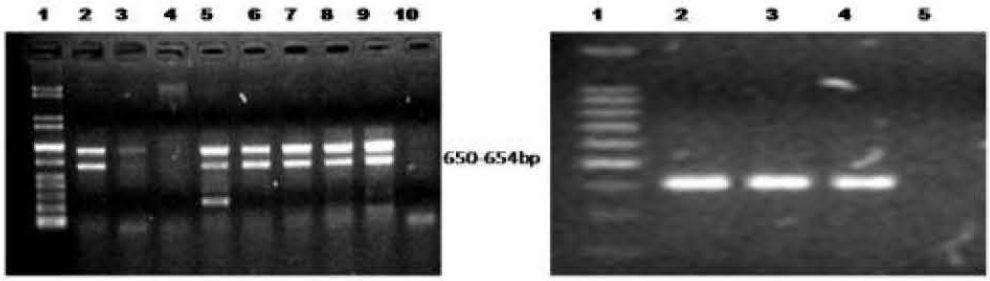

Fig.4a.p450-4 fragments of GA3 Fig.4b Fum 5 genes

Fig. 4: PCR amplification of various genes using extracted DNA
Fig. 4a. PCR amplication of the *cpr* genes of GA3 biosynthetic gene cluster region from Fusarium. Species,
Lane 1-1kb ladder, lane 2- *F. verticillioises;* lane 5- *F. verticillioises*; lane 6- 8*Gibberella fujikuroi*; lane 10-positive control
Fig. 4b. PCR amplication of PKS (Polyketide synthase) region *fum 5* gene from Fusarium sps.
Lane 1-1kb ladder, Lane 2- *F. graminearum*; Lane 3- *F. equseti*; Lane 4- *F. oxysporum*; lane 5positive control

Using the purified DNAs as templates the obtained DNA is quite sufficient to do PCR like trans elongation factor in Fusarium sps. which was presently used in the species discrimination. 5to10ng of Fusarium DNA was used to all 5 Fusarium species mentioned above produced amplicons of 700bp of size of TEF gene (Fig. 2) and sequence analysis yielded efficient way of species discrimination. Similarly ITS 4and5 were used for species determination for *Aspergillus sps* (Fig. 3). Similar results were observed when 5-10ng of DNA of Fusarium species were subjected to PCR analysis with GA3 production involvement genes like gibberellins biosynthetic genes (cpr) and with polyketitde synthase genes (PKS) fum5 with Fusarium sps.(Fig. 4a, 4b)

All the above results indicated t the quality and quantity of DNA obtained are very good and comparable with available methods of extraction. The simple extraction method is cost-effective, employment of major equipments and toxygenic chemicals were minimized and its effective even in less fungal biomass (5mg) used. DNA extraction from *Fusarium* and other species grown in liquid media also yielded about the same amounts of DNA.

DISCUSSION

All the available methods of DNA extraction are time consuming and rather costly *(18-20)* numerous reports have described procedures for the extraction and purification of fungal DNA. Many of these are modifications of the CTAB method originally developed for plant tissue extraction [21] or employ direct sample extraction with organic solvent as the principal means of denaturing and eliminating contaminating protein [22]. DNA extraction form mycorrhizal fungi by Manian *et al.* [23] involves purification through columns. The major challenge for isolation of DNA of good quality and quantity from fungi lies in breaking the rigid cell walls, as they are often resistant to traditional DNA extraction procedures [24]. DNA extraction from filamentous fungi posses difficulties because of its high polysaccharide contents [25,26]. The use of liquid nitrogen in some extraction methods also effective against the breaking the fungal cell wall content, but in our present method the usage of such chemicals were completely eliminated.

The present findings help in the way of rapid extraction of fungal DNA from Fusarium and other species like, *Verticillium* sps. *Aspergillus* and *R.solani.* Though the DNA extraction of Fusarium is said to be simple but the available methods are too laborious and more time consuming but our present method proves that the total time taken to extract any fungal DNA must be within one hour. Since the *Aspergillus sps.* are said to be more sporulating the extraction of DNA from fungal spores are very complicated but our lysis method proves to be more efficient on sporulating fungus also. The major drawback of CTAB extraction is growing of fungus in liquid media for more than 1 week and involvement of toxigenic chemicals like chloroform, isoproponal and Ethanol etc. in this procedure we completely eliminated the usage of such chemicals.

The previous report about the use of microwave treatment is proved to be efficient over *M. grisea* fungus only [13]. In our present method, we obtained good results over other fungus also that too addition of two more steps like lysis buffer treatment and hot water treatment favours better extraction. The efficient extraction limits were observed with fungal biomass of less than 5mg of mycelium. The method described herein is efficient to prepare DNA from the filamentous fungus resulting in DNA sufficiently pure for PCR analyses. The extracted DNA was high enough to perform all kind of PCR-based reactions and also can be used for other DNA based techniques like Southern blot, Northern blot, DNA library construction etc.,

ACKNOWLEDGEMENTS

The work was supported by the Italian Ministry for Environment Territory and Sea (Frame work programme "Sustainable Agriculture"). Under mutual collaboration agreement between University of Madras, India and Italian Ministry for University and Research and by the Compagnia di San Paolo, Turin.

REFERENCES

1. Ashktorab, H. and R.J. Cohen, 1992. Facile isolation of genomic DNA from filamentous fungi. Biotechnique., 13: 198-200.
2. O'Donnel, K., H.I. Nirenberg, T. Aoki and E. Cigelnik, 2000. A multigene phylogeny of the *Gibberella fujikuroi* species complex: Detection of additional phylogenetically distinct species. Mycosci., 41: 61-78.
3. Sreenivasa, M.Y., R.S. Dass, A.P. Charith Raj and G.R. Janardhana, 2006. Molecular detection of fumonisin-producing *Fusarium* species of freshly harvested maize kernels using Polymerase Chain Reaction (PCR). Taiwania., 51: 1-10.

4. Laday M. and A. Szecsi, 2001. Distinct electrophotetic isozyme profies of *Fusarium grminearum* nd closely related species. Systematic and Appl. Microbiol., 24: 67-75.
5. Leach J., D.B. Finkelstein and J.A. Ramboek, 1986. Rapid miniprep of DNA from filamentous fungi. Fun. Gen. Newslet., 33: 32-33.
6. Chihlar, R.L. and P.S. Sypherd, 1980. The organisation of the ribosomal RNA genes in the fungus *Mucor racemosus*. Nucl Acids Res., 8: 793-804.
7. Cubeta, M.A., E. Echandi, T. Abernethy and R. Vilgalys, 1991. Characterization of anastomosis groups of binucleate *Rhizoctonia* species using restriction analysis of an amplified ribosomal RNA. Phytopathol., 81: 1395-1400.
8. Garber, R.C and O.C. Yoder, 1983. Isolation of DNA from filamentous fungi and separation into nuclear, mitochondrial, ribosomal and plasmid components. Anal Biochem., 135: 416-422.
9. Raeder, U. and P. Broda, 1986. Rapid preparation of DNA from filamentous fungi. Appl. Microbiol., 1: 17-20.
10. Moller, E.M., G. Bahnweg, H. Sandermann and H.H. Geiger, 1992. A simple and efficient protocol for isolation of high molecular weight DANN from filamentous fungi, fruit bodies and infected plant tissue. Nucleic. Acid. Res., 20: 6115-6116.
11. Hultner, M.L. and J.E. Cleaver, 1994. A bacterial plasmid DNA mini-prep using microwave lysis. Biotechnique., 16: 990-992.
12. Fujikawa, H.H., H. Ushioda and Y. Kudo, 1992. Kinetics of *Escherichia coli* destruction by microwave irradiation. Appl Environ Microbiol., 58: 920-924.
13. Tendulkar, S.R., B. Archna Gupta and B.B. Bharat Chattoo, 2003. A simple protocol for isolation of fungal DNA. Biotech Let., 25: 1941-1944.
14. Goodwin, D.C. and S.B. Lee, 1993. Rapid, microwave mini-prep of total genomic DNA from fungi, plants, protists and animals for PCR. Biotechnique., 15: 438-444.
15. O'Donnell, K., H.C. Kistler, E. Cigelnik and R.C. Ploetz, 1998. Multiple evolutionary origins of the fungus causing Panama disease of banana: Concordant evidence from nuclear and mitochondrial gene genealogies. Proc of the National Aca. of Sci. of the USA., 95: 2044-2049.
16. Baird, R., H.K, Abbas, G. Windham, P. Williams, S. Baird, P. Ma, R. Kelley, L. Hawkins and M. Scruggs, 2008. Identification of Select Fumonisin Forming *Fusarium* Species Using PCR Applications of the Polyketide Synthase Gene and its Relationship to Fumonisin Production *in vitro*. Int. J. Mol. Sci., 9: 554-570.
17. Geiser, D.M., S. Kang, I. Makalowska, N. Veeraraghavan, T.J. Ward, N. Zhang, G.A. Kuldau, K and O'Donnell, 2004. FUSARIUM-ID v. 1.0: A DNA sequence database for identifying Fusarium. Eur. J. of Pl. Pathol., 110: 473-479.
18. Wilson, K., 1990. Preparation of genomic DNA from bacteria. In: Current protocols in molecular biology (Ausubel FM and Brent R, eds.) Greene Publ. Assoc. and Wiley Interscience, New York., pp: 241-245.
19. Syn, C.K. and S. Swarup, 2000. A scalable protocol for the isolation of large-sized genomic DNA within an hour from several bacteria. Anal Biochem., 278: 86-90.
20. Sambrook, J. and D.W. Russel, 2001. Rapid isolation of yeast DNA. In: Molecular cloning, a laboratory manual (Sambrook J and Russel DW, eds.). Cold Spring Harbor Laboratory, New York., pp: 631-632.
21. Saghai-Maroof., 1984. Ribosomal DNA spacer-length polymorphisim in barley. Proc. Natl Acad Sci USA., 81: 8014-8018.
22. Blin and Stafford., 1976. A general method for isolation of high molecular weight DNA from eukaryotes. Nucl. Acids. Res., 3: 2303-2308.
23. Manian, S., S. Sreenivasaprasad and P.R. Mills, 2001. DNA extraction method for PCR in mycorrhizal fungi. Let. Appl. Microbiol., 33: 307-310.
24. Fredricks, D.N., C. Smith and A. Meier, 2005. Comparison of six DNA extraction methods for recovery of fungal DNA as assessed by quantitative PCR. J. Clin. Microbiol., 43: 5122-5128.
25. Zhang, D., Y. Yang, L.A. Castlebury and C.E. Cerniglia, 1996. A method for the large scale isolation of high transformation efficiency fungal genomic DNA. FEMS Microbiol Lett., 145: 261-265.
26. Muller, F.M., K.E. Werner, M. Kasai and A. Francesconi, 1998. Rapid extraction of genomic DNA from medically important yeasts and filamentous fungi by high-speed cell disruption. J. Clin. Microbiol., 36: 1625-1629.