INTERNATIONAL JOURNAL OF SCIENCE AND NATURE
© 2004 - 2011 Society for Science and Nature (SFSN). All rights reserved
www.scienceandnature.org

ECOLOGY, CHEMICAL DIVERSITY AND MOLECULAR PROFILING OF *ASPERGILLUS SPECIES* FROM PAPER AND PULP POLLUTED SOIL

[1]Usha.V., [1]Vijayalakshmi.S., [1]Riyaz Ahmad Rather and [2]Rajagopal. K.
[1]Research Scholar, Dept. Of Biotechnology, VELS University, India.
[2]Professor & Head, Dept. Of Biotechnology, VELS University, India.

ABSTRACT
Most of the industries dump their liquid waste in streams and river that the introduction of contaminants through pulp and paper mill effluent is one of the major environmental bugbears in India, contributing to soil and water pollution. The present study was aimed in exploiting the diversity of fungi inhabiting the polluted and non – polluted sites near Pugalur paper mill, Karur district, Tamilnadu, India. The results revealed the abundant stress tolerance by *Aspergillus sp.* with their high density populations in the polluted soil. Owing to the physico – chemical analysis of the soil, the higher electrical conductivity and alkaline nature could interfere in stress adaptations of the fungi for survival. Also the chemical analysis inferred the qualitative presence of phenolics and terpenoids among the isolates of *Aspergillus sp., (A. fumigatus, A.niger and A.terreus)* from the polluted site. Enzyme screening via plate assay methods also aided in inferring the production of laccase, amylase, cellulase, protease and tyrosinase respectively. RFLP analysis of the DNA and SDS – PAGE analysis of the total cell protein revealed polymorphic patterns and diverse molecular profiles of *Aspergillus* isolates from the different ecologies that insight into molecular studies of stress tolerant genes and proteins impacted by the pollutants being realized in the affected site.

KEYWORDS: ***Aspergillus*,** Protein profile, RFLP, Tyrosinase.

INTRODUCTION

Fungi thrive in environment shunned by higher organisms. Fungi can exploit marginal living conditions in large part because they produce unusual enzymes capable of performing chemically difficult reaction that some of these enzymes have already been harnessed in pulp and paper processing (biopulping and biobleaching), and in the synthesis of fine chemicals (biocatalysis), etc., (5). Reports of fungal enzyme have been started in the 19th century (1, 13). Few important enzymes concentrated in this study includes: Laccase, Cellulase, Amylase, Protease and Tyrosinase being employed in production of sugar syrups, juices, etc., and also in starch removal from fabrics; in azo dye reduction, major strategy concerned in removal of dye pollutants from paper and textile industries, etc., (10).

Environment has known to play a very important role in morphological, structural, in turn in their gene expression characters of mycobiota that restriction patterns and their polymorphisms could well reveal the molecular insights into their gene characterization. For the purpose of characterization and classification, representative strains of the same fungal species were subjected to the DNA fingerprinting technique (RFLP). Further, PAGE analysis of proteins aids in analyzing for a comparative study in the production of proteins concerning their adaptation and survival to different environments.

Also, the physico – chemical parameters of the soil ecology could impact on the persistence of pollutants that might infer the production of various stress metabolites of fungi being revealed by chemical analysis. Although fungi are known to grow in different environments and produce various compounds including enzymes, scant attention has been given to isolation of filamentous fungi from paper and pulp effluent polluted soil.

Hence, the present study is aimed to determine the distribution of fungus in paper and pulps polluted soil and exploit their potential regarding enzyme and other chemical metabolite production under stressed environment. Also, molecular profiling via RFLP analysis of genomic DNA and SDS – PAGE protein profile of the identical fungal isolates from different environments, aids in gene and protein characterization which also might insight into stress tolerant phenomena studies that can thence forth be put upon in future.

MATERIALS AND METHODS

Soil samples were collected from the polluted sites near Pugalur paper mill, Karur district, Tamilnadu. Non – polluted (Garden) soil samples were also collected. The surface deposit was removed to a depth of 15cm and the exposed soil was collected to a depth of 10 cm. The samples were stored in thin- walled polythene bags in a refrigerator and analyzed for their physico – chemical parameters with in 48 hours of collection.

Media and glass wares including petridish were sterilized in an autoclave at 121°C for 15 minutes. Analytical grade chemicals (Sigma – Aldrich, India) were used for the preparation of both liquid and solid media. Two methods were used for the isolation of filamentous fungi from soil (Soil dilution and War cup method) (6).

In soil dilution method, 1 gm of soil was taken with 10ml of sterile distilled water and from this serial dilutions were made. 1ml of the final dilution (10^{-4}) was pipetted out on Czapek Dox Agar (CDA) media amended with an antibiotic, chloramphenicol (12 mg/100 ml media). The plates were incubated at 30°C for 7 days.

In war cup method, 1gm of soil sample was spread in the bottom of the sterile petriplate to which molten and cooled

CDA medium was poured over the soil and incubated at 30°C for 7 days.
Fungi that appeared on agar plates were then isolated and maintained as pure cultures on an agar slant. The fungi were identified using Lactophenol cotton blue stain using the keys provided by Barnett & Hunter, 1972 (2). The density and percentage of frequency was calculated using the following formula:

Distribution of Fungi:

$$\text{Density} = \frac{\text{Total number of colonies of species in all plates}}{\text{Total number of plates}}$$

$$\text{Percentage frequency} = \frac{\text{Total number of plates in which occurs}}{\text{Total number of plates.}} \times 100$$

The inoculum was raised using solid state fermentation in Erlenmeyer flask containing 5gm of carbon source (mixture of wheat bran and paddy straw in the ratio of 2:6) with 20ml of basal salt solution and trace elements (NH_4NO_3, KH_2PO_4, K_2HPO_4 $CaCl_2.2H_2O$, $MgSO_4.7H_2O$, $FeSO_4.7H_2O$, $MnSO_4.H_2O$). The pH of the medium was 6, being adjusted using 1N HCL and 1M NaOH. The inoculum was maintained in the media for 48 hours at 27°C.

Fungus grown in solid state fermentation were transferred to the petridishes - basic (90mm diameter) containing the basal medium. The mycelial discs (around 5 mm diameter) were taken from the growing edge of colony on the basal medium. Tests were conducted for screening at 27°C. Production of extra cellular enzyme was determined semi quantitively by incorporating respective substrates into the medium or by addition of substrates or reagents after a specified period of growth (11). The appearance of coloured or clear zones was recorded in arbitrary units.

From the fungal cultures – *Aspergillus species* were selected; broth cultures were obtained in Potato dextrose broth (incubated for 2 weeks at 26^{0}C at 150 rpm). The culture filtrate was filtered, extracted using ethyl acetate and the crude extract was analysed for various chemical metabolites (4). The fungal matt was subjected for DNA and Protein extraction procedures. Four different techniques were adopted for the extraction of DNA from *Aspergillus* fungi (3, 12, 14, 17). Protein was extracted via Phosphate buffer saline method. The DNA content were quantified and subjected for RFLP analysis – using Bam HI enzyme while protein was characterized via SDS – PAGE profiling (9).

RESULTS AND DISCUSSION

Physical and chemical parameters of the polluted and garden soil samples were analysed that revealed an alkaline pH and higher levels of EC, organic carbon, phosphorous, potassium, calcium in polluted soil (Tab.1).

TABLE 1: Physico –Cchemical analysis of soil sample

S.No.	Parameters studied	Results	
		Polluted soil	Garden soil
1.	pH	7.2	6.7
2.	Electrical conductance	1.41 mmhos/cm	0.23mmhos/cm
3.	Moisture content	38%	9.3%
4.	Available nitrogen as N_2	0.06%	0.04%
5.	Organic carbon	3.1%	0.40%
6.	Calcium	7400 µg/g	2900 µg/g
7.	Magnesium	800 µg/g	600 µg/g
8.	Phosphorous	182 µg/g	138 µg/g
9.	Potassium	70 µg/g	42 µg/g
10.	Bulk density	0.2% g/cm^2	0.8% g/cm^2
11.	Total alkalinity	3.4 meq/100g	1.9 meq/100g

The polluted soil and garden soil samples were screened for the presence of filamentous fungi by Warcup and Soil dilution methods after 7days of incubation at 30±1°C. Soil sample harbored some fungal species (Fig. 1 & 2) that most of them were the species of *Aspergillus.*

Among the Aspergillus genus, *A. niger, A. fumigatus and A. terreus* were the most abundant fungi. Other three filamentous fungi isolated from the soil were *Penicillium sp, Curvularia lunata* and *Fusarium oxysporum.* Garden soil possessed more fungi than the polluted soil (Tab. 2).

The percentage frequency and density were calculated for fungal isolates that revealed *A .niger* and *A. fumigatus* were higher in both the soil samples. However, the density and percentage frequency of *F. oxysporum.,* was least in garden soil. Fungi produce extracellular enzymes such as cellulase, protease, amylase, pectinase, etc., which enable them to survive in different environments (8). In the present study, we screened the potency of *A. fumigatus*, *A. niger* and *A. terreus* isolated from the two different soils for the production of five different extra cellular enzymes. *A. fumigatus* and *A. terreus* isolated from the two different soils and *A. niger* from the garden soil produced all the five enzymes tested in this study (Tab. 3), (Fig. 3) where as *A. niger* isolate from polluted soil produced only 4 enzymes except tyrosinase (Tab. 3).

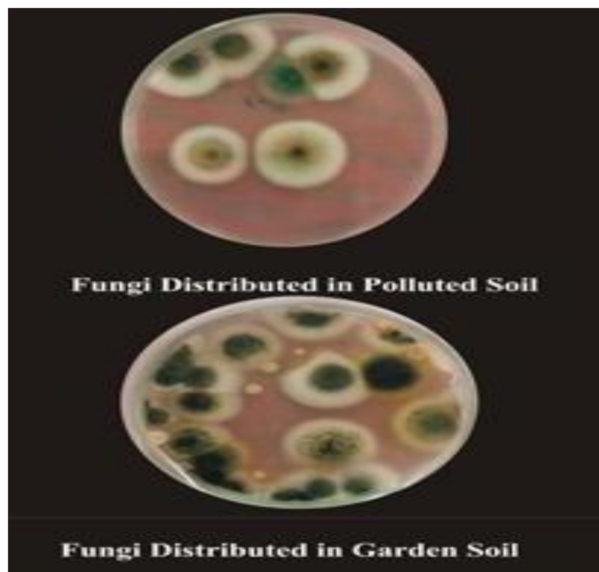

FIG. 1 Distribution of Fungi from the Soil samples

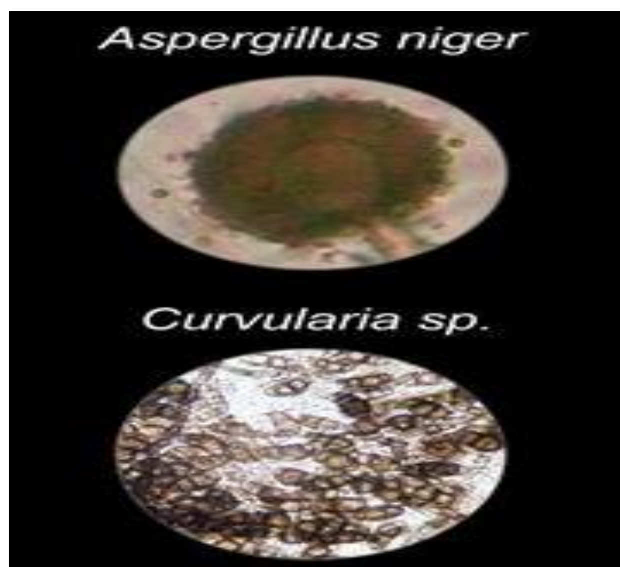

FIG. 2 Fungi isolated from the soil samples

TABLE 2: Occurrence of fungi in polluted and garden soil

S.NO	Name of fungus	% Frequency		DENSITY	
		Polluted soil	Garden soil	Polluted soil	Garden soil
1.	*Aspergillus fumigatus*	100%	100%	5.6	7.2
2.	*Aspergillus niger*	100%	100%	4.5	6.2
3.	*Aspergillus terreus*	100%	100%	3.2	4.5
4.	*Penicillium sp.*	_	100%	_	4.2
5.	*Curvularia lunata.*	_	80%	_	3.0
6.	*Fusarium oxysporum.*	_	80%	_	2.6

TABLE 3: Extracellular enzyme production by *A. fumigatus, A. terreus*, & *A. niger* isolated from polluted and garden soil

S.no.	Enzyme	*Aspergillus fumigatus*		*Aspergillus niger*		*Aspergillus terreus*	
		Polluted soil	Garden soil	Polluted soil	Garden soil	Polluted soil	Garden soil
1.	Amylase	+	+	+	+	+	+
2.	Protease	+	+	+	+	+	+
3.	Laccase	+	+	+	+	+	+
4.	Cellulase	+	+	+	+	+	+
5.	Tyrosinase	+	+	_	+	+	+

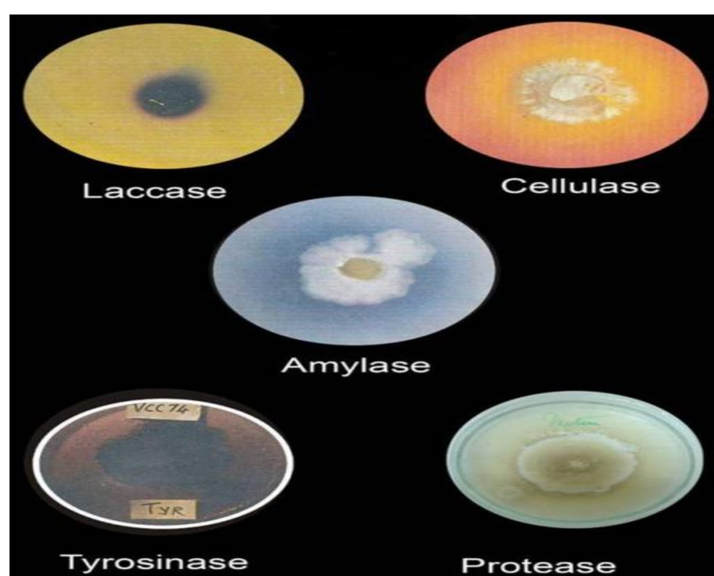

FIG. 3 Screening of fungi for Enzyme production

Although fungi are known for their ability to tolerate and grow in different environment there have been limited reports of fungi from paper and pulp effluent polluted soil. Our study shows that filamentous fungi were although limited in number in polluted soil, they could survive in such environment. *Aspergillus* was the most dominant genus. Among Aspergillus genus, *A. fumigatus* was found to be the denser and dominant species (Tab.2) suggesting that this fungus tolerated well the polluted conditions. These results are in consonance with that of Tresner & Hayes (16) and Suryanarayanan *et al.*(15).

Colonization of an environment by fungi is dependent on successful utilization of the substrate which can only be achieved by the production of various types of enzymes by a fungus. *A. fumigatus, A. terreus* and *A. niger* isolated from paper and pulp effluent polluted soil and garden soil were studied for the production of five different enzymes. All the isolates were found to produce a spectrum of enzymes.

Chemical diversity in an organism can be a chemotaxonomic characteristic of a particular genus or species. The quality of chemical compounds (secondary metabolites) produced may vary dramatically among isolates which are growing in different environments. The ethyl acetate extract was subjected to different chemical analysis that showed the presence of carbohydrates,alkaloids, and proteins in all the isolates while phenolics and terpenoids were detected only in isolates from polluted soil (Tab. 4).

TABLE 4: Chemical analysis of *Aspergillus fumigatus, Aspergillus terreus & Aspergillus niger* isolated from polluted and garden soil

S.no.	Chemical group & test	*Aspergillus fumigatus*		*Aspergillus niger*		*Aspergillus terreus*	
		Polluted soil	Garden soil	Polluted soil	Garden soil	Polluted soil	Garden soil
1.	Alkaloids (Dragendorff's test)	+	+	+	+	+	+
2.	Carbohydrates (Molisch's test)	+	+	+	+	+	+
3.	Steroids (Lieberman Burchand test)	_	_	+	_	_	_
4.	Terpenoids (Lieberman Burchand test)	+	_	+	_	+	_
5.	Tannins (Ferric chloride test)	+	+	_	+	+	+
6.	Proteins (Ninhydrin test)	+	+	+	+	+	+
7.	Phenolic compounds (Ferric chloride test)	_	_	+	_	+	_

Steroids were present only in *A. niger* from polluted soil. Hence considerable variances in chemical profile could be observed indicative of stress metabolites induced by pollutants, can also be applied in chemotaxonomy.

The DNA extracted from six different fungus were restricted with enzyme Bam H1 and the results showed that *A. niger* and *A. terreus* isolated from polluted soil had similar band pattern (Fig. 4 & 5) and no restricted band pattern for *A. fumigatus* from polluted soil. Also, *A. niger* isolated from garden soil showed similar band pattern as *A. niger* of polluted soil. However band pattern of other species from the two different environments showed variations.

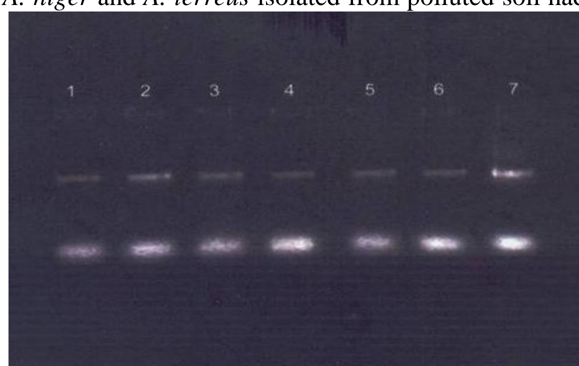

FIGURE 4. Agarose Gel Electrophoresis of DNA of *Aspergillus species* isolated from two different environment

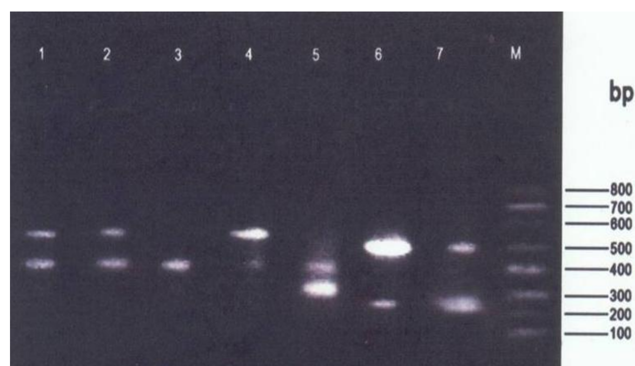

FIGURE 5. Bam HI - RFLP Analysis of DNA of *Aspergillus species* isolated from two different environments.

(Lane 1: Aspergillus niger@; Lane 2: Aspergillus terreus @;(Lane1: Aspergillus niger@; Lane 2: Aspergillus terreus@
Lane 3: Aspergillus fumigatus@; Lane 4: Aspergillus niger# Lane3: Aspergillus fumigatus@;Lane 4: Aspergillus niger #
Lane 5: Aspergillus terreus #; Lane 6: Aspergillus fumigatus#;Lane5: Aspergillus terreus#;Lane6: Aspergillus fumigatus #
Lane 7: Yeast chromosomal DNA.Lane 7: Yeast chromosomal DNA. @ - polluted soil isolate ; # - garden soil isolate) @ - polluted soil isolate ; # - garden soil isolate)

Concerning the SDS – PAGE protein profile, all the six *Aspergillus* species produced two similar proteins near 66KD. None of the isolates produced protein above 97.4KD (Fig. 6). *A. terreus* & *A. fumigatus* produced a protein with 97.4 KD is very prominent as the bands are very thick. Also, the production of 43 KD protein by the polluted soil isolates can be observed. Significant difference in the protein pattern of these two isolates of different environment could be observed upon comparison (Fig. 6). These results show that these organisms produced different types of proteins for their survival.

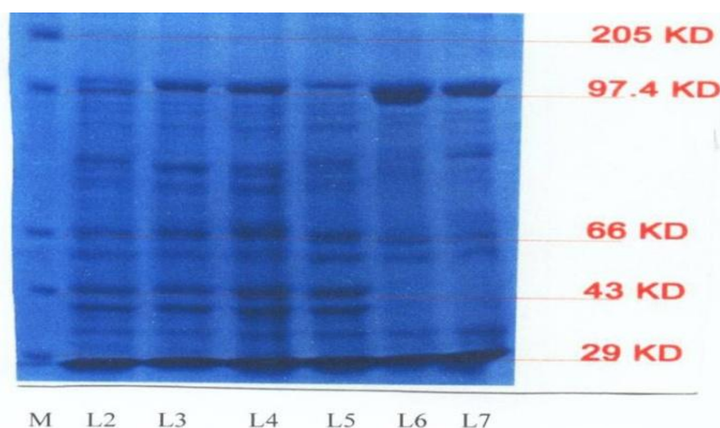

Fig. 6. Protein profile of Aspergillus species isolated from two different environments
(Lane 1:Marker protein ; Lane 2: Aspergillus niger @; Lane 3: Aspergillus terreus @;Lane 4: Aspergillus fumigatus @; Lane 5: Aspergillus niger # ; Lane 6: Aspergillus terreus #;Lane 7: Aspergillus fumigatus #. @ - polluted soil; # - garden soil)

In the present study the cellulase and protease production by the *Aspergillus sp.,* assumed to be responsible for the degradation of cellulose present in the paper and pulp polluted soil, whereas their production was also seen in garden soil. However, the fungus isolated from same soil differs in their enzyme profile reported by Harvey & McNeil, 2007 (7). Thus it is obvious that *A. fumigatus, A. terreus* and *A. niger* isolated from two different soils have evolved a panoply of adaptations which includes mechanisms for producing different enzymes and chemicals to overcome competition with other soil organisms and surviving harsh environmental conditions.
Hence, our study shows that paper and pulp polluted soil harbors restricted number of fungi perhaps due to the selection pressure and *Aspergillus* is the most dominant fungus in such environment. *A. fumigatus, A. terreus* and *A. niger* elaborate different enzymes in culture. Hence, this kind of organism growing in harsh environments could be exploited for metabolites production including enzymes. Further, variation in the RFLP and protein profile could be seen. Hence, organisms isolated from polluted soil had different molecular pattern than garden soil. Hence environment plays a very vital role in the organism's morphological and molecular characters.

ACKNOWLEDGEMENT
Authors thank the Vel's management for their assistance and facilities aided for the project.

REFERENCES
1. Anustrup, K. (1979) Applied Biochemistry and Bioengineering: Enzyme Technology. Vol. 2. Academic Press, New York, San Francisco, London.
2. Barnett, H.L. and Hunter, B.B. (1972) Illustrated genera of Imperfect fungi, Burgress publishing company, Pp. 241.
3. Chow, T.Y.K and Kafer, E. (1993) A rapid method for isolation of total nucleic acids from *Aspergillus nidulans*. Fungal Genet. Newsl. 40, 25-27.
4. Frisvad, J. C., Anderson, B., and Thrane, U. (2007) The use of secondary metabolite profiling in chemotaxonomy of filamentous fungi. Mycol Res. 112, 2, 231 -40.
5. Fungal Genomics Project, Concordia University, (2005) Enzymes & Industry Applications. Available at: https://fungalgenomics.concordia.ca/home/index.php.
6. Girivasan, K. P., Rajagopal, K., Muruganandam, V., and Suryanarayanan, T. S. (1998) Isolation of fungi from tropical peat of Southern India. Current Science. 74,4, 359 – 362.
7. Harvey, L. M. and McNeil, B. (2007) The effects of bioprocess parameters on extracellular proteases in a recombinant *Aspergillus niger* B1-D. Appl. Microbiol. Biotechnol. 78, 2, 333 - 341.
8. Kaur, J., Chadha, B.S., Kumar, B.A. and Saini, H.S. (2007) Purification and characterization of two endoglucanases from *Melanocarpus* sp. MTCC 3922. Bioresource Technology, 98, 1, 74-81.
9. Laemmli, U. K. (1970) Cleavage of structural protein during the assembly of head of bacteriophage T4. Nature. 227, 680-685.
10. Nakamura, H., Kubota, H. and Kono, T. (2001) Method of deinking waste paper using cellulase without lowering paper strength and method of

evaluating the same. United States Patent 7297224.

11. Pointing, S. B., Parungao, M. M. and Hyde, K. D. (2003) Production of wood-decay enzymes, mass loss and lignin solubilization in wood by tropical *Xylariaceae*. Mycological Research 107, 231-235.

12. Punekar, N.S., Kumar, S. V., Jayashri, T. N. and Anuradha, R. (2003) Isolation of genomic DNA from acetone-dried *Aspergillus* mycelia. Fungal Genet. Newsl. 50, 15 -17.

13. Rajagopal, K. and Suryanarayanan, T. S. (2000) Isolation of endophytic fungi from leaves of neem *(Azadirachta indica* A. Juss.). Current Science 78, 1375-1378.

14. Saghai-Maroof, M.A., Soliman K, M., Jorgensen, R. A. and Allard, R. W. (1984) Ribosomal DNA spacer length polymorphism in barley: Mendelian inheritance, chromosomal location, and population dynamics. Proc. Natl. Acad. Sci. USAs 81, 8014–8018.

15. Suryanarayanan TS, Muruganandam V, Rajamurugan J. and Prakash G. (1991) Biodegradation of petroleum hydrocarbons by filamentous fungi. In Fungi and Biotechnology-Recent Advances (ed.H.C. Dube): pp. 127-135. Today and Tomorrow's Printers and Publishers, New Delhi.

16. Tresner, H. D. and Hayes, J.A. (1971) Sodium Chloride Tolerance of Terrestrial Fungi Appl. Environ Microbiol. 22(2), 210-213.

17. Zhang, Z., Li, R., Li, D., and Wang, D. (1997) Typing of common dermatophytes by Random Amplification of polymorphic DNA Jpn. J. Med. Mycol. 38, 239-246.