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Original Article

Exploration of unique relation among industrial fungi by statistical analysis

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Abstract

This work was carried out to explore the relation among thermophilic cellulolytic fungi, which are of industrial importance. There was no report found about the genetic relationship of fungi, which are used to produce industrial enzymes. So the aim of the study was to observe the similarity among different cellulolytic fungi on genetic level, which will provide the background to understand the correlation among cellulase producing systems of these fungi. Eleven (11) fungi were studied for genetic diversity using the Random Amplified Polymorphic DNA (RAPD) a PCR based molecular marker system. In this regard twenty universal decamers used for RAPD resulted in 1527 numbers of bands observed during comparison of all wild strains. Maximum polymorphism was generated with GLA-07. Average numbers of bands per 20 primers were 65-72. An Interesting feature of the study was the similarity of *Humicola insolens* with *Torula thermophile,* more than with the other members of the *Humicola* family. This genetic pattern affects the physical structure of the fungi. Spores of *Torula thermophila* are more related to *Humicola insolens* than to its own family. Similarity between the two was found to be 57.8%, whereas between *Humicola lanuginosa* (*Thermomysis lanuginosus*) and *Humicola grisea* it was 57.3%. Apart from this, similarity between *Talaromyces dupontii* and *Rhizomucor pusillus* was 51.5%. Least similarity was found in *Rhizomucor pusillus* and *Humicola grisea,* which was 18.7% and *Chaetomium thermophile* and *Sporotrichum thermophile,* which was 18.3%. Genetic similarity matrix was constructed on the basis of Nei and Li's index.

Keywords: genetic diversity, cellulolytic fungi, DNA fingerprinting, RAPD

1. Introduction

The fungi constitute a most fascinating group of organisms exhibiting great diversity in form, structure, habit, life history and mode of nutritional and mycelial tropic stage, which adequately distinguish the fungi as separate kingdom (Hawksworth *et al.*, 1983). Microbial sources such as fungi are well recognized to produce a wide variety of chemical structures, several of which are most valuable pharmaceuticals, agrochemicals and industrial products such as enzymes. The world of fungi provides a fascinating and almost endless source of biological diversity, which is a rich source for exploitation. Fungi interact with their hosts, and also with abiotic variables in the environment (Manoharachary *et al.*, 2005).

Thermophilic fungi are a small assemblage in mycota that have a minimum temperature of growth at or above 20°C and a maximum temperature of growth extending up to 60 to 62°C. As the only representatives of eukaryotic organisms that can grow at temperatures above 45°C, the thermophilic fungi are valuable experimental systems for investigation of mechanisms that allow growth at moderately high temperature yet limit their growth beyond 60 to 62°C (Cooney and Emerson, 1964).

The properties of their enzymes show differences not only among species but also among strains of the same species. Genes of thermophilic fungi encoding lipase, protease, xylanase, and cellulase have been cloned and over expressed in heterologous fungi, and pure crystalline proteins have been obtained for elucidation of the mechanisms of their

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intrinsic thermostability and catalysis (Maheshwari *et al.*, 2000).

The current interest in the production of energy from biomass has provided fresh impetus for research in the area of cellulose degradation (Wang *et al.*, 1978). The cellulases from thermophiles with the ability to operate at temperatures of 55°C and higher offer the advantages of an increased rate of reaction and a stable enzyme system (Bellamy, 1977; Rosenberg, 1975).

Actually in some cases, the use of enzymes of mesophilic organisms can be a disadvantage, once they generally undergo denaturation in temperature higher than 55°C, resulting in low efficiency of the system. The employment of thermo stable enzyme to carry the processes is advantageous because it contributes to increase technical and economical viability of the process (Gomes *et al.,* 1994).

Genetic diversity in populations of a fungus some time causes major problems. Molecular methods offer a more rapid, more sensitive and more certain means of detecting differences within populations than is often possible when identification is based on morphological or physiological characteristics (Weber *et al.*, 2005).

Within the last decade, technological advancement has increasingly supported the use of genetics in determining population diversity. A large number of methods are available for the assessment of genetic variability, diversity and relatedness among germplasms as well as for molecular fingerprinting. Morphological and biochemical markers (protein-based techniques) are influenced by the environment, but DNA based techniques represent reliable tools and obviate many of the standard problems associated with other techniques. They allow also a high throughput of material for DNA typing (Miqdadi *et al.*, 2006).

Similar colonial morphologies of many isolates can cause problems in laboratories where the fungus is being studied as contamination of a culture. For these reasons it would be useful to have a quick and easy means of distinguishing between fungal isolates that are morphologically identical. Since all other methods are relatively labor intensive and time consuming and none of them distinguish between all of the isolates tested other than RAPD markers (Agnes *et al.*, 1992).

The ease and simplicity of the RAPD technique make it ideal for genetic mapping and DNA fingerprinting, with particular utility in the field of population genetics. In many instances, only a small number of primers is necessary to identify polymorphism among species (Williams *et al.*, 1990).

Random amplified polymorphic DNA (RAPD) is a truly multiplex PCR based molecular marker system (Williams *et al.*, 1990; Welsh and McClelland, 1990). RAPD is a reliable method when performed with practice and care, moreover, its repeatability or reproducibility is actually high (Hedrick, 1992). It has been used extensively for genetic studies, for example, analysis of genetic variation in bacteria, fungi and plants (Wang *et al.*, 1993; Bidochka *et al*., 1994; Mailer *et al*., 1994) and construction of the first linkage maps for certain plant species (Lodhi *et al.*, 1995; Yang and Quiros, 1995).

Analysis of randomly amplified polymorphic DNA (RAPD) is widely used to investigate variability among microorganisms (Novo *et al.*, 1996). This technique is fast, cheap and easy to perform, and requires only small amounts of DNA that is available even from dried materials and does not require any previous sequence information (Simpson *et al.*, 2002).

There are no reports found on phylogenetic evaluation of thermophilic cellulolytic fungi at molecular level. This work is an effort to study the genetic diversity among various cellulolytic thermophilic fungi of Pakistan origin (Latif *et al.*, 1995), by using the Randomly amplified polymorphic DNA (RAPD) technique.

In this regard, evaluation of band pattern of various thermophilic fungi was carried out by comparing the RAPD profile on the basis of the presence or absence of each band. A similarity matrix was generated by using Nei and Li's coefficient of similarity and a dendrogram was generated by using Un-weighted Pair Group Method of Arithmetic Means (UPGMA) (Nei and Li, 1997)

2. Materials and Methods

2.1 Selection of thermophilic fungi

Eleven (11) cellulase producing fungi were purified for the study. The fungal strains used are shown in Table 1.

2.2 Culture purification and maintenance

The cultures were purified by using plates of Eggin's and Pugh (1962) media using cellulose as carbon source. The plates were cultured at 45°C. Wherever required, the dilution plate method was used along with antibiotics to remove the contaminating fungi and bacteria. The pure cultures of thermophilic cellulolytic fungi were screened out by culturing again and again on media containing cellulose as carbon source. Purification was confirmed by microscope (Zeiss, Germany).

Table 1. Thermophilic cellulolytic fungal strains.

Number	Fungi	Abbreviations
1	*Humicola lanuginosa*	H.l
2	*Humicola insolens*	H.i
3	*Humicola grisea*	H.g
4	*Malbranchia pulchella*	M.p
5	*Torula thermophila*	T.t
6	*Talaromyces dupontii*	T.d
7	*Sporotricum thermophile*	S.t
8	*Rhizomucor pusillus*	R.p
9	*Paecilomyces thermophila*	P.t
10	*Aspergillus fumigatus*	A.f
11	*Chaetomium thermophile*	C.t

All the cultures were finally maintained on Potato dextrose agar (PDA) medium. Pure cultures of fungi were inoculated in Eggin's and Pugh media to gain a thick mass of fungal mycelium for DNA isolation.

2.3 DNA isolation

The protocol for fungal DNA isolation reported by Cenis (1992) was used with some modifications accordingly to achieve best results. An amount of 0.3 g of fungal mat (on dry mass basis) was taken, chopped and ground with the help of pestle mortal in liquid nitrogen. 5mL of extraction buffer (200 mM Tris HCl of pH 8.5, 250 mM NaCl, 25 mM EDTA, 0.5% SDS) and 2.5 mL of 3M sodium acetate of pH 5.2 was added (for 0.3 g pad) in a 50 mL centrifuge tube and gently mixed to wet the entire powdered pad. The mixture was placed in refrigerator at -20°C temperature for 20 min. It was centrifuged in tabletop centrifuge for 10 min. at 8000 rpm. Supernatant was transferred to a new tube and an equal volume of isopropanol was added. High molecular weight DNA spooled out on gentle mixing and was further incubated at room temperature for 5 min. It was again centrifuged in tabletop centrifuge to obtain the DNA pellet. After discarding the supernatant the DNA pellet was washed with 70% ethanol thrice and then with absolute alcohol to remove salts and water. The pellet was dried and redissolved in 500 µl double distilled, deionized, autoclaved water. RNAse was used to remove RNA from the DNA. Phenol treatment was necessitated to remove RNAse. DNA obtained by this method was used in RAPD-PCR.

2.4 Optimizations for PCR

Different concentrations of $MgCl_2$ (3 to 6 µl of 25 mM $MgCl_2$), template DNA (0.25 to 5.0 µl) and primer (0.5 and 1 µl of 1 µg/µl) were used for optimization of PCR amplifications. Selected concentrations of each were further used in PCR.

2.5 Polymerase Chain Reaction (PCR)

RAPD-PCR reaction was carried out using 20 universal "Gene Link" primers from which 10 (GLA-01 to GLA-10) belonged to A-series and the other 10 (GLB-01 to GLB-10) to B-series of primers (Table 2). Each PCR reaction mixture (50 µl) contained 0.25 µl Template DNA, 1.00 µl (1µg/µl) Primer, 5.00 µl $MgCl_2$, 5.00 µl 10 x PCR buffer, 1.00 µl dNTPs (0.25ul each), 0.50 µl Taq polymerase, 2.50 µl Gelatin, 34.75 µl ddd water. PCR reactions were performed according to Williams *et al*. (1993). Amplifications were carried out in a thermocycler (MJ Mini-BioRad) set on the following parameters: 5 min of denaturation at 95°C followed by 40 cycles of 1 min at 94°C, 1 min at 36°C, 2 min at 72°C followed by 10 min at 72°C of final extension. The RAPD products after PCR were separated by electrophoresis on 1.5% agarose gel in 1X TAE (Tris acetic acid EDTA) buffer using ethedium bromide staining and visualized under UV light.

2.6 Statistical analysis

Nei and Li's coefficient (Nei and Li, 1979) was used to calculate the relatedness of the studied species among each other. Nei and Li's co-efficient was calculated by the following statistical equation

$$F = 2Nxy/(Nx + Ny)$$

Where, F is the similarity coefficient, Nx and Ny are the numbers of fragments from population x and y respectively, Nxy is the number of fragments shared by the two populations.

Data was analyzed by comparing the RAPD profile on the basis of the presence or absence (1 or 0 respectively) of each reproducible DNA band. A similarity matrix was generated using Nei and Li's coefficient of similarity and a dendrogram was generated using Unweighted Pair Group Method of Arithmetic Means (UPGMA) (Nei and Li, 1979).

Table 2. List of primers used for the study.

No.	Oligo's name	Sequence 5′-3	No.	Oligo's name	Sequence 5′-3′
1	GLA-01	CAGGCCCTTC	11	GLB-01	GTTTCGCTCC
2	GLA-02	TGCCGAGCTG	12	GLB-02	TGATCCCTGG
3	GLA-03	AGTCAGCCAC	13	GLB-03	CATCCCCCTG
4	GLA-04	AATCGGGCTG	14	GLB-04	GGACTGGAGT
5	GLA-05	AGGGGTCTTG	15	GLB-05	TGCGCCCTTC
6	GLA-06	GGTCCCTGAC	16	GLB-06	TGCTCTGCCC
7	GLA-07	GAAACGGGTG	17	GLB-07	GGTGACGCAG
8	GLA-08	GTGACGTAGG	18	GLB-08	GTCCACACGG
9	GLA-09	GGGTAACGCC	19	GLB-09	TGGGGGACTC
10	GLA-10	GTGATCGCAG	20	GLB-10	CTGCTGGGAC

3. Results and Discussion

Analysis of randomly amplified polymorphic DNA (RAPD) is widely used to investigate variability among microorganisms (Novo *et al.*, 1996). This technique is fast, cheap and easy to perform, and requires only small amounts of DNA that is available even from dried materials and does not require any previous sequence information (Simpson *et al.*, 2002).

Bhat and Maheshwari (1987) conducted a comparative study of the cellulase system of various strains of *Sporotrichum thermophile* and showed appreciable differences between them either in thei growth pattern on various media or in the production of cellulolytic enzymes. All strains tested were able to consume the cellulose substrate in liquid cultures at 50°C in 6 days but the cellulolytic activities of culture filtrates were quite variable. But no comparison on DNA level was given in these studies.

The present study aimed to compare the cellulolytic fungi on genetic level. The DNAs isolated from eleven fungal strains were found to be of high molecular weight as determined by agarose gel electrophoresis. These DNAs were used for the RAPD-PCR. RAPD technique was applied to determine the genetic diversity among thermophilic cellulolytic fungi. For this study a total of 20 primers was used, 10 from GLA (Gene Link) series and 10 from GLB series (Table 2). Variation in band profile was observed with each primer when used for PCR amplification of the genomic DNA of eleven different fungi and bands with sufficient intensity were scored.

RAPD conditions were optimized to get clear and good amplification by varying concentrations of Mg^{2+} ions, primers and DNA. The alterations in the different parameters such as template DNA, concentration of $MgCl_2$, and primer concentration tested had varying degrees of influence on the RAPD patterns and its reproducibility. It was observed that by increasing amount of DNA the number of bands in PCR reaction decreased. In the current report we are presenting only the results with optimized concentrations of Mg^{2+}, primers and template DNA.

Demeke and Adams (1992) reported that DNA concentration is particularly important when large numbers of samples have to be analyzed. In fact the polysaccharides, which are common contaminants in DNA preparations, are unlikely to affect the PCR reactions.

Magnesium is an essential component of PCR reactions and affects the quality of RAPD profiles obtained (Munthaly *et al.*, 1992). It is known to affect primer annealing and template denaturation, enzyme activity and fidelity and the formation of primer-dimer artefacts (Saiki, 1988). Typically $MgCl_2$ concentrations range from 1-8mM in most RAPD analyses reported in the literature. In this study different concentrations (3, 4, 5 and 6 µl) of $MgCl_2$ (25 mM/1 mL of stock) were used to get clear and good amplification; 3 µl and 4 µl did not show any amplification. A very good band pattern was observed by using 5 µl of $MgCl_2$ while 6 µl $MgCl_2$ did not gave any clear band pattern. Thus, 5 µl $MgCl_2$ was chosen for PCR reaction. This is very low concentration of $MgCl_2$ used for RAPD analysis.

As Williams *et al.* (1993) reported, generally, increasing amounts of Mg^{2+} will result in the accumulation of non-specific amplification products, although insufficient Mg^{2+} will reduce the yield

Total numbers of bands observed in this study were 1527. Maximum polymorphism generated with GLA-07 (Figure 2). Average numbers of bands per 20 primers were 65-72. This average was higher for A-series primers than B-series of primers.

Observation of the similarity matrix reveals a very out of the ordinary behavior among *Humicola* species. Maximum similarity was found in *Torula thermophile* and *Humicola insolens* of 57.8%, whereas, between *Humicola lanuginosa* and *Humicola grisea* was 57.3%. However, the interesting feature of study showed *H. insolens* to be much closer in band pattern similarity with *Torula thermophila* than *Humicola lanuginosa* and *Humicola grisea* (Figure 6). Ellis and Griffiths (1976) described that the thallospores of *Torula thermophila* arise in a manner different from that of the blastospores produced by other species of *Torula* and are structurally more closely related to the spores produced by *Humicola insolens*. So *Torula thermophila* is genetically more similar to *Humicola insolence*. Similar band pattern of *Torula thermophile* and *Humicola insolens* can be observed in Figure 1 and 2.

Apart from this, similarity between *Talaromyces dupontii* and *Rhizomucor pusillus* was 51.5% (similar band pattern in Figure 5). The least similarity was found in *Rhizomucor pusillus* and *Humicola grisea*, which was 18.7% and *Chaetomium thermophile* and *Sporotrichum thermophile*, which was 18.3% (Table 3).

There was a significant level of polymorphism observed for various species. Figure 3 is a representative figure to show the maximum level of polymorphism among

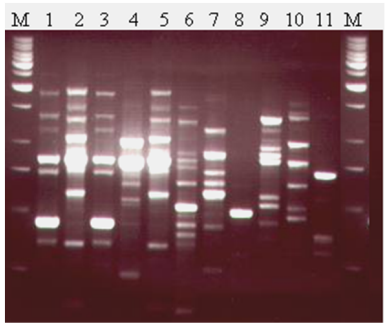

Figure 1. PCR of fungal DNA with Primer GLA-04. Numbers 1 to 11 are named as: *Humicola lanuginose* 1; *Humicola insolens* 2; *Humicola grisea* 3; *Malbranchia pulchella* 4; *Torula thermophila* 5; *Talaromyces dupontii* 6; *Sporotricum thermophile* 7; *Rhizomucor pusillus* 8; *Paecilomyces thermophila* 9; *Aspergillus fumigatus* 10; *Chaetomium thermophile* 11.

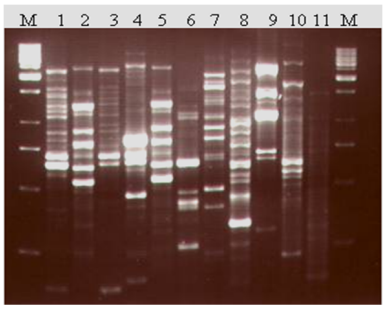

Figure 2. PCR of fungal DNA with Primer GLA-07. Numbers 1 to 11 are named as: *Humicola lanuginosa* 1; *Humicola insolens* 2; *Humicola grisea* 3; *Malbranchia pulchella* 4; *Torula thermophila* 5; *Talaromyces dupontii* 6; *Sporotricum thermophile* 7; *Rhizomucor pusillus* 8; *Paecilomyces thermophila* 9; *Aspergillus fumigatus* 10; *Chaetomium thermophile* 11.

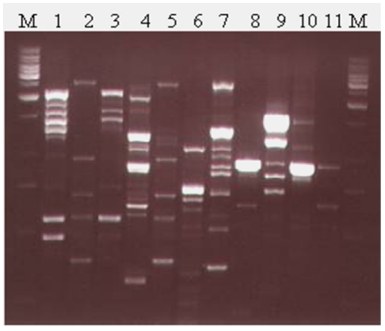

Figure 3. PCR of fungal DNA with Primer GLA-08. Numbers 1 to 11 are named as: *Humicola lanuginosa* 1; *Humicola insolens* 2; *Humicola grisea* 3; *Malbranchia pulchella* 4; *Torula thermophila* 5; *Talaromyces dupontii* 6; *Sporotricum thermophile* 7; *Rhizomucor pusillus* 8; *Paecilomyces thermophila* 9; *Aspergillus fumigatus* 10; *Chaetomium thermophile* 11.

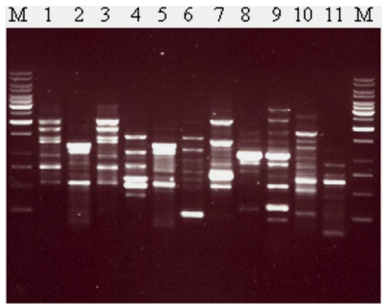

Figure 4. PCR of fungal DNA with Primer GLB-02. Numbers 1 to 11 are named as: *Humicola lanuginosa* 1; *Humicola insolens* 2; *Humicola grisea* 3; *Malbranchia pulchella* 4; *Torula thermophila* 5; *Talaromyces dupontii* 6; *Sporotricum thermophile* 7; *Rhizomucor pusillus* 8; *Paecilomyces thermophila* 9; *Aspergillus fumigatus* 10; *Chaetomium thermophile* 11.

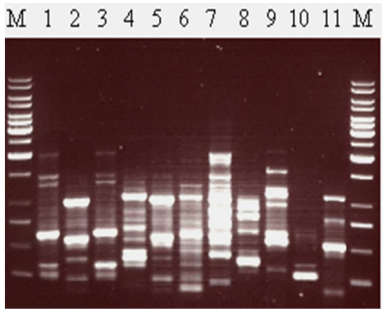

Figure 5. PCR of fungal DNA with Primer GLB-07. Numbers 1 to 11 are named as: *Humicola lanuginosa* 1; *Humicola insolens* 2; *Humicola grisea* 3; *Malbranchia pulchella* 4; *Torula thermophila* 5; *Talaromyces dupontii* 6; *Sporotricum thermophile* 7; *Rhizomucor pusillus* 8; *Paecilomyces thermophila* 9; *Aspergillus fumigatus* 10; *Chaetomium thermophile* 11.

Table 3. Similarity matrix of different thermophilic cellulolytic fungi for the various primers used for the RAPD

Organism	H.l	H.i	H.g	M.p	T.t	T.d	S.t	R.p	P.t	A.f	C.t
H.l	1.000										
H.i	0.423	1.000									
H.g	0.573	0.298	1.000								
M.p	0.220	0.321	0.316	1.000							
T.t	0.494	0.578	0.401	0.378	1.000						
T.d	0.355	0.392	0.360	0.515	0.456	1.000					
S.t	0.280	0.332	0.250	0.345	0.301	0.391	1.000				
R.p	0.236	0.268	0.187	0.305	0.287	0.323	0.329	1.000			
P.t	0.359	0.334	0.298	0.312	0.411	0.407	0.357	0.233	1.000		
A.f	0.300	0.305	0.282	0.342	0.308	0.344	0.346	0.221	0.361	1.000	
C.t	0.229	0.294	0.212	0.206	0.321	0.324	0.183	0.266	0.259	0.248	1.000

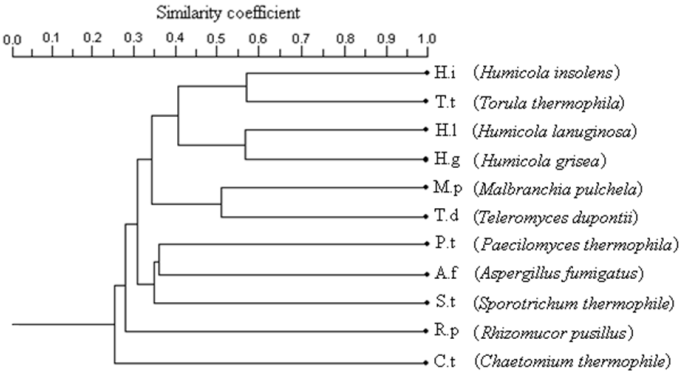

Figure 6. Dendrogram of different thermophilic cellulolytic fungi by using RAPD

fungal strains with primer GLA-08. So this primer can also be used as a marker to check cross contamination among these fungal strains. Some of the species on the other hand showed considerable level of similarities such as *H. Lanuginosa* to *H. grisea* (Figure 4). Moreover, correlation exists among all the fungal strains observed in study.

Unweighted Pair Group Method of Arithmetic Means (UPGMA) was used successfully in this study to compare RAPD pattern of fungi. The comparison of each profile for each primer was performed on the bases of presence (1) vs. absence (0) of the RAPD product of the same size. RAPD is informative enough to screen population. Nei and Li's coefficient (Nei and Li, 1979) was used to calculate the relatedness of the studied species among each other. A dendrogram was made on the basis of similarity coefficient of fungal strains.

Our repeated experiments showed that RAPD results can be transferred from pure research to practical applications. These studies will enable the scientists to evaluate the specific or non-specific group of fungi for the similarities and dissimilarities among their genome. This will be helpful in elucidating the behavior of closely related fungi for their characteristic study of enzymes or primary and secondary metabolites.

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