Pak. J. Bot., 43(5): 2621-2625, 2011.

DISTRIBUTION OF CELLULOLYTIC-THERMOPHILIC FUNGI ON VARIOUS SUBSTRATES AND GEOGRAPHIC LOCATIONS IN PAKISTAN

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Abstract

Eighty seven different strains belonging to seven different types of thermophilic-cellulolytic fungi such as *Aspergillus fumigatus, Chaetomium thermophile* var. *dissitum, Humicola grisea* var. *thermoidea, Humicola insolens, Sporotrichum thermophile, Talaromyces duponti* and *Torula thermophila* were isolated from different habitats of Sheikhupura, Khanewal, Sadiqabad and Jacobabad. *A. fumigatus* showed 88-100% frequency of occurrence in all habitats, but other cultures showed different frequency of occurrence in different habitats ranging from 33-100%. Results indicated that soil as habitat and Sheikhupura as a locality are the rich source of such fungi in Pakistan. Diversity indices showed that complete diversity exists. However, 100% diversity was not observed in any case. *C. thermophile* showed habitat specificity to soil and garden compost while *H. insolens* to textiles waste and herbivore, dung. Among 87 isolates *H. insolens* TAS-13 showed better cellulolytic potential.

Introduction

Among fungi, thermophilic strains are the chief components that grow in heaped masses of plant material, piles of agriculture, forestry products and other accumulations of organic matter where the warm, humid and aerobic environment provides the basic conditions for their development. These strains constitute a heterogeneous physiological group of various genera in the phycomycetes, ascomycetes and Deutromycotina (fungi imperfecti) with a minimum at or above 20°C and a maximum temperature range of up to 62°C for their growth. They are the only representatives of mycoflora that can grow at temperature above 45°C. Since, thermophilic fungi grow at elevated temperature, but they offer faster growth rates as compared with mesophiles (Mouchacca, 1997; Ashraf *et al.,* 2007).

Thermophilic fungi have a powerful ability to degrade polysaccharide constituents of biomass like cellulose and are the potential source of cellulolytic enzymes with scientific and commercial interest. These fungi can make the process more economical due to their thermostable enzymes, high rate of cellulolysis and ability to saccharify under non-aseptic conditions (Haggerdal *et al*., 1980; Merchant *et al*., 1988; Maheshwari *et al*., 2000; Sohail *et al.,* 2009). The presented work was aimed at the (i) isolation of cellulolytic-thermophilic fungi and (ii) to assess their richness and diversity pattern in Pakistan along with their cellulolytic potential.

Materials and Methods

Study area and sampling: Samples from 7 different habitats viz., soil from rhizosphere, garden compost, herbivore dung, decomposting bagasse piles, textile wastes, wheat straw and rice straw heaps were collected from four localities of Pakistan i.e., Sheikhupura [Lat. 31.41:17N (31.6881), Long. 73.53:11E (73.88640)], Khanewal [Lat. 30.20:03N (30.3342), Long. 71.51:55E (71.8652)], Sadiqabad [Lat. 28.22:00N (28.3668), Long. 70.17:35E (70.2932)] and Jacobabad [Lat. 22.22:00N (28.3668), Long. 68.29:48E (68.490)] during summer season with temperature ranging from 38-49°C.

Incubation, observation and data analysis: All the samples were moistened (1:1 ratio) with distilled water (sterilized in autoclave at 15 lb/in^2 and 121°C for 15 minutes) and mixed to confine homogeneity. These samples were packed in polythene bags and incubated at 50±1°C for about three days. Cellulolytic thermophilic fungal strains were isolated from moist samples making serial dilutions by plate method after Warcup (1950). The fungal cultures were further purified from bacterial contaminants by using 10 mg/L combination of penicillin and streptomycin (1:1 ratio) in the Petri plate medium. All the isolates of thermophilic fungi were identified by microscopic examination under compound microscope after Cooney & Emerson (1964), Domsch *et al*., (1980), Onion *et al*., (1986) and Koneman *et al*., (1991). Independent colonies of each identified isolate were picked up and transferred to potato dextrose agar (PDA) slants for culture maintenance.

The percent frequency and diversity indices of culture's occurrence were determined after Magurran (1988) by recording the time fold appearance of the species (Table 1), grown on Eggins & Pugh (1962) medium supplemented with 1.0% cellulose powder after five days of incubation at 45±1°C (Maheshwari *et al*., 2000).

$$\text{Frequency of occurrence (\%)} = \frac{\text{Total number of plates in which specific fungi occur}}{\text{Total number of plates for specific sample}} \times 100$$

Screening for cellulolytic potential: Tansey (1971) plate screening method was used to select high titre cellulase producing strains. The plate medium contained Eggins & Pugh (1962) mineral salts solution in addition with (g/L); agar (20), Sigmacell-101 (5.0) as sole carbon source, Triton X-100 (1.0 ml) and Rose Bengal (0.01) as an antibacterial agent. The strains were selected on the basis of cellulose degradation ability, which was indicated by the formation of clear relative zone. Relative zone of cellulose hydrolysis was measured according to the equation 02.

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Table 1. Diversity measure models.

Measure	Formula	Key
Shannon's index (*H*)	$H = -\sum_{i=1}^{s} p_i \ln p_i$	Where p_i is the proportion of *S* made up of the ith species (estimated using n_i/N). $H_{max} = lnS$, which show maximum diversity
Shannon evenness/ Equitability (*E*) OR Lloyd and Ghelardi (*J*)	$E = H/\ln S$ OR $J = H/H_{max}$	The Shanon evenness index presumes that the most equatible community could have equal numbers of all OTUs. Lloyd and Ghelardi (*J*) is a refinement of this. The *E* or *J* presumes that the most equitable community could have equal numbers of all the species. Maximum value is 1 which indicate community have equal numbers in all study areas.
Simpson's index (*D*)	$D = \sum_{i=1}^{s} p_i^2$	For the full community *D* is simply Σpi^2, where p_i is the proportion of *S* made up of the ith species. The proportion of species i relative to the total number of species (p_i) is calculated and squared
Simpson evenness (*d*)	$D = 1/D$	The *d* presumes that the most equitable community could have equal numbers of all the species. Maximum value is equal to *S*, which indicates community have equal numbers in all study areas.
Simpson equitability (*ED*)	$ED = D/D_{max}$	Simpson's index (*D*) and expressing it as a proportion of the maximum value *D* that could assume, if individuals in the community were completely evenly distributed (D_{max}), which equals *S*.

Unless stated the key, formulae from Magurran (1988)

$$\text{Relative zone of cellulolytic hydrolysis (mm)} = \frac{\text{Total diameter (mm)}}{\text{Colony size (mm)}}$$

Statistical analysis: Diversity indices of culture's occurrence were determined by recording the time fold appearance of the species grown after five days of incubation at 45±1ºC using models as Table 1. Computer software (Costat, 3.03 Berkeley, CA 94701) was used to compare the size for relative zone of cellulose hydrolysis after Snedecor & Cochrane (1980). The significant difference among parallel three replicates has been presented as Duncan's multiple range tests in the form of probability <p> values.

Results and Discussion

Present efforts were made to isolate and identify different cellulolytic thermophilic fungi from different habitat and localities of Pakistan. Eighty-seven cultures of thermophilic fungi were isolated and among these, 18 cultures of *Aspergillus fumigatus*, 02 cultures of *Chaetomium thermophile* var. *dissitum*, 15 cultures of *Humicola grisea* var. *thermoidea*, 03 cultures of *Humicola insolens*, 17 cultures of *Sporotrichum thermophile*, 21 cultures of *Talaromyces duponti* and 11 cultures of *Torula thermophila* were identified. Among all these, *A. fumigatus* showed 88-100% frequency of occurrence in all habitats, but other cultures showed different frequency of occurrence in different habitats ranging from 33-100%. It was observed that Sheikhupura and Khanewal are rich in having habitats containing various genera of thermophilic fungi.

The diversity indices provide a summary statistic of the diversity of a fungal community containing maximum number of thermophilic fungi (Pielou & Arnason, 1965). Results presented shows that *A. fumigatus*, *H. grisea*, *S. thermophile*, *T. duponti* and *T. thermophila* were in abundance in different localities, indicating that all these cultures are evenly distributed in these localities. Moreover, these cultures are diversified in the light of the fact that complete diversity exists, if the values of ln*S* and *H* are more or less same. But, if the *H* value is less then it means diversity is uneven. On the other hand, *S. thermophile* and *T. duponti* were in higher numbers among different localities so all the cultures are diversified. However, 100% diversity is not seen in any case (Tables 2 & 3). A fungal community that has more species have a greater diversity index than a community of similar evenness with fewer species. While, the Shannon's index introduces a problem for classification and it is a proportional abundance index, which is considered primarily as richness index. It seeks to crystallize richness and evenness into a single figure (Lloyd & Ghelardi, 1964; Magurran, 1988; Tom *et al*., 2003). In this respect, all the cultures are less diversified among different localities except *C. thermophile*, whose value is exactly one and among different habitats, *C. thermophile* and *H. insolens* are diversified. While, zero value for Shannon's Equitability indicates minimum diversity index and value near about to one means maximum diversity index (Tom *et al*., 2003; Goulart *et al*., 2005). So results indicated that all the cultures in different habitats and localities are more or less diversified. It is fact that a community with greater evenness also has a larger diversity index than a community of the same richness with lower evenness.

Table 2. Diversity indices of thermophilic fungi in different localities.

Name of species	Habitat				Total	*S*	*lnS*	*H*	*E or J*	*D*	*d*	*ED*
	Sheikhupura	**Khanewal**	**Sadiqabad**	**Jacobabad**								
A. fumigatus	04	06	05	03	18	04	1.386	1.330	0.960	0.256	3.910	0.977
C. thermophile	02	----	----	----	02	01	0.000	0.000	0.000	1.000	1.000	1.000
H. grisea	04	05	04	02	15	04	1.386	1.326	0.956	0.266	3.760	0.940
H. insolens	01	01	----	01	03	03	1.099	1.089	0.990	0.333	3.000	1.000
S. thermophile	07	05	03	02	17	04	1.386	1.270	0.916	0.295	3.389	0.974
T. duponti	07	05	05	04	21	04	1.386	1.358	0.980	0.259	3.860	0.965
T. thermophila	01	04	03	03	11	04	1.386	1.276	0.920	0.285	3.500	0.875
Total	**26**	**26**	**20**	**15**	**87**							

Table 3. Diversity indices of thermophilic fungi on different habitats.

Name of species	Habitat							Total	*S*	*lnS*	*H*	*E or J*	*D*	*d*	*ED*
	Soil	**Garden compost**	**Decompos-ing bagasse**	**Textile waste**	**Herbivore dung**	**Wheat straw**	**Rice straw**								
A. fumigatus	04	01	04	02	----	04	03	18	06	1.790	1.70	0.95	0.188	5.30	0.884
C. thermophile	01	01	----	-----	----	-----	----	02	02	0.690	0.68	0.98	0.500	2.00	1.000
H. grisea	02	04	----	01	03	03	02	15	06	1.790	1.69	0.94	0.190	5.24	0.870
H. insolens	----	----	----	01	02	----	----	03	02	0.690	0.63	0.91	0.550	1.80	0.910
S. thermophile	04	03	03	02	02	02	01	17	07	1.945	1.86	0.96	0.160	6.27	0.890
T. duponti	03	03	02	03	02	04	04	21	07	1.945	1.92	0.99	0.151	6.61	0.940
T. thermophila	03	02	01	----	04	----	01	11	05	1.610	1.47	0.92	0.250	3.93	0.780
Total	**17**	**14**	**10**	**09**	**13**	**13**	**11**	**87**							

Strains were screened out for cellulolytic potential on the basis of diameter of clear zone produced due to cellulose hydrolysis by different cultures of the same species. These results indicated that among the cultures of *A. fumigatus*, TAS-10; among *C. thermophile*, TAS-02; among *H. grisea*, TAS-81; among *H. insolens* TAS-13; among *S. thermophile*, TAS-73; among *T. duponti*, TAS-76 and among *T. thermophila*, TAS-82 were the best cellulase producers giving 1.70, 1.40, 1.66, 1.90, 1.34, 1.45 and 1.25 mm zone, respectively. Among the 87 isolated cultures of fungal strains, *H. insolens* TAS-13 showed bigger zone of cellulose hydrolysis (Table 4). This strain has the ability to utilize cellulose efficiently as carbon source by its powerful protein secretary system, enriched with cellulase enzyme as compared to the all other isolates.

Conclusion

Cellulolytic-thermophilic fungal strains are highly diversified and have potential to utilize cellulosic biomass efficiently. From this work, it was also concluded that diversity indices provide important information about rarity and commonness of a fungal culture in a habitat or in a particular locality.

Acknowledgements

We are highly thankful to Higher Education Commission of Pakistan for providing financial support.

Table 4. Screening of different thermophilic species of a genus for best cellulolytic ability on the basis of relative zone of cellulose hydrolysis.

Sr. No.	*A. fumigatus*		*C. thermophile* var. *dissitum*		*H. grisea* var. *thermoidea*		*H. insolens*		*S. thermophile*		*T. duponti*		*T. thermophila*	
	Cultures (TAS-)	Relative zone of cellulose hydrolysis (mm)	Cultures (TAS-)	Relative zone of cellulose hydrolysis (mm)	Cultures (TAS-)	Relative zone of cellulose hydrolysis (mm)	Cultures (TAS-)	Relative zone of cellulose hydrolysis (mm)	Cultures (TAS-)	Relative zone of cellulose hydrolysis (mm)	Cultures (TAS-)	Relative zone of cellulose hydrolysis (mm)	Cultures (TAS-)	Relative zone of cellulose hydrolysis (mm)
1.	01	1.60^{b}	02	1.40^{a}	03	1.30^{e}	13	1.90^{a}	04	1.20^{cd}	05	1.10^{h}	18	1.10^{c}
2.	10	1.70^{a}	06	1.38^{b}	07	1.25^{h}	43	1.71^{b}	08	1.30^{ab}	09	1.21^{de}	31	1.17^{abc}
3.	19	1.50^{c}			20	1.60^{b}	80	1.57^{c}	11	1.20^{de}	12	1.20^{de}	35	1.22^{ab}
4.	23	1.30^{ef}			24	1.44^{cd}			14	1.30^{ab}	15	1.20^{de}	38	1.10^{c}
5.	27	1.20^{g}			28	1.46^{c}			16	1.30^{ab}	17	1.40^{b}	47	1.12^{c}
6.	32	1.30^{ef}			33	1.21^{h}			21	1.20^{cd}	22	1.20^{de}	56	1.13^{bc}
7.	36	1.53^{c}			40	1.31^{e}			25	1.21^{cd}	26	1.33^{c}	60	1.12^{bc}
8.	39	1.30^{ef}			44	1.40^{d}			29	1.30^{ab}	30	1.21^{de}	65	1.14^{ab}
9.	48	1.31^{e}			49	1.34^{e}			34	1.11^{e}	42	1.22^{de}	74	1.11^{c}
10.	51	1.24^{fg}			57	1.22^{gh}			37	1.32^{ab}	46	1.25^{d}	82	1.25^{a}
11.	53	1.19^{g}			64	1.31^{e}			41	1.16^{de}	50	1.12^{gh}	87	1.16^{abc}
12.	61	1.33^{e}			67	1.32^{e}			45	1.32^{ab}	52	1.20^{de}		
13.	66	1.43^{d}			70	1.33^{e}			54	1.16^{de}	55	1.14^{fg}		
14.	69	1.52^{c}			75	1.26^{ef}			58	1.10^{e}	59	1.11^{gh}		
15.	72	1.22^{g}			81	1.66^{a}			62	1.21^{cd}	63	1.20^{de}		
16.	77	1.62^{b}							73	1.34^{a}	68	1.21^{de}		
17.	78	1.34^{e}							84	1.25^{bc}	71	1.10^{gh}		
18.	83	1.59^{b}									76	1.45^{a}		
19.											79	1.12^{h}		
20.											85	1.32^{c}		
21.											86	1.17^{ef}		
LSD		**0.064**		**0.018**		**0.042**		**0.133**		**0.068**		**0.046**		**0.087**

Each value is an average of three replicates and numbers followed by different letters differ significantly at $p \leq 0.05$

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(Received for publication 27 September 2010)