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Study of cellulolytic soil fungi and two nova species and new medium

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Abstract: This study is aimed at identifying and determining the percentage of occurrence frequency of cellulose decomposing soil fungi. The soil samples were inoculated into culture plates prepared in Sabouraud medium under sterilized conditions and incubated at 30 °C for 4 to 7 d. The identified fungal species were incubated in self-designed cellulose medium for testing their cellulolytic ability. Forty-two species, including 2 nova species, representing sixteen genera showed growth and sporulation in the cellulose medium. Most of the isolated species were from genus *Aspergillus* and *Penicillium*. *Aspergillus niger* and *Mucor hiemalis* showed highest occurrence frequency (45% and 36% respectively), as these species were collected from about 80% of soil samples. Being agar free and cheaper, the new fungal medium designed showed results equivalent to Sabouraud medium.

Key words: Cellulolytic soil fungi, Cellulose medium, Nova species, Taxonomy
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INTRODUCTION

Study of fungi from the taxonomic point of view is of practical value and the importance of taxonomy is evident from a paper whose author mentioned 20 cellulolytic species of genus *Cladosporium* by number instead of name (Ghahfarokhi *et al*., 2004). Apart from their disease causing nature (Wainwright, 1995), their beneficial effects and increasing role in the industrial area (Ander *et al*., 1996; Bergquist *et al*., 2003; Gupta and Soni, 2000; Henriksson *et al*., 2000; Mehta and Gupta, 1991; 1992; Nevalainen and Te'o, 2003), fungi are General Manager in Nutrients Recycling Department of Nature. As a decomposer, they play a most important role in our economy because fertility of soil greatly depends on microbial activity. The diversity of soil microbes depends on the chemistry, texture and water holding capacity of soil; the success of fungi to reach and colonize a patch of soil is mainly due to their competitive saprophytic ability, expressed by fast mycelial growth, spores production, presence of an efficient and extensive system of powerful enzymes and tolerance to antibiotics (Garrett, 1963; Wainwright, 1995), salinity (Malik and Sandhu, 1973; Moustafa, 1975; Ortuno *et al*., 1977), heavy metals (Lebedeva *et al*., 1999), chemicals/fungicides (Brazauskiene, 1998; El-Said, 2001; Taiwo and Oso, 1997) and temperature (Fenice *et al*., 1997; 1998; Redman *et al*., 1999; Robinson and Morris, 1984; Robinson, 2001).

Plant materials subjected to microbial decay in soil contain various substances that are chemically and physically heterogeneous. Cellulose is the most abundant constituent, ranging from 15%~60% of the organic matter dry weight. It provides easily available energy source to soil microflora and makes complex plant constituents available to other microorganisms for decomposition. A wide variety of microbes including bacteria, fungi, actinomycetes and protozoa are involved in the decomposition of cellulose, fungi have generally considered to be the main organisms responsible (Cowling, 1958; Gas-

coigne and Gascoigne, 1958; Siu, 1951). So the rate of utilization of plant materials and its transformation into humus is largely dependent on the active soil mycoflora. This is the reason why scientists are studying the fungi at molecular level (Nevalainen and Penttilä, 2003; Shimosaka *et al*., 1996), trying to discover more cellulolytic fungi (Ariunaa and Temuulen, 2001), developing new techniques to take advantage of the effect of celluloses (Norenko *et al*., 1994; Steiner *et al*., 1994; Szakäcs-Dobozi *et al*., 1985) and are developing mutant strains to enhance the production of cellulases (Chand *et al*., 2005; Gupta *et al*., 1981).

Various culture media containing all the essential food substances, are useful for fungal studies although none of them are ideal substratum. Their deficiency is evident by the finding that it is impossible to use artificial media to cultivate certain fungi, notably the rusts, downy mildews and powdery mildews. Moreover, many fungi remain sterile in culture, others cannot be induced to complete their normal developmental cycle, some are manifestly teratological, and some require vitamins or growth factors. A satisfactory medium contains glucose (5 to 10 g), KNO_3 (0.5 g), asparagine or glutamic acid (1 to 2 g), KH_2PO_4 (1 g), $MgSO_4$ (0.5 g), microelements (Fe, Mn, Zn) in traces, and distilled water (1 L). Vitamins thiamine (100 μg), biotin (5 μg), and pyridoxine (100 μg) may be added routinely to the deficient species (Lilly and Barnett, 1951). Therefore, mycologists prefer a potato-dextrose agar medium containing 5 to 10 g glucose, 1 to 2 g yeast extract, 20~50 g agar and 1 L distilled water. This medium is easy to make and the pH need not be adjusted. Sabouraud medium is another good one (Difco Laboratories, 1972). For the detection of cellulolytic ability, the fungal species are incubated in medium containing cellulose. There are various techniques for isolating fungi (Kelman, 1967; Stevens, 1947), but the simplest one is dilution plate method and the lifting of conidia from sporulating conidiophores. This simple methodology often yields contamination free cultures. Fungal species growing in habitats with abundance of bacteria require the use of antibiotics in culture medium.

The present study was aimed at determining the soil mycoflora responsible for cellulose degradation in addition to their isolation, identification and occurrence frequency percentage. For this purpose, soil samples were collected from different localities of District Rawalpindi, to which no attention was paid in the past.

MATERIALS AND METHODS

The soil samples taken from the top 20 cm of the soil were brought to laboratory in sterilized polythene bags. The cultural plates were prepared from autoclaved (at 120 °C under 15 lbs pressure for 20 min) Sabouraud medium (65 g/L distilled water). Then 1 g of each soil sample was dissolved in 10 ml of distilled water and from each sample ten dilutions were prepared. For checking the bacterial growth 500 mg/L of penicillin was added after the Sabouraud medium was cooled. After inoculation of the soil samples in laminar flow chamber, the culture plates were incubated for 4~7 d at 30 °C. After growth, the fungal species were further isolated by dilution plate and single spore culture method and then subjected to taxonomic studies. For identification "A Manual of Soil Fungi" by Gilman (1971) was followed. For photography Nikon AFX II microscope was used. Finally, the cellulolytic ability of each species was tested in two types of cellulose medium (one containing ground pepers, the other containing cotton, both agar free), prepared by modifying the E and P cellulose medium proposed by Eggins and Pugh (1962). In both cases an about 10 mm thick substratum was placed in petri dishes and then soaked with solution containing L-asparagine (0.5 g), $MgSO_4{\cdot}7H_2O$ (0.2 g), $CaCl_2$ (0.1 g), KH_2PO_4 (1 g), $(NH_4)_2SO_4$ (0.5 g) and distilled water (1 L). All the materials were sterilized before inoculation into the culture medium. Occurrence frequency of different fungi was determined collectively for all samples by using Warcup (1950)'s soil-plate method. In addition a new fungal medium with results equivalent to Sabouraud's, but cheaper and free from agar, was also designed and applied in this study (Table 1).

The composition of used medium is listed in Table 1.

RESULTS AND DISCUSSION

Forty-two fungal species representing 16 genera

Table 1 Composition of medium

No.	Ingredient	Weight
1	Wheat flour	60 g
2	Meat extract	100 g
3	L-asparagine	0.5 g
4	$MgSO_4 \cdot 7H_2O$	0.2 g
5	$CaCl_2$	0.1 g
6	KH_2PO_4	1 g
7	$(NH_4)_2SO_4$	0.5 g
8	$CuCl_2$	0.5 mg
9	Mn	0.5 mg
10	$ZnCl_2$	0.5 mg
11	Vitamin-C	500 mg
12	Folic acid	150 μg
13	Vitamin-B_1	15 mg
14	Vitamnin-B_2	15 mg
15	Nicotinamide	100 mg
16	Vitamin-B_6	20 mg
17	Vitamin-B_{12}	12 μg
18	Pentothenic acid	20 mg
19	Distilled water+meat extract	1 L

were isolated (from soil samples of 22 localities), identified, and successfully grown on cellulose medium. Nine species belonged to genus *Penicillium* and 8 to *Aspergillus*. Four species belonged to each genus of *Monocillium* and *Fusarium*, three species to genus *Mucor*, two species to each of genus *Rhizopus*, *Cladosporium* and *Verticillium*. While the rest of the genera *Acremonium*, *Alternaria*, *Botrytis*, *Curvularia*, *Drechslera*, *Monacrosporium*, *Monilia* and *Trichoderma* were represented by a single species. The isolation of various fungal species showed that the soil of district Rawalpindi-Islamabad (Pakistan) is quite rich in mycoflora. The interesting thing is that all the localities in present study fall into two categories of climatic conditions: cold-hilly (with minimum temperature of −6 °C) and warm-plain areas (with maximum temperature of 46 °C). The isolation of more species of genus *Aspergillus* and *Penicillium* are probably due to their capability for producing a diverse range of antibiotics and mycotoxins (El-Said, 2001; Wainwright, 1995) that protect them from other soil organisms and may also hinder the growth of other fungal species, apart from their extensive enzyme system (Ali-Shtayeh *et al*., 1989). The dominance of these two genera in the present study accorded with the results of El-Said (2001). These are also at the top in salinity tolerance (Radwan *et al*., 1984) and divergent evolution.

The occurrence frequency of these fungal species is given in the Table 2. The results have also been expressed as percent of soil samples inhibited by any given species. The former parameter gives information on the predominance of any species in the investigated area, while the later index indicates the relative distribution of different species in the soil. *Aspergillus niger* and *Mucor hiemalis* showed occurrence frequency of 45% and 36% respectively, as these two species were isolated from about 80% of the soil samples. Whereas the lowest occurrence frequency was shown by *A. koningi*, *C. lunata*, *D. hawaiiensis*, *F. sambucinum*, *F. semitectum*, *M. geophilus* and *R. oryzae*, as these were collected from not more than 5% of soil samples. While fungi (*T. lignorum*, *V. candelabrum*, *A. luchuensis*, *A. flavus* and *A. fumigatus* etc.) showing moderate occurrence frequencies were collected from 18% to 27% of soil samples. These results showed that a positive correlation exists between occurrence frequency percentage and the percentage of soil samples from which fungal species were collected (Fig.1). The significance of this correlation is that within a specific area we can determine the distribution of a particular fungal species from its occurrence frequency. We have tried to relate occurrence frequency with sporulation, growth rate on culture and cellulose medium, and conidial size, but results have shown no correlation. For example, abundant sporulation was observed in species of *Aspergillus*, *Penicillium*, *Trichoderma*, *Cladosporium*, *Mucor*, *Rhizopus* and *Monilia*, while less sporulation was found in species of *Monacrosporium* and *Botrytis*. In the case of *Rhizopus* and *Monilia* sporulation is abundant but their occurrence frequency is low, like *Monacrosporium* and *Botrytis*, and accordingly these were isolated from fewer soil samples. Similarly, fast growth rate was observed in genus *Mucor*, *Aspergillus*, *Penicillium* and *Trichoderma species*, and slow in growth rate *V. candelabrum* and *C. cladosporioides*. Though these two species (*V. candelabrum* and *C. cladosporioides*) were isolated from the same percentage of soil samples (18%) and have slow growth rate, but the occurrence frequency of the former is almost double that of the later. This shows that not

only one fungal characteristic/ability is responsible for the high occurrence frequency and distribution, but various characteristics (like sporulation, growth rate, enzyme system, food preferences and tolerance to various stress conditions), their degree and different combinations are ultimately responsible for the high occurrence frequency and distribution.

Ten species were collected from both types of localities with much variation in climatic conditions. These include *Aspergillus flavus*, *Aspergillus fumigatus*, *Aspergillus niger*, *Aspergillus versicolor*, *Cladosporium cladosporioides*, *Monocillium* sp.1, *Monocillium* sp.4, *Mucor hiemalis*, *Penicillium* sp.2 (nova) and *Trichoderma lignorum*. Their isolation from both types of localities and especially those one with highest occurrence frequency shows that these are physiologically and genetically more versatile, not only in coping with the harshness of the climate but also have an extensive enzyme system to degrade organic matter of various nature and have strong resistance to soil pollutants like heavy metals, herbicides, pesticides and antibiotics produced by other fungi and/or plants (Shalaby *et al*., 2002; Balasubramanian *et al*., 1970; Baig *et al*., 2003; Garrett, 1963; Lebedeva *et al*., 1999; Taiwo and Oso, 1997; Wainwright, 1995). These fungi have also been reported from saline soils (Malik and Sandhu, 1973; Moustafa, 1975; Ortuno *et al*., 1977). As regards climatic conditions, these species have both types of genes coding for proteins to act as shock absorber for extremely low as well as high temperatures. For example *Aspergillus niger*, *Aspergillus fumigatus*, and certain *Penicillium* species can grow at 35 °C after one week tolerance in 55 °C (but did not grow at 55 °C) as reported by Redman *et al*.(1999). However, the mechanism of action of such genes and their practical application requires further study and these could be transferred to crops for cultivation in areas with severe winters, instead of vernalization treatment of seeds every year (Robinson *et al*., 1998). Regarding *Aspergillus niger*, it is a most diverse species and has also been isolated from water resources, with our results according with those of Nasser (2003) and Gochenaur (1975) in occurrence frequency, but the highest occurrence frequency of *Mucor hiemalis* is a new finding in our results. However, certain species like *C. lunata*, *D. hawaiiensis* and *M. elegans* were isolated from a single

Table 2 Percentage of occurrence frequency of various fungi isolated from different soils

	Occurrence frequency (%)	Samples* (%)
Fungi from hot-plain area		
Aspergillus luchuensis	12.12	27.27
Aspergillus koningi	0.76	4.55
Aspergillus sydowi	7.58	27.27
Botrytis pilulifera	1.52	4.55
Cladosporium elatum	1.52	9.10
Curvularia lunata	0.76	4.55
Drechslera hawaiiensis	0.76	4.55
Fusarium sambucinum	0.76	4.55
Fusarium semitectum	0.76	4.55
Monacrosporium elegans	1.52	4.55
Monocillium sp.2	3.79	9.10
Monocillium sp.3	1.52	4.55
Penicillium sp.1 (nova)	2.27	4.55
Penicillium decumbens	5.30	9.10
Penicillium duclauxi	4.55	9.10
Penicillium islanidicum	3.03	4.55
Penicillium notatum	6.06	13.64
Penicillium puberulum	4.55	9.10
Penicillium rubrum	3.79	9.10
Rhizopus oryzae	0.76	4.55
Verticillium candelabrum	12.88	18.18
Verticillium cellulose	4.55	9.10
Fungi from cold-hilly area		
Acremonium stromaticum	5.30	1.52
Alternation tenuis	2.27	1.52
Aspergillus funiculosus	9.85	18.18
Fusarium dimenrum	1.52	9.10
Fusarium solani	9.10	18.18
Monilia geophila	4.55	9.10
Mucor geophilus	0.76	4.55
Mucor pusillus	8.33	13.64
Penicillium lilacinum	1.52	4.55
Rhizopus arrhizus	1.52	4.55
Fungi from both areas		
Aspergillus flavus	10.61	22.73
Aspergillus fumigatus	9.85	18.18
Aspergillus niger	45.46	81.00
Aspergillus versicolor	3.79	9.10
Cladosporium cladosporioides	6.82	18.18
Monocillium sp.1	5.30	13.64
Monocillium sp.4	3.03	9.10
Mucor hiemalis	36.36	77.00
Penicillium sp.2 (nova)	3.79	9.10
Trichoderma lignorum	19.70	27.27

*: Percentage of samples from which species was isolated

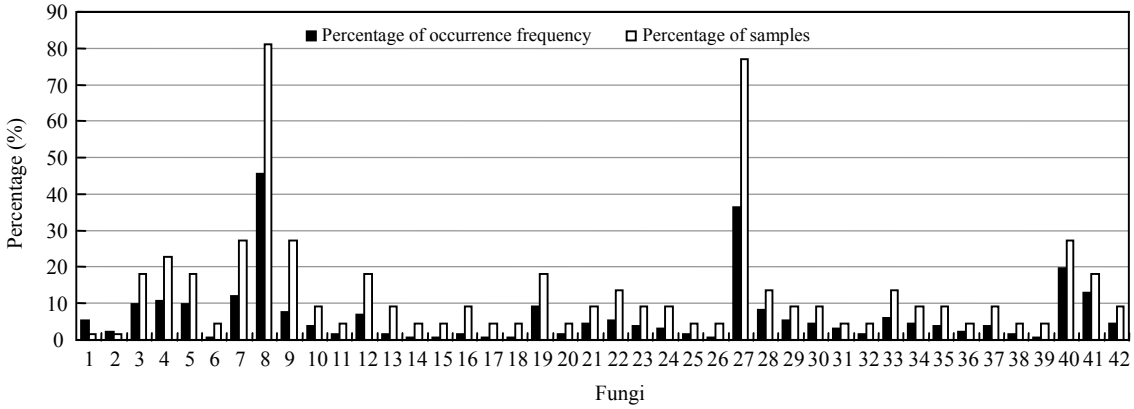

Fig.1 Relationship between percentage of occurrence frequency and percentage of samples from which species was isolated

1: *Acremonium stromaticum*; 2: *Alternation tenuis*; 3: *Aspergillus funiculosus*; 4: *Aspergillus flavus*; 5: *Aspergillus fumigatus*; 6: *Aspergillus koningi*; 7: *Aspergillus luchuensis*; 8: *Aspergillus niger*; 9: *Aspergillus sydowi*; 10: *Aspergillus versicolor*; 11: *Botrytis pilulifera*; 12: *Cladosporium cladosporioides*; 13: *Cladosporium elatum*; 14: *Curvularia lunata*; 15: *Drechslera hawaiiensis*; 16: *Fusarium dimenrum*; 17: *Fusarium sambucinum*; 18: *Fusarium semitectum*; 19: *Fusarium solani*; 20: *Monacrosporium elegans*; 21: *Monilia geophila*; 22: *Monocillium* sp.1; 23: *Monocillium* sp.2; 24: *Monocillium* sp.4; 25: *Monocillium* sp.3; 26: *Mucor geophilus*; 27: *Mucor hiemalis*; 28: *Mucor pusillus*; 29: *Penicillium decumbens*; 30: *Penicillium duclauxi*; 31: *Penicillium islanidicum*; 32: *Penicillium lilacinum*; 33: *Penicillium notatum*; 34: *Penicillium puberulum*; 35: *Penicillium rubrum*; 36: *Penicillium* sp.1 (nova); 37: *Penicillium* sp.2 (nova); 38: *Rhizopus arrhizus*; 39: *Rhizopus oryzae*; 40: *Trichoderma lignorum*; 41: *Verticillium candelabrum*; 42: *Verticillium cellulose*

locality, perhaps because they prefer a particular type of organic matter present in that locality or they lack competitive saprophytic ability to fight against antibiotics/toxic substances produced by other soil mycoflora and/or plants.

Two species, *Penicillium* sp.1 and *Penicillium* sp.2 showed great variations in their conidial size than that mentioned in "A Manual of Soil Fungi" By Gilman (1971) for *Penicillium charlesii* and *Penicillium purprogenum* respectively. *Penicillium* sp.1 also showed variation in color and texture of conidia. In the present study conidia were smooth and hyaline instead of rough and green as mentioned in "A Manual of Soil Fungi" by Gilman (Table 3). The conidial characteristics like shape, size, color, texture, septation and pattern of production/formation are so most important and constant taxonomic characteristics that various fungal species (for example *Fusarium* species) have been differentiated based on the conidial difference of even 1 μm (Gilman, 1971). These variations are testified to their "nova" ness. Taxonomic studies are essential from time to time because evolutionary process is not static and as changes in the global environment are occurring continuously, but at much faster pace than that in the past, so the changes in microbes at gene level in the

Table 3 Variation in conidial size, color and texture

Fungal species	Size in present study	Size in manual of Gilman
Penicillium sp.1	8 μm×4 μm hyaline, smooth	(2.5~3) μm×(2~2.5) μm green, rough
Penicillium sp.2	1.5~2 μm	(3.4~3.8) μm×(2~2.5) μm

emergence of new species being in harmony with the changing environment are not surprising. So these should be regarded as *Penicillium ahdi* (sps.1 nova) and *Penicillium shaze* (sps.2 nova) as shown in Figs.2 and 3, respectively. However, four species belonging to genus *Monocillium* remained unidentified due to lack of access to literature.

Description of new species

1. *P. ahdi*

Colonies white, floccose, spreading fast, becoming gray with age; conidiophores (35~45) μm×5 μm, dichotomously branched, septate, arise as short branches from erect aerial hyphae. Branches of conidiophores tapering gradually with very small vesicle at apex that bears a group of phialides; Penicilli typically in single verticals of phialides borne on branches which maintain the identity of each vertical, each bearing a terminal monoverticillate penicillus;

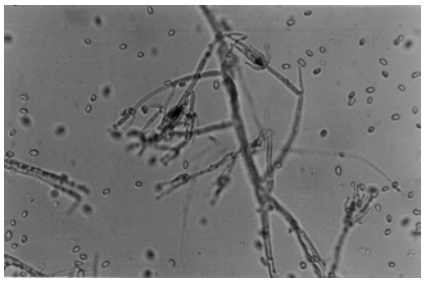

Fig.2 *Penicillium ahdi* (200×)

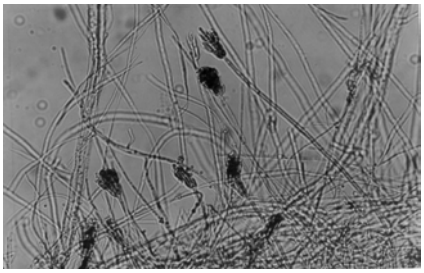

Fig.3 *Penicillium shaze* (200×)

Phialides are flask shaped 12.5~15 μm long; Conidia smooth, hyaline, ovate 8 μm×4 μm.

2. *P. shaze*

Colonies velvety, slowly spreading, zonate having grey to greenish center, then with yellow ring and margins white, never developing coremia, ropiness absent, producing deep red pigment in the medium; Conidiophore smooth, erect, septate, 100~300 μm long; Penicilli of more than one series of elements. Penicilli typically consisting of one symmetrical verticil of metulae, bearing symmetrical verticil of phialides; Metulae 12 μm×2.5 μm, phialides 9 μm×2.5 μm; Conidia smooth, greenish, globose 1.5~2 μm in diameter.

For testing the cellulolytic activity the fungal species were inoculated onto two types of self prepared cellulose medium: one containing ground pepers the other containing cotton but both free of agar; instead of the cellulose medium proposed by Eggins and Pugh (1962). The objection to this medium is the presence of agar in it that makes it difficult to discriminate between cellulolytic and non-cellulolytic fungal species especially when one has no facility for isolating and testing cellulases, because the fungi also have the ability for degradation and use agar, as all the species in the present study have shown growth on medium containing agar along with essential nutrients. However, cellulose medium proposed by Eggins and Pugh is the best for isolating cellulases from cellulolytic fungi and so it should be called as "cellulase isolating medium" for cellulose loving fungi instead of "cellulose medium". On the other hand the growth and sporulation in a medium having nothing other than cellulose clearly elucidates the cellulolytic nature of fungal species.

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