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Some studies on thermophilic and thermotolerant fungi from Lahore, Pakistan.

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Abstract
An attempt has been made to isolate thermophilic and thermotolerant fungi from eight different substrates. A total of twelve different fungal species were isolated from compost, farmyard manure, leaf mold, decaying wood, mixed substrate (Farmyard + leaf mold), field soil and seeds of *Cicer arietinum* (Chickpea) and *Vigna mungo* (Black Gram). The isolated fungi comprised of four species of *Aspergillus, (A. fumigatus, A. nidulans, A. versicolor and A. sparsus*), two species of *Mycocladus (Mycocladus blakesleeanus, Mycocladus corymbifera)* and one species each of *Scytalidium (Scytalidium thermophilum), Humicola, (Humicola grisea* var. thermoidea*), Thermomyces (Thermomyces lanuginosus), Mucor (Mucor fragilis) Rhizomucor* sp. and *Chaetomium (Chaetomium thermophlium).* Maximum species richness (6 species) was recorded in the case of compost and 4 species in each leaf mold, farmyard manure and mix substratum while it reduce to one species in the case of field soil and seeds of Peas chick and mash bean.
Keywords:

Introduction
Among the eukaryotic organisms, only a few species of fungi have the ability to thrive at temperatures above 45 °C. Such fungi comprise thermophilic and thermotolerant forms, which are arbitrarily distinguished on the basis of their minimum and maximum growth temperatures. The thermophilic fungi have a growth temperature minimum at or above 20°C and maximum extending up to 60-62°C (Maheshwari *et al.* 2000) and the thermotolerant forms have a temperature range of growth from below 20 to ~55°C (Cooney and Emerson, 1964). Thermophilic fungi are the chief components of the mycoflora that develops in heaped masses of plant material, piles of agricultural and forestry products,stored food grains and other accumulations of organic matter wherein the warm, humid, and aerobic environment provide the basic conditions for their development. Thermophilic fungi are potential source of enzyme with scientific and commercial interest. They have a powerful ability to degrade polysaccharide constituents of biomass. The properties of their enzymes show differences not only among species but also among strains of the same species. Their extracellular enzymes display temperature optima for activity that are close to or above the optimum temperature for the growth of other organisms and, in general, are more heat stable than those of the mesophilic fungi, (Maheshwari, 1997). The ability of *Thermomyces lanuginosus* to produce high levels of cellulase-free thermostable xylanase has made the fungus an attractive source of thermostable xylanase with prospects as one of the bleach-boosting agents in pulp-paper industry and as an additive in the baking industry (Singh, 2003). From Pakistan the efforts to isolate and characterize thermophilic or thermotolerant fungi are scanty. Qureshi (1977) and Qureshi *et al.*,(1980a,b) reported 28 species of thermophilic and thermotolerant fungi from Pakistan and studied their cellulytic and amylolytic activities.

In the present study we have attempted to achieve two objectives. Firstly, the isolation of thermophillic and thermotolerant fungi from potential substrates followed by their identification and characterization at the species level. Secondly, the species richness was correlated with the substrate type.

Materials and Methods
The fungi were isolated from various substrates like compost, farmyard manure, and leaf mold, wood, mixed substrate (farmyard manure+ leaf mold), cowpea seed, mash beans and cultivated field soil. Samples were collected

in pre-sterilized polythene bags and were stored in refrigerator at about 45 °C for further study. For each sample, date, and site of sampling was recorded. Isolation and further culturing of fungi from the substrate sample was carried out by different methods depending upon the type and nature of substrate.

Moist chamber for dung, compost, leaf and farmyard manure

Moist chambers were constructed in plastic jars, 8 cm in diam. and 12 cm high. A layer of moist hay was spread out at the bottom and was covered with filter paper (Fig 1). These chambers were incubated at 45 °C. Fungal growth was visible within 4-5 days of incubation. Isolation and further sub-culturing (purification) was done on three different media viz., Dung agar medium (DAM), 1% Malt extract agar (MEA) and Yeast glucose agar (YGA). These chambers were regularly studied for one month at intervals of four days and emerging fungi were transferred on agar medium.

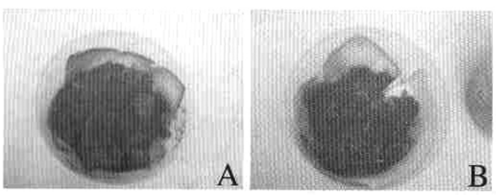

Fig 1. A: farmyard manure **B:** leaf mold in chambers.

Moist sterilized filter paper technique for seeds and grains

For the isolation of fungi from seeds, sterilized glass petriplates (9 cm in diam.) were taken. Moistened filter papers were placed in petriplate @ 2 filter papers per plate. Nine (9) seeds of cowpeas and mash bean were surface sterilized before placing them on the moist filter papers aseptically. The petriplates were incubated at 45°C for a period of 04 days. The fungi appearing on the seeds were aseptically transferred to culture media plates of MEA and YGA and pure culture were obtained.

Dilution plate method

This technique was used for the isolation of fungus from the soil. One gram of field soil was taken and mixed in 9ml of sterilized water; well shaken, and then was placed undisturbed until all the soil particles settled down. One ml of this suspension was added in another 9ml sterilized water tube. The same steps were repeated six times and as a result 07 serial dilutions were obtained. 0.5 ml suspension from each tube was inoculated 1% MEA. After 5 days the fungal growth appeared. Inoculum from actively growing margins of the colony was taken and transferred on fresh agar media plates for pure cultures.

All isolates were studied morphologically and comprehensive description of each isolate was prepared. Identification was done by comparing this data with the authentic synoptic keys published by Raper & Fennel (1965), Cooney and Emerson (1964), and Dix & Webster (1995).

Results and Discussion

Aspergillus fumigatus Fresenius
Bieträge zur Mykologie p.81 (1863)

Colony olive green powdery rapid growing (5.2 cm) on czpecks at 25 and 45 °C reverse dull green. *Conidial head* abundant, columnar, green. *Conidiophore* smooth walled, 50 x -9 µm. *Vesicle* pyriform, dull green 16-21 x 15-21µm. *Sterigmata* uniserate, dark green, ampuliform, 2x4µm. *Conidia* rough walled 2-3 µm (Plate 2G).
Specimen examined: Compost, farmyard manure and leaf mold, field soil, mixed substrate, wood. (FCBP # 768)

Aspergillus sparsus **Raper & Thom**
Mycologia. 36 : 572-574 (1944)

Colony bluish gray, powdery, rapid growing (5.8 cm) on YGA at 45 °C after four days with white zones reverse creamy. *Conidial head* abundant, radiate, grayish. *Conidiophore* smooth walled, hyaline 3-7µm wide. *Vesicle* globose, bluish green 15-24 x 12-19 µm. *Sterigmata* uniserate, phialides ampuliform 2x3µm. *Conidia*: rounded, rough walled, 2 µm (Plate 2F).
Specimen examined: Farmyard manure and leaf mold, field soil. (FCBP # 854)

Aspergillus versicolor (vuill)Tiraboschi
Thom and church. The Aspergilli. Pp.142-144 (1926)
(= *sterignatocystis versicolor*. Vuil 1903, Mirsky, Thēsade mēdecine, Nancy. No.27 Pp15)

Colony fawn color, velvety, slow growing on Czepecks and YGA at 25 and 45 °C with different shade of brown, reverse white. *Conidial head* radiate to loosely columnar. *Conidiophores* smooth, 5 µm wide. *Vesicle* globose, 10-16 x 12-16 µm. *Sterigmeta* biserate, ampuliform 2x4 µm. *Conidia* rounded, rough walled, 3µm diam.

Hülle cell rounded, abundant, 16 μm in diameter (Plate 2J).
Specimen examined: Leaf mold

Aspergillus nidulans (Eidam)Wint.
Rab Crypt.F1.1(2) : 62 (1884)
(= *sterigmatocystis nidulans* Eidam in Cohn 1883 Beitr. Boil.Pflanz. 3:392-411)

Colony green, powdery, rapid growing on Czepecks at 25 and 45 °C. *Conidial head* abundant, loosely columnar. *Conidiophore* smooth walled 3-6μm in diameter. *Vesicle* globose, light bluish green 10-21 x 9-16 μm. *Sterigmeta* biserate, phialides ampuliform, 6x9μm. *Conidia* rough walled 2-3 μm. Hülle cell: rounded abundant 13-21μm in diameter (Plate 2H).
Specimen examined: Mixed substrate

Thermomyces lanuginosus Tsikl.
Annls inst. Pasteur, Paris 13 : 500-504 (1899)

Colony radish white, filamentous, rapid growing on MEA at 45 °C, reverse black (Plate 2B). *conidiophores* thin, hyaline. *Conidia* rounded, double walled, intercalary spore absent, singly on conidiophore
Specimen examined: Compost, farmyard and leaf manure.

Humicola grisea var. thermoidea Traaen 1914

Colony radish to black, filamentous, rapid growing on MEA at 45 °C, reverse black. *conidiophores* thin, hyaline 8-10 μm in thickness. *Conidia* rounded rough walled intercalary spore absent singly on conidiophore, 6-8 μm in diameter (Plate 2D).

Specimen examined: Compost, farmyard manure and leaf mold. (FCBP # 740)

Scytalidium thermophilum Cooney & Emerson
Austwick (1976)

Conidia: globose smooth wall 6-12 μm in diameter produce in chain lacking ascocarp. (Plate 2A)
Specimen examined: Compost.

Chaetomium thermophilum Trans.
Bot mycol. Soc. 33 (1-2) : 95 (1950)

The fruiting body contains appendages which help in identification. *Ascocarp* rounded black 25x35 μm. *Ascospores*: rounded to ovoid dark brown 8x6 μm. (Plate 2C)
Specimen examined: Compost.

Mucor fragilis Bainier
Ann.sci. Nat. 6sr.19 : 208 (1910)

Colony dirty white, fluffy, rapid growing on YGA at 45 °C reverses creamy. *Sporangiophore* hyaline, smooth walled, coenocytic 18 μm. *Columella* present, bell shaped, apophysis absent, 31 x 33 μm. *Sporangiospore* rounded 4 μm in diameter (Plate 2E).
Specimen examined: Cowpeas. (FCBP # 808)

Mycocladus blakesleeanus (Lender) Mirza Khan, Begum & shugufta,
Mucarales of Pak.: 94 (1979)

Colony off white, fluffy, rapid growing on YGA at 45 °C reverse creamy. *Sporangiophore* hyaline, thick and smooth walled, coenocytic. *Columella* present, bell shaped, apophysate 34-48 x 40-57 μm, size of sporangia + Columella 34-61 x 33-58 μm. *Sporangiospore*: irregular ellipsoid double walled 2.5-3 μm in diameter (Plate 2I).
Specimen examined: *Phaseolus mungo* seed (FCBP # 845)

Mycocladus corymbifera (cohn) Mirza,
(1972)

Colony white, cottony, rapid growing on YGA at 45 °C reverse creamy. *Sporangiophore* hyaline, branched, septum present at base 65-90x5-8μm. *Columella* dark grey, subglobose, apophysate 35-40 x 15-25 μm. *Sporangiospore*: ellipsoid, double walled, 5-3 μm in diameter (Plate 2L).
Specimen examined: Compost. (FCBP # 865)

Rhizomucor sp.

Colony: white, cotton, rapid growing on YGA at 45 °C reverse creamy. *Sporangiophore*: hyaline, smooth walled 65-90 x 5-8 μm. *sporangia* in whorls. *Columella* dark gray, bell shaped, without apophysis, 13-20 x 9-15 μm. *Sporangiospore*: ellipsoid, 2-3μm in diameter (Plate 2K).
Specimen examined: Compost, horse and goat dung. (FCBP# 847)

The thermophilic and thermotolerant fungi were isolated from different substrates including compost, farmyard manure (FMY), leaf mold, and mix substrate (Farmyard + leaf mold), cowpea seeds, field soil and mash beans. The YGA and MEA were found to be the best media for isolating the thermophilic fungi. The Czepecks dox agar medium had been excellent to study the morphological and microscopic characters of *Aspergillus* species. Nearly all the substrates yielded a variety of fungi. However, Maximum species richness (6 species) was recorded in the case of compost followed by (4 species each) leaf manure, farmyard manure and mix substratum. While it reduced to one species in the case of field soil and seeds of chickpea and mash bean (Fig. 2).

Most frequently observed species were of the genus *Thermomyces*. Three *Aspergillus* species and one species each of *Mucor* and *Chaetomium*, which are not thermophilic but may be included in the category of the thermotolerant fungi, are also reported in the present study. These species i.e., *Aspergillus nidulans, A. versicolor* and *A. sparsus* have been isolated for the first time in the present screening.

To recognize the thermophilic and thermotolerant species, if accompanied by morphological and cultural information, appears to be the most useful and logical procedure to define the unique ecological character of thermophily. Cooney and Emerson (1964) taxonomic treatments have been followed in these studies. Since it is the only thorough study available of thermophilic fungi. The subsequent studies like the one conducted by Maheshwari (1997) basically focused on the physiology of thermophilic and thermotolerant fungi.

To check the thermophilism of these fungi, the biomass produced the absorbance of spore suspension and the number of spore/ml at different temperatures is being studied in the next phase of research along with enzyme activity test. It is hoped that the advanced study being carried out would throw some light on the basis of this extreme tolerance by this group of fungi.

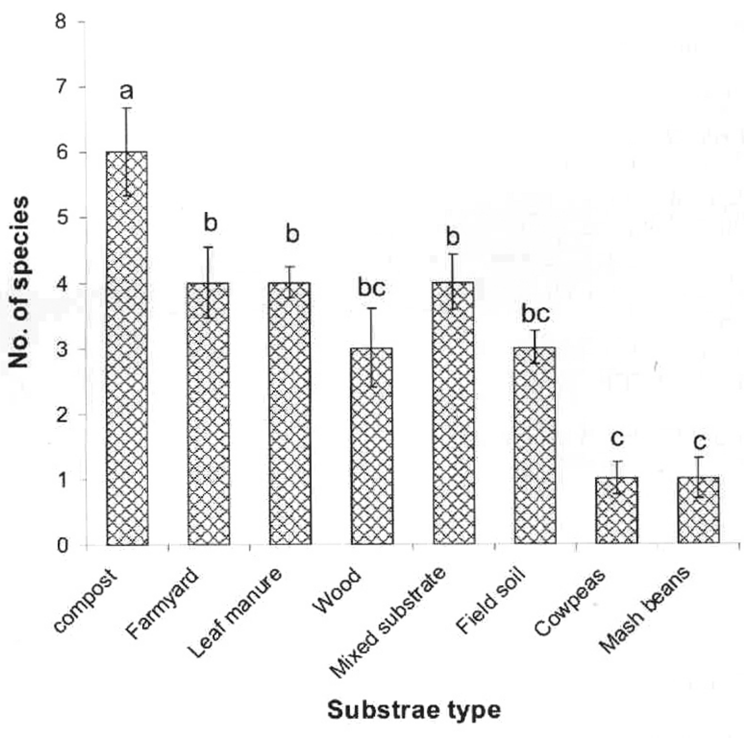

Fig. 2. Richness of different substrates for the Thermophilic and thermotolerant Fungi.

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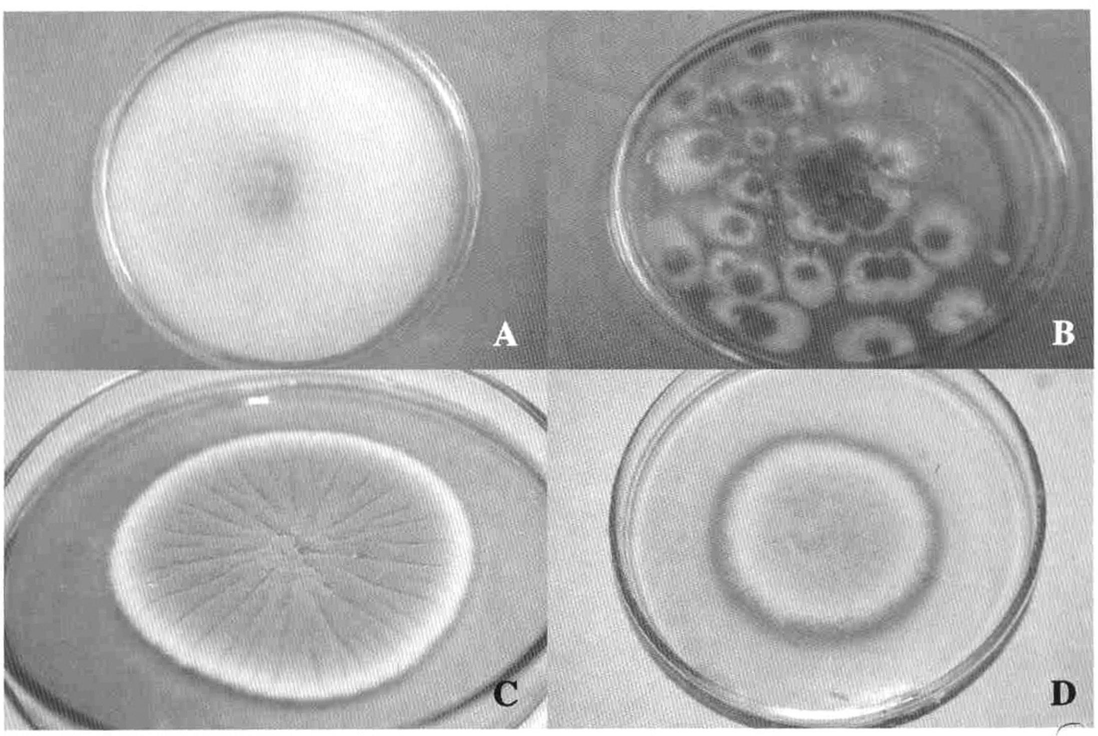

Plate 1*:* **A:** *Rizomucor sp.;* **B:** *Aspergillus nidulans,* **C:** *Aspergillus sparsus,* **D:** *Thermomyces lanuginosus,*

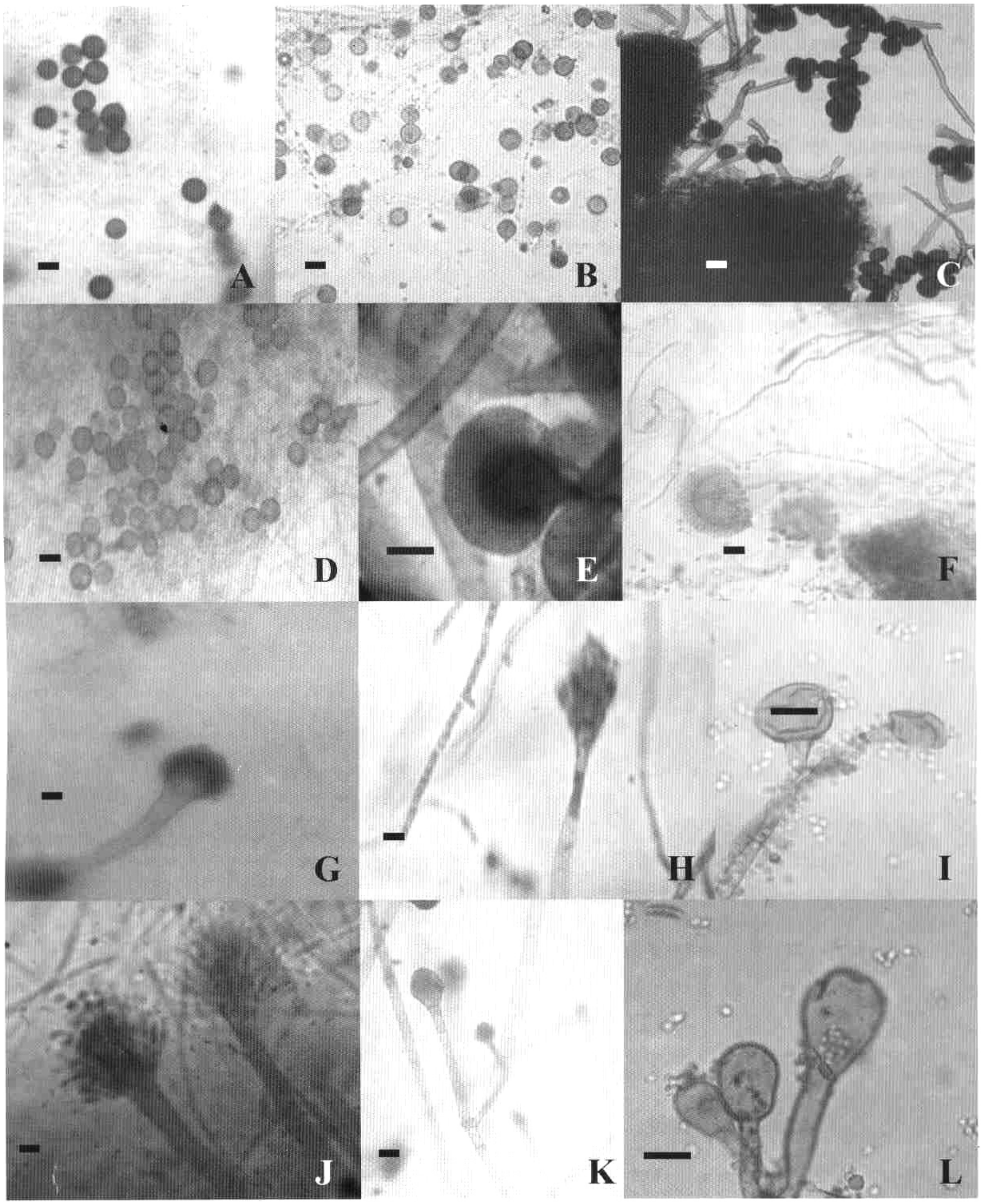

Plate 2. A: *Scytalidium thermophilum*; B: *Thermomyces lanuginosus*; C: *Chaetomium thermophlium*; D: *Humicola grisea var. thermoidea*; E: *Mucor fragilis;* F: *Aspergillus sparsus;* G: *Aspergillus fumigatus*; H: *Aspergillus nidulans;* I: *Mycocladus blakesleeanus;* J: *Aspergillus versicolor;* K: *Rizomucor sp.;* L: *Mycocladus corymbifera)* (bar=10 µm).