Thermophilous fungi from temperate soils of northern India

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Temperate soils of northern India were studied for thermophilic and thermotolerant fungi and 19 species belonging to 14 genera were isolated. Ten species were thermophilic and six were thermotolerant. *Chaetomium senegalense* (Ascomycetes) and *Myceliophthora fergusii* (anamorphic ascomycetes) are reported for the first time from India. The fungal "Colony Forming Units" (CFUs) in temperate soils were investigated by examining soil cores. Studies on importance value index (IVI) and numerical values for population numbers indicated that a higher number of CFUs and isolates occurred in Shimla soil. The temperature of the soil from which all fungi were isolated did not correspond with their optimum axenic growth temperature.

Key Words: colony forming units, importance value index, soil cores, thermal tolerance

Introduction

Microbial species exist in many environments that experience extremes of temperature, pH, chemical content and/or pressure. This occurrence is due to certain genetic and/or physiological adaptations (Cooney and Emerson, 1964; Dix and Webester, 1995; Aguilar, 1996; Stetter, 1999). Of the three domains of life, most of the thermophilic species that have been described belong to Archaea or Eubacteria (Barns *et al*., 1996). Eubacteria are less temperature tolerant as compared to other groups. The maximum temperature limit for Eukaryota has been found to be 62ºC. (Tansey and Brock, 1978). Fungi are considered to be thermophilic if they grow at or above 50ºC and fail to grow at or below 20ºC (Cooney and Emerson, 1964).

There are fewer than 50 species of thermophilic fungi (Mouchacca, 1997). Thermophilic fungi are common in soils and in habitats wherever organic matter heats up for any reason. In recent years thermophilic fungi have been isolated from manure, compost, industrial coal mine soils, beach sands, nuclear reactor effluents, dead sea valley soils and desert soils of Saudi Arabia

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(Redman *et al*., 1999). In these habitats, thermophiles may occur either as resting propagules or as active mycelia depending on the availability of nutrients and favorable environmental conditions.

Generally, there is an inverse relationship between biological diversity and the amount of adaptation required to survive in a specific habitat. Contrary to possible expectations, thermophiles are more frequently isolated from temperate soils than tropical soils (Ellis and Keans, 1981). Thus, we expected that temperate soils of north India would have more fungal diversity with regard to thermophilic fungi and hence are more amenable to our overall aim of characterizing soil fungal communities and their responses to environmental change. The objective of this study were (i) to determine the diversity of culturable thermophilic and/or thermotolerant fungal species in temperate soils of northern India, (ii) to determine the optimal *in vitro* growth conditions for these species, and (iii) to determine importance value index (IVI) for each fungal species.

Materials and methods

Characterization of sites

Thermophilous fungi were cultured from different soil samples collected from four temperate soils (viz. Manali, Mussoorie, Nainital and Shimla). These sites exhibited heterogeneity with regard to temperature, pH, moisture content, altitude and vegetation cover (Table 1). We selected these sites because their eurythermal conditions and dense vegetation suggested a high fungal diversity and hence would be interesting to characterize.

Soil Cores

Soil samples were collected in sterile polyethylene bags with the help of a 5 cm diameter stainless steel corer pre-sterilized with 95% ethanol and 15 cm soil cores were obtained. Ten soil cores were taken from a single site, mixed thoroughly, stored at 4ºC, and processed next day. Soil was prepared by suspending 6 ml (~5 g) of soil in 100 ml of sterile distilled water, vortexing the suspension for 15 minutes, and allowing the debris to settle. After removing the aliquots for culturing, the pH values of the suspensions were determined with the help of a laboratory digital pH meter.

Table 1. Physical and biological parameters of soil samples examined for thermophilic fungi.

Sites	Parameters				
	Altitude (in meters)	Moisture contents (%)	pH	CFU/g (dry wt.)	Number of species isolated
Manali	1871	17.2	6.3	491	7
Mussoorie	2042	29.3	7.3	271	11
Nainital	1938	17.6	6.7	43	7
Shimla	2202	26.3	5.8	1456	12

CFU= Colony Forming Units

Isolation, growth and identification of fungi

A serial dilution agar plating method was employed for the isolation of soil mycota. Soil suspensions were diluted to obtain 20-30 CFUs per plate. All fungi were isolated by plating soil suspensions on YpSs medium (Yeast extract, 4 g; K_2HPO_4, 1 g; $MgSO_4.7H_2O$, 0.5 g; Soluble starch, 15 g; Agar, 20 g; Distilled water, 750 ml; Tap water, 250 ml). The medium contained 50-mg/L streptomycin, which was added to cooled medium after autoclaving to inhibit bacterial growth. For primary isolations Rose bengal (50 mg/L) was also added to the medium. Five plates from each soil sample were incubated for 24 to 96 hours at 45ºC, and each morphologically unique fungal colony was subcultured on YpSs media. All of the fungal species observed grew on YpSs medium, which was used for subsequent experiments and for storage in slants.

All fungal species were identified by microscopic analysis by using taxonomic guides and standard procedures (Raper and Thom, 1949; Ames, 1961; Cooney and Emerson, 1964; Barnett and Hunter, 1972; Samson and Tansey, 1977; and Domsch *et al*., 1980). Unidentified species were distinguished on the basis of absence of any fruiting bodies and were designated as sterile mycelia. The absence of species designation for one isolate of *Aspergillus* (Table 2) does not mean that the organism is a new species, but rather reflect difficulties in taxonomic identification. Identification of some of the isolates was confirmed by the International Mycological Institute (IMI), Egham, Surrey, UK and these isolates are maintained at IMI.

In vitro growth condition and temperature optima of fungi

The temperature optima of various fungal isolates were determined by measuring the colony diameters after incubation of YpSs plates for 24 to 120

hours at 15, 20, 25, 35, 45, 55, 60 and 65ºC. Before incubation, the inoculated plates were kept in sterile polyethylene bags, to prevent contamination and drying of the medium at higher incubation temperatures. The bags were opened twice daily to aerate the cultures. For temperatures above 35ºC a beaker of sterilized water was also placed in each of the incubators containing the inoculated plates to avoid desiccation. The species that grew at 50ºC but not at or below 20ºC were considered thermophilic (Cooney and Emerson, 1964). Growth rates (kd) were determined with the following equation: kd = D/T, where D is the experimentally determined average diameter of the fungal colony in mm exclusive of the diameter of the inoculum (8 mm) and T = time period.

Thermal tolerance of fungi

Mycelial discs (8 mm) without spores from cultures grown at 35ºC were transferred to YpSs plates and incubated at 55ºC for 7 days; then they were again incubated at 35ºC. Colony diameters were measured prior to the temperature shift and at 72, 96 and 120 hours after the shift. The organisms that did not grow at 55ºC but grew after the shift back to 35ºC were considered thermotolerant organisms, and the organisms that grew at 55ºC were considered as thermophilic organisms.

Importance value index (IVI)

The numbers of fungal CFUs were determined for each isolate. A relative importance value index (IVI) for each species was calculated by adding the average frequency, average relative density and the presence value, Singh and Sandhu (1986).

Results

Culturable fungi in temperate soils

The analysis of thermophilous fungi in temperate soils of North Indian region (Manali, Mussoorie, Nainital and Shimla) was carried out. In these soils the average maximum annual temperature is approximately 20ºC, which is the minimum temperature, required for the growth of thermophilic fungi (Cooney and Emerson, 1964). From these soils we cultured 19 different species belonging to 14 genera of thermophilic and thermotolerant fungi on YpSs plates at 45ºC. All of the species that grew at 45ºC were members of the

Ascomycetes, anamorphic ascomycetes or Zygomycetes; no members of the Basidiomycetes were isolated. The optimal growth temperature for most of these fungi was 45ºC when the organisms were grown on YpSs medium. Six species, *Aspergillus fumigatus*, *Chaetomium senegalense*, *Chrysosporium tropicum, Emericella nidulans*, *Penicillium chrysogenum* and *Rhizopus microsporus* had thermotolerant profiles since they did not grow at 55ºC but grew when they were incubated at 35ºC after exposure to 55ºC for 7 days. Ten other species (*Chaetomium thermophile*, *Humicola grisea*, *H. insolens*, *Malbranchea sulfurea*, *Myceliophthora fergusii, Rhizomucor pusillus*, *Thermoascus aurantiacus*, *Thermomyces lanuginosus*, *Torula thermophila* and *Stilbella thermophila*) grew at 55ºC but not below 20ºC. These species were classified as thermophiles (Cooney and Emerson, 1964).

Number of fungal CFUs in soil cores

The average number of fungal CFUs of all the soil samples was 447 (Table 1). We isolated various fungal species listed in Table 2 from different study sites. The data for numbers of species and importance value index (calculated by adding the average frequency, average relative density and the presence value) of different thermophilic fungi at the different study sites is also presented in Table 2. The fungi were less frequent at Nainital and Mussoorie; average CFUs being 43 and 271, respectively, whereas the soils of Shimla and Mussoorie were rich both qualitatively and quantitatively. The average CFUs of thermophilic fungi was highest at Shimla (1,456) followed by Mussoorie (491). *Aspergillus fumigatus* and *Humicola insolens* were common to all the study sites. *Thermomyces lanuginosus* and *Torula thermophila* were isolated from 75% of the study sites. *Chaetomium senegalense* and *Myceliophthora fergussii* were isolated far the first time from Shimla soil and are new records for India. *Chaetomium thermophile*, a frequent colonizer of composts (Kumar and Aneja, 1999a), was less frequent and isolated from Nainital soil only.

The soils at Shimla, Manali and Nainital were slightly acidic (Table 1) and they varied significantly with respect to altitude, pH and organic carbon (data not shown) contents. A total of 12 fungal species were isolated from Shimla soil, whereas 7 fungal species were isolated each from Manali and Nainital. In contrast the pH of Mussoorie soil was slightly alkaline (pH 7.3). However, there was no apparent relationship between soil pH and the number of CFUs. The soils had snow cover at least once in a year and moisture content was much higher in the winter than in the summer months. The moisture content of various soils shown in Table 1 is that of sampling period, *i.e.* in the

month of October. A positive correlation between the number of fungal species and the altitude is established, the number of fungal species isolated were more at higher altitudes.

Discussion

Physical analysis of soil samples revealed that significant variations in soil pH and moisture contents occurred at different sites of north India. Soil samples of Shimla, Nainital and Manali were acidic whereas the soil of Mussoorie is in alkaline range. The relationship between the number of fungal species and altitude was mainly due to the high moisture content and high organic carbon in the soil samples. Waksman and his co-workers reported isolation of thermophilic fungi from soils as early as 1939. Before, several reports on the occurrence of thermophilic fungi in soil had occurred in the literature (Tansey and Brock, 1978; Johri, 1980; and Dix and Webster, 1995). Reports from the habitat in question are scarce (Sandhu and Singh, 1981). In fact the temperate soils of Himalayan region have not been exploited for the occurrence of thermophilic fungi. The frequencies of the species that we isolated corresponded with the altitude. The highest number of fungal CFU occurred in Shimla soil, which is at an altitude of 2202 meters. We cultured 10 true thermophilic fungi and six thermotolerant organisms from the four study sites. Two species viz., *Chaetomium senegalense* (ascomycetes) and *Myceliophthora fergusii* (anamorphic ascomycetes) were recorded for the first time from India. These species were isolated from Shimla soil only and were never isolated from other sites.

The fungi isolated from temperate soils, when compared to those of tropical climates, revealed that they were qualitatively similar (Maheshwari, 1968; Hedges and Rangaswamy, 1969; Johri and Thakre, 1975; Thakre and Johri, 1976; Sundram, 1977; Subrahmanyam, 1980; Sandhu and Singh, 1981; Singh and Sandhu, 1986; Maheshwari *et al.*, 1987). This finding shows that a reservoir of thermophilic and thermotolerant fungi always exists in the temperate soils. Despite the high temperatures, which they require for growth, thermophiles occur in temperate soils where they grow as temperature allows. In these ecological niches, the sun can easily warm the soil above 20ºC on sunny days. Insolation from the sun and widespread occurrence of other habitats suitable for the growth of these molds are the cause of their ubiquity. The average importance value index of *A. fumigatus* was highest. An interesting feature of *A. fumigatus* is that a small amount of fungal biomass can produce a large number of readily air-borne spores which are the cause of its widespread occurrence (Tansey and Flierman, 1978). Thus the species

observed in the present study are worldwide in distribution and there appears no fungal mycota characteristic of the region.

The study revealed a variety of thermophilous fungi, yet it may not represent the whole spectrum of fungal diversity in soils. This may be due to the inhibition of the development of propagules of slow-growing fungi by the competitive and faster-growing fungi, or some species are not represented since their population numbers are too low. Our previous study (Kumar and Aneja, 1999b) has shown that the growth rates of different thermophilic fungi differ at their growth optimum temperature. Since the optimum conditions for growth do not prevail at most times in these soils, rare species are probably less successful thermophiles in terms of adaptation to temperate environments and in competition with other species. The fungal CFUs was greater in soils of cooler climate as compared to those of tropical climates (Kumar, 1996). This could be due to high moisture contents in soils of temperate region. On the contrary, in tropical soils, low moisture contents in summer months may be responsible for physiological stress and desiccation of the spores of these fungi. Our own studies and a review of the literature (Archana and Satyanarayana, 1999) convince us that careful study of the thermophilic fungi present in a particular heated habitat can usually be expected to yield undescribed species. This is important for industrial microbiologists, who are increasingly using thermophilic microorganisms for large-scale fermentations, because they reduce the need for cooling during growth. This reduces costs as compared with the use of mesophiles, where growth would stop if the heat of metabolism were not removed. Additional studies performed with molecular biology based techniques (LaMontagne *et al*., 2002), such as PCR-amplifiable DNA followed by denaturing gradient gel electrophoresis, should reveal whether culturable fungal species are, indeed, the predominant fungal species in these soils and whether such soils harbor uncultured fungi which can provide a more accurate assessment of the microbial biodiversity of these unique habitats.

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Table 2. Occurrence and importance value index (IVI) of thermophilic fungi in temperate soils of north India.

Species	Optimal *in vitro* growth temp. (^{0}C) [a]	Growth rate (mm/h) [a]	Temp class [b]	*In vitro* temp range (^{0}C)	Importance Value Index (IVI)[c] SITES			
					Manali	Mussoorie	Nainital	Shimla
Aspergillus fumigatus	35-45	0.38	TT	15-55	221	198	249	241
Aspergillus sp.	ND	ND	ND	ND	82	99	-	56
Chaetomium thermophile	45	1.13	TP	25-60	-	-	135	-
Chaetomium senegalense	35	0.29	TT	15-50	-	-	-	114
Chaetomium luteum	ND	ND	ND	ND	-	-	-	54
Chrysosporium tropicum	35	1.7	TT	15-45	-	33	-	-
Emericella nidulans	35	0.30	TT	15-55	-	107	-	-
Emericella rugulosa	ND	ND	ND	ND	-	-	-	33
Humicola grisea	55	0.32	TP	20-55	121	-	72	-
Humicola insolens	55	0.35	TP	20-55	229	87	155	87
Malbranchea sulfurea	45	0.54	TP	25-55	-	44	-	-
Penicillium chrysogenum	35	0.22	TT	15-45	214	44	-	-
Myceliophthora fergusii	45	1.9	TP	25-45	-	-	-	48
Rhizomucor pusillus	35-45	0.85	TP	25-60	-	96	-	45
Rhizopus microsporus	35-45	1.10	TT	15-55	-	103	-	31
Thermoascus aurantiacus	35	1.70	TP	20-60	-	-	93	31
Thermomyces lanuginosus	45	0.71	TP	20-60	227	-	61	140
Torula thermophila	45	1.13	TP	25-55	83	34	143	-
Stilbella thermophila	45	0.35	TP	25-55	-	-	-	30
Sterile mycelia	ND	ND	ND	ND	-	42	-	-

[a]Determined on YpSs medium

[b]TP, thermophilic (organisms grew at or above 50^0 C); TT, thermotolerant (organisms survived incubation temperature of 55^{0}C for 7 days); ND, Not determined.

[c]IVI = Average frequency + Average relative density + Presence value