SOIL AMENDMENTS AND ENRICHMENT MEDIA IN THE ECOLOGY OF THERMOPHILIC FUNGI*

by B. N. JOHRI** *and* R. P. THAKRE**, *Department of Botany, University of Saugar, Sagar,* 470003 (*M.P.*)

(*Received 10 October, 1975; after revision 23 December 1975*)

Thermophilic mycoflora was isolated from soils of Chandameta, Parasia and Newton Chikly Collieries in Chhindwara district of M.P. using soil plate method. In order to facilitate recovery of fungi, soil was amended with glucose, cellulose, ammonium nitrate, ammonium phosphate and asparagine and incubated at 45°C prior to plating; alternatively, unamended but incubated soil was plated on ten enrichment media. The thermophilic mycoflora consisted of thirteen fungi belonging to Zygomycetes, Ascomycetes and Deuteromycetes.

INTRODUCTION

Thermophilic fungi occur in a variety of self-heated materials, prominent amongst which are the composts (Kane and Mullins 1973), wood-chips (Ofosu-Asiedu and Smith 1973), soil and seed grains (Taber and Pettit 1975). The mycoflora recovered from such diverse habitats has, however, yielded no more than two dozen purely thermophilic forms. It was thought that low number of thermophiles known may well be the result of limited techniques applied and in order to test the validity of this assumption the thermophilic mycoflora of coal mine soils in Chhindwara District of Madhya Pradesh was studied with the help of inorganic/organic amendments and enrichment media.

MATERIALS AND METHODS

The sampling sites were located in the vicinity of three collieries, viz. Chandameta, Parasia, and Newton Chikly in Chhindwara District of Madhya Pradesh; the soils are designated as S_1, S_2, and S_3 in the text. This site was chosen for obvious reasons, the prime one being the heating due to self combustion. These coalfields are mainly confined to the upper valleys of the Pench and Kanhan rivers on the Satpura plateau. The average temperature of the district is 21.1°C and the annual rainfall approaches an average of 64 cm.

The collection spots were cleaned of all the superficial deposits and a pit of 15 × 15 cm was dug. The soil was loosened, collected in sterile polythene bags and brought to the laboratory for further use. Ten soil samples were collected from each

*Paper presented at the symposium on "Ecology of Soil Microorganisms" held in the Department of Botany, University of Saugar on December 27 to 29, 1974 (Convenor—Prof. S. B. Saksena, FNA).

***Present address:* Department of Bio-Sciences, University of Bhopal, Bhopal (M.P.).

of the three coalfields and after chemical analysis, samples of one locality were mixed together to provide three composite samples. Thermophilic mycoflora was isolated by direct plating, amendments and enrichment media. Amendments in the form of glucose (10 per cent w/w), cellulose powder (10 per cent, w/w), asparagine (5 per cent, w/w), ammonium nitrate (5 per cent w/w) and ammonium phosphate (5 per cent, w/w) were added and the soil samples were incubated at 45°C; isolations were made at intervals of 3, 6, 12, 18, 24 and 30 days on Emerson's Y_pS_s agar. Alternatively, unamended soil samples were incubated at 45°C and the platings were made on the following media for the recovery of thermophiles:

Emerson's Y_pS_s agar —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, 1 litre.

Emerson's Y_pS_s *agar* containing 5% glucose.

Yeast starch medium — soluble starch, 5 g; yeast extract, 2 g; K_2HPO_4, 1 g; $MgSO_4.7H_2O$, 0.5 g; microelement solution, 1 ml; agar, 20 g; distilled water to make 1 litre.

One ml microelement solution contained the following: $Fe(NO_3)_2.9H_2O$, 200 μg; $CuSO_4.5H_2O$, 38 μg; $ZnSO_4.7H_2O$, 225 μg; $MnSO_4.4H_2O$, 24 μg; $Na_2MoO_4.2H_2O$, 40 μg.

Yeast cellulose medium — yeast extract, 4 g; K_2HPO_4, 1 g; $MgSO_4.7H_2O$, 2 g; powdered cellulose, 10 g; agar, 20 g, distilled water, 1 litre.

Malt extract agar — malt extract 20 g; powdered cellulose, 10 g; agar, 20 g; distilled water, 1 litre.

Abram's medium — NH_4NO_3, 3 g; K_2HPO_4, 2 g; KH_2PO_4, 2.5 g; $MgSO_4.7H_2O$, 2 g; agar, 20 g; distilled water, 1 litre.

Abram's medium containing 3% NH_4NO_3.

Abram's medium containing 2.5% K_2HPO_4.

Abram's medium containing 5% cellulose powder.

Glucose-asparagine agar — glucose, 10 g; asparagine, 5 g; KH_2PO_4, 1 g; $MgSO_4.7HO_2$, 0.5 g; agar, 20 g; distilled water, 1 litre.

Soil plate method (Warcup 1950) was used for all isolations. Four replicates were used for each treatment and per cent frequency was calculated on the basis of observations recorded at six intervals.

RESULTS

Though observations for the appearance of thermophilic mycoflora were recorded at six different intervals, the salient features dealt with below take into account only overall frequency. The data for soil amendments are given in Table I and those for enrichment media in Table II.

Soil amendments

Unamended soil samples yielded a limited number of thermophilic fungi; most prominent were, *Absidia corymbifera, Rhizopus microsporus, R. rhizopodiformis* and *Torula thermophila*. Addition of glucose to the soils resulted in the appearance of several new forms, viz., *Sporotrichum* sp., *Thermoascus aurantiacus*,

TABLE 1

Frequency of occurrence of thermophilic fungi in unamended and amended soils

Fungi recorded	Unamended soil			Soil+Glucose			Soil+$(NH_4)_3PO_4$			Soil+NH_4NO_3			Soil+Asparagine			Soil+Cellulose		
	S_1	S_2	S_3	S_1	S_2	S_3	S_1	S_2	S_3	S_1	S_2	S_3	S_1	S_2	S_3	S_1	S_2	S_3
Absidia corymbifera	4.2	17.0	17.0	17.0	60.0	21.0	8.3	—	—	—	—	—	—	8.3	—	—	—	—
Achaetomium macrosporum	—	—	—	—	—	—	17.0	17.0	8.3	66.0	—	—	21.0	8.3	17.0	—	29.0	33.3
Aspergillus fumigatus	100.0	71.0	75.0	87.0	54.2	62.5	83.3	91.7	33.3	29.0	33.3	17.0	—	—	—	33.3	33.3	33.3
Humicola grisea	—	25.0	—	—	4.2	—	—	—	—	—	8.3	12.5	—	—	—	8.3	21.0	33.3
Aspergillus nidulans	46.0	12.5	—	—	33.3	8.3	—	—	21.0	37.5	25.0	33.3	—	—	—	—	—	—
Penicillium sp.	—	—	—	—	8.3	8.3	17.0	17.0	17.0	—	8.3	33.3	37.5	—	—	—	—	—
Rhizopus microsporus	12.5	25.0	25.0	8.3	17.0	17.0	—	—	—	8.3	—	—	8.3	—	8.3	—	—	—
Rhizopus rhizopodiformis	33.3	25.0	29.0	21.0	33.3	8.3	17.0	—	—	—	—	—	—	—	—	17.0	17.0	17.0
Sporotrichum sp.	—	—	—	—	33.3	21.0	—	—	—	—	—	—	17.0	8.0	8.3	—	—	—
Thermoascus aurantiacus	—	—	—	—	8.3	8.3	17.0	—	—	80.0	—	17.0	33.3	33.3	25.0	—	—	—
Thermomyces lanuginosus	—	—	—	—	21.0	33.3	—	—	—	—	—	—	—	—	—	25.0	42.0	25.0
Thielavia minor	—	—	—	—	—	21.0	12.5	8.3	21.0	8.3	—	4.2	4.2	12.5	8.3	—	—	—
Torula thermophila	37.5	4.2	21.0	8.3	17.0	17.0	8.3	—	—	8.3	17.0	12.5	—	17.0	17.0	17.0	—	—

—No fungal colony recorded at any of the six incubation periods.

Thermomyces lanuginosus, and *Thielavia minor*. It should be noted that only five fungi were recovered from S_1 whereas others appeared in S_2 and S_3 samples. Ammonium phosphate-amended soil suppressed the appearance of *Humicola grisea*, *Rhizopus microsporus*, *Sporotricum* sp; and *Thermomyces lanuginosus*; *Absidia corymbifera* could be isolated from S_1 only but all three soils exhibited *Achaetomium macrosporum*. *Aspergillus fumigatus*, a ubiquitous mould and known thermotolerant occurred regularly in fairly high frequency. Ammonium nitrate in the soil suppressed *Absidia corymbifera*, *Rhizopus rhizopodiformis*, *Sporotrichum* sp. and *Thermomyces lanuginosus*; *Achaetomium macrosporum*, however, occurred at a high frequency (66.0 per cent) in S_1. Asparagine and cellulose both, were poor substrates for amendments since no more than half of the total thermophilic mycoflora could be recovered from these samples.

Enrichment media

The selection of enrichment media was based upon the nutritional constituents, osmotic potential and the nature of C/N sources. Emerson's Y_pS_8 agar allowed all but *A. macrosporum*, *Penicillium* sp., and *Torula thermophila* to appear in the isolation plates. Addition of 5 per cent glucose somewhat compensated this nutritional deficiency. Yeast-starch and yeast-cellulose media were quite comparable in the pattern of thermophilic mycoflora except for some minor details; absence of *Thermoascus aurantiacus* in the latter was prominent. Malt extract medium turned out to be a poor choice since six fungal forms could be recovered. Abram's synthetic medium also did not allow strongly thermophilic *T. aurantiacus* and *Thermomyces lanuginosus* to appear in the plates. Amongst those present, *A. macrosporum*, *R. microsporus* and *Torula thermophila* were recovered from S_1 sample only. Addition of 3 per cent NH_4NO_3 or 2.5 per cent K_2HPO_4 changed the pattern of mycoflora only slightly. *Penicillium* sp., however, did not appear on all these media; this mould was also absent in collulose-amended Abram's medium. *Achaetomium macrosporum*, *Aspergillus nidulans*, *Rhizopus rhizopodiformis*, *Sporotrichum* sp. and *Thermomyces lanuginosus* did not appear on glucose-asparagine plates.

The overall frequency (per cent) for different taxonomic groups is as follows: mucoraceous—6.0, 7.5, 9.0; ascomycetous—7.0, 10.7, 9.7; deuteromycetous—15.4, 18.9 and 15.6 for S_1, S_2 and S_3 respectively. Thus, in spite of obvious differences in the pattern of thermophilic mycoflora from the three coal mine localities, there appeared to be little variation in the total average presence of these fungi.

Discussion

Soil as a source of thermophilic mycoflora has been ably exploited by Apinis (1963), Eggins and Malik (1969) and Evans (1971*a*). These reports are, however, confined to temperate conditions and a common observation made by these authors was that thermophiles mostly occurred in surface layers. Apinis recorded 20 fungal forms from alluvial soils while Evans recovered thirty-two thermotolerant and thermophilic moulds from coal spoil tips. The coal mine soils, in contrast, resulted in isolation of only thirteen thermophilic fungi and this low number has apparently resulted from the fact that the sampling sites were close to the colliery area and thus

TABLE

Occurrence of thermophilic fungi

Fungi recorded	Y_pS_s agar			Y_pS_s with 5% glucose			Yeast starch			Yeast cellulose		
	S_1	S_2	S_3	S_1	S_2	S_3	S_1	S_2	S_3	S_1	S_2	S_3
Absidia corymbifera	—	8.3	8.3	—	—	17.0	—	—	17.0	17.0	17.0	17.0
Achaetomium macrosporum	—	—	—	17.0	42.0	29.0	—	—	—	—	—	—
Aspergillus fumigatus	96.6	66.6	50.0	96.0	75.0	83.3	92.0	75.0	83.3	100.0	87.5	71.0
Aspergillus nidulans	—	25.0	29.0	25.0	8.3	12.5	25.0	25.0	12.5	—	25.0	—
Humicola grisea	—	8.3	21.0	—	8.3	—	—	17.0	8.3	8.3	33.3	29.0
Penicillium sp.	—	—	—	—	—	—	—	—	—	21.0	21.0	—
Rhizopus microsporus	8.3	—	—	—	—	8.3	—	—	17.0	—	8.3	17.0
Rhizopus rhizopodiformis	12.5	8.3	25.0	—	—	—	—	—	—	—	—	—
Sporotrichum sp.	8.3	54.0	—	12.5	21.0	33.3	17.0	17.0	8.3	—	—	—
Thermoascus aurantiacus	—	4.2	—	21.0	—	—	—	17.0	8.3	—	—	—
Thermomyces lanuginosus	17.0	17.0	25.0	17.0	33.3	—	8.3	33.3	8.3	29.0	12.5	—
Thielavia minor	—	58.0	37.5	8.3	29.0	42.0	—	25.0	—	—	—	—
Torula thermophila	—	—	—	8.3	—	—	—	—	—	—	—	—

— No fungal colony recorded at any of the six incubation periods.

devoid of plant cover. In general, grasses alone were present in the sampling area which, therefore, experienced excessive heating and lack of a thick litter layer did not provide sufficient nutrients for the growth and sustenance of the microbes. These observations are in agreement with those of Evans (1971*a*).

The distributional pattern of mycoflora isolated from cellulose and glucose-amended soils resembled closely with the observations noted by Eggins and Malik (1969) in a pasture land soil. The use of enrichment media not only allowed isolation of a variety of thermophiles but also provided an indication of their nutritional behaviour. Thus, *Absidia corymbifera, Rhizopus microsporus* and *R. rhizopodiformis* did not tolerate high levels of inorganic nitrogen and were, therefore, absent on Abram's medium supplemented with 3 per cent NH_4NO_3. *Rhizopus rhizopodiformis* appeared to be comparatively less osmotolerant than the other two mucoraceous members. The fastidious nature of *Achaetomium macrosporum* was evident from the fact that this mould did not appear on more than half of the enrichment media. The species of *Penicillum* was invariably found to be associated with *Thermoascus aurantiacus* in the isolation plates and inhibited the latter to a considerable degree; the only other thermophile so far known to show antagonism is *Malbranchea pulchella* var. *sulfurea* (Emerson 1968). The ability of thermophiles to appear on complex media containing starch and cellulose suggests the presence of strong amylolytic and cellulolytic machinery and thus points towards their industrial exploitation (Fergus 1969); such

II

on enrichment media (%)

Malt extract			Abram's medium (AM)			AM with 3% NH_4NO_3			AM with 2.5% K_2HPO_4			AM with 5% cellulose			Glucose-asparagine		
S_1	S_2	S_3	S_1	S_2	S_3	S_1	S_2	S_3	S_1	S_2	S_3	S_1	S_2	S_3	S_1	S_2	S_3
—	17.0	17.0	17.0	17.0	17.0	—	—	—	—	—	25.0	21.0	17.0	25.0	—	17.0	17.0
—	—	—	29.0	—	—	29.0	—	8.3	8.3	17.0	8.3	—	—	8.3	—	—	—
96.0	83.3	66.6	33.3	25.0	33.3	62.0	66.6	33.3	46.0	46.0	33.3	83.3	66.6	42.0	100.0	75.0	75.0
—	—	—	—	—	29.0	12.5	8.3	—	—	17.0	—	—	—	—	—	—	—
—	—	—	8.3	17.0	25.0	8.3	21.0	25.0	—	—	—	—	—	8.3	8.3	8.3	4.2
—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	25.0	37.5	42.0
—	—	8.3	8.3	—	—	—	—	—	—	—	8.3	8.3	12.5	8.3	—	—	8.3
17.0	17.0	—	—	17.0	8.3	—	—	—	17.0	—	—	25.0	—	33.3	—	—	—
—	46.0	42.0	—	50.0	46.0	—	17.0	50.0	8.3	58.0	4.2	—	17.0	29.0	—	—	—
—	—	—	—	—	—	—	8.3	8.3	8.3	17.0	17.0	—	8.3	—	21.0	42.0	17.0
37.5	17.0	17.0	—	—	—	—	—	17.0	—	8.3	—	—	12.5	50.0	—	—	—
—	—	—	42.0	33.3	17.0	42.0	37.5	25.0	21.0	17.0	8.3	4.2	8.3	8.3	17.0	33.3	62.0
—	—	—	17.0	—	—	33.3	29.0	17.0	17.0	—	8.3	—	17.0	8.3	—	62.0	50.0

complex media also suppress fast-growing sugar-loving forms and allow the slow colonizers to express themselves.

The narrow margin in the per cent presence of individual thermophile was not unexpected since soils of the three sampling sites differed only marginally in their physical and chemical characteristics. The regular appearance of slow-growing *Sporotrichum* sp. in all the three soils appears to have resulted from the slow pace of natural microbial competition during the period of incubation. These observations are in contrast to those recorded by Apinis (1963) for alluvial soils where frequency of occurrence of *A fumigatus, Absidia ramosa, Byssochlamys nivea* and *Sporotrichum thermophile* were comparable. The coal mine soils on the contrary appear to be dominated by the thermotolerant *A. fumigatus*. The frequency levels of *Thermoascus aurantiacus* and *Thermomyces lanuginosus* in alluvial and coal mine soils, however, compare favourably. Similar observations for relatively high incidence of thermophiles from wheat straw compost and coal spoil tips are existent in the literature (Chang and Hudson 1967; Evans 1971*b*).

ACKNOWLEDGEMENTS

The authors are thankful to Prof. S. B. Saksena, FNA, for providing the laboratory facilities.

REFERENCES

Apinis, A. E. (1963). Occurrence of thermophilous microfungi in certain alluvial soils near Nottingham. *Nova Hedwigia,* **5,** 57–78.

Chang, Y., and Hudson, J. J. (1967). The fungi of wheat straw compost. I. Ecological studies. *Trans. Br. mycol. Soc.*, **50,** 649–666.

Eggins, H. O. W., and Malik, K. A. (1969). The occurrence of thermophilic cellulolytic fungi in a pasture land soil. *Antonie von Leuwenhoek J. Microbiol. Serol.*, **35,** 178–184.

Emerson, R. (1968). Thermophiles. *In:* The Fungi, Vol. III, 105–128, (*ed. by* Aimsworth and Sumsman). Academic Press, London and New York.

Evans, H. C. (1971*a*). Thermophilous fungi of coal spoil tips I. Taxonomy. *Trans. Br. mycol. Soc.*, **57,** 241–253.

———— (1971*b*). Thermophilous fungi of coal spoil tips. III. Seasonal and spatial occurrence. *Trans. Br. mycol. Soc.*, **57,** 267–272.

Fergus, C. L. (1969). The cellulolytic activity of thermophilic fungi and actinomycetes. *Mycologia,* **61,** 120–129.

Kane, B. E., and Mullins, J. T. (1973). Thermophilic fungi in a municipal waste compost system. *Mycologia,* **65,** 1087–1100.

Ofosu-Asiedu, A., and Smith, R. S. (1973) Degradation of three softwoods by thermophilic and thermotolerant fungi. *Mycologia,* **65,** 240–244.

Taber, R. A., and Pettit, R. E. (1975). Occurrence of thermophilic microorganisms in peanuts and peanut soil. *Mycologia,* **67,** 157–161.

Warcup, J. H. (1950). Soil plate method for isolation of fungi from soil. *Nature, Lond.*, **166,** 117.