THE BIODEGRADATION OF CERTAIN COMPONENTS OF TOWN WASTE BY THERMOPHILIC FUNGI

A thesis presented in partial fulfilment of the requirements for the degree of Doctor of Philosophy.

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SUMMARY

An investigation to elucidate the possible role played by the thermophilic fungi in the process of town waste composting was carried out since previous observations had suggested that several species were highly cellulolytic but that very few species had ever been isolated from composting town waste. Biochemical studies on a small town waste windrow confirmed that a high percentage of cellulose was utilised, and that the pH of the waste rose to alkaline values during the thermophilic phase of composting. This suggested that those cellulolytic thermophiles which had been isolated from composting town waste were successful in colonising this substrate because they were able to adapt to environmental parameters imposed by such a system. This was partially confirmed by a study of the cellulolytic activity of the twelve cellulolytic thermophiles in isolation, on cellulose perfused with pH-buffered salts, by their inter-reactions on cellulose, and by a study of their C_x cellulase enzymes. The results indicated that the six cellulolytic thermophiles isolated from composting town waste could grow, sporulate, produce active cellulase enzymes and compete together successfully on cellulose under conditions similar to those imposed by the town waste composting process. Suggestions for future work were outlined. The activity of the thermophilic fungi towards synthetic polymers and plasticisers was studied since these materials are found in increasing quantities within town waste. Polyethylene and polyvinyl chloride were non-biodegradable and a process in which these recalcitrant molecules could be recycled was described. All nineteen thermophiles tested were shown to actively produce esterase enzymes when grown upon ester plasticisers. Finally, all thermophilic fungi under test were able to produce humus substances which were similar in composition to substances isolated from composted town waste.

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CHAPTER 1

CHAPTER 1

Introduction

> "Today, the environment is being polluted, as never before, by the accumulation of solid wastes - a staggering burden born of affluence, nurtured by rising populations, fostered by technology, and all but neglected by society."
>
> W. E. Gilbertson (1969)

Town refuse is solid waste arising from urban activity which is assisted to the final place of disposal by organised collection facilities. Disposal is usually carried out by one of several approved methods which include incineration, landfilling, pyrolysis and composting. The composting of town waste represents a most significant method for the reuse of solid waste since the organic fraction is converted into a stable, humus-like substance which is considered to have valuable soil conditioning properties. The work presented in this thesis will be an investigation into the biodegradation of two important isolated components of town waste, cellulose and "plastics", by a group of microfungi, the thermophilic fungi, which are considered to play an important role in the decomposition of organic material during the composting process.

Refuse - the magnitude of the problem

In 1970 a Government Report was issued (HMSO 1970) which

represented the findings of a working party concerned with refuse disposal in this country. Their findings indicated that refuse collected in the years 1966/67 was disposed of by local authorities in the following manner:-

(a) Direct tipping on land (90.4%)

(b) Separation and Incineration (7.6%)

(c) Direct incineration (0.7%)

(d) Pulverisation (1.0%) and

(e) Composting (0.3%).

Figures, in comparison, for the U.S.A. show that 24% of their town waste remains uncollected, 6% is incinerated, 70% is tipped - with about 2% of this being previously pulverised, but very little is composted (Tinker 1972). The government report goes on to state that over 50% of the authorities they approached proposed to continue controlled tipping, and 20% were considering incineration because of difficulties in obtaining tipping sites. Tipping in fact seems to be a "thing of the future and not of the past" - the only problem being cost of transportation, since in some areas refuse has to be transported quite considerable distances to the tipping site (Abrahams 1969, Clutterbuck 1971).

Composting in this country is practised only on a small scale. Some plants, such as the one at Chesterfield, add sewage sludge to increase the moisture content of their composting town waste; however as a process, composting of town refuse is expensive and there is no widescale demand (in this country) for the product (HMSO 1970). These comments do not apply however in countries such as Holland where town waste composting has been carried out on a large scale for many years.

The situation in America is that 360 million tons of solid waste is produced per year (Breidenbach and Eldredge 1969, Schulze 1971) and plain dumping and landfilling are still the most widely used methods for its disposal. There are 13,626 landfilling sites in the U.S.A. and 260 incinerators (Schulze 1971) but apparently this author and Stickelberger (1971) think that only 5 - 10% of these landfills are considered satisfactory from a sanitary point of view. Schulze goes on to say that from a practical viewpoint composting does not really exist as a solid waste disposal method in the U.S.A., but makes a plea that since most of the waste is potentially biodegradable, composting should be carried out on a larger scale. Inorganics could still be added to produce a fertiliser, and he considers that the only way to produce humus is by composting.

Why then are authorities reluctant to compost their town waste? It is suggested that as a process it is expensive and in some countries there is little demand for the product, but are other factors also involved?

Table 1 shows the changing composition of British refuse since 1888 (HMSO 1970, Tinker 1972). Several noticeable factors stand out when one examines this table:- (a) The advent of smokeless fuel together with oil, gas and electricity for domestic heating has reduced the amount of dust, ash and cinders in refuse over the past decade. (b) the largest increase in refuse volume has resulted from the packaging industry with large increases in the amounts of paper and plastics within town refuse. The increase in cellulose content would seem to make refuse an ideal substrate for the biological composting process. (c) The volume of glass waste is expected to remain fairly constant over the next 10 years since much glass is in the form of returnable bottles (HMSO 1970). The advent

TABLE 1

Showing the changing % composition of household refuse in the U.K. since 1888, and the projected composition by 1980 if present trends continue.

	1888	1935/6	1963	1967	1968	1980
Dust, Ash, Cinders	81	57	39	31	22	12
Paper & Cardboard	1	14	23	30	37	43
Glass	2	3	9	8	9	9
Metal	1	4	8	8	9	9
Plastics	-	-	-	1	1	5
Rags & Textiles	1	2	3	2	2	3
Vegetable & Organic	13	14	13	16	18	17
Miscellaneous	1	6	5	4	2	2

of plastic disposable milk and beer bottles and the plastic lined cardboard containers for milk are also helping to keep the amounts of glass waste constant, but they are contributing greatly to the ever increasing amounts of plastics waste in refuse.

Cellulose presents no problem in the composting process since it is readily degraded by microorganisms, however plastics are relatively inert materials biologically and will not rot away during the composting process. Moreover they are difficult to separate off mechanically from town waste, unlike the inorganic materials also present within town waste.

Staudinger (1970) found that in 1968 the consumption of plastics in the U.K. was 1,105,000 tons and that plastics used in packaging represented 250,000 tons i.e. 22% of consumption. From figures based on refuse collected by the City of Birmingham, plastics in collected refuse amounted to 1.12% of the total domestic refuse collected in 1968, and because of their low density and voluminosity represented 3 - 5% of the domestic refuse volum When one considers the packaging sector it can be seen that the polyolefins as a group constitute the major proportion of all plastics used by the packaging industry (Table 2). Of these polymers, 50% were used in the form of films and thin sheets, 18% as bottles and containers, 10% as moulded containers, 9% as closures, 5% as coatings, 5% as crates and boxes and 3% as other forms including foams (Staudinger 1970). It has been estimated that by 1980, if present trends continue, the projected amounts of plastics in town refuse will have increased to 5% by weight, with just over 50% being polyolefins, 20% P.V.C., under 20% polystyrenes and the rest miscellaneous materials (Staudinger 1970, HMSO 1971, Anon 1971, Tinker 1972).

TABLE 2

From Staudinger (1970) showing U.K.
consumption of plastics used in packaging in 1968

Polyolefins (including 8% polypropylene)	74%
Polystyrenes	16%
P.V.C.	4%
Thermosetts	3%
Miscellaneous	3%

The figures presented here are being echoed throughout the Western World at the present time. Figures for Germany and the U. S. A. show increasing amounts of plastics in refuse (Abrahams 1969, Staudinger 1970). Are existing disposal methods suitable for removing or recycling this sort of waste? Plastics will not rot away in the soil, and because they are not easily compressed can produce cavities and air pockets within the soil making such a landfill unsuitable for supporting roads and building constructions. Even with pulverisation it is unlikely that reclaimed land containing large quantities of plastics can be used for anything else but landscaping (Staudinger 1970). The incineration of plastics waste has its problems also (Cheater 1970). Polyethylene drips on burning, as does polypropylene, Polyamides and ABS plastics with resultant clogging up of incinerators. Polystyrene develops dense sooty smoke and Polymethyl methacrylate produces aromatic odours. The halogenated plastics P.V.C. and PTFE give off HCl and HF gas which react readily with the incinerator walls causing corrosion (Rolfe 1969, Staudinger 1970). Apart from these effects there is also the danger of atmospheric pollution by smoke and gases released from burning plastics. New furnaces are being designed however to cope with the problems involved in the complete combustion of plastics waste, and pyrolysis plants may be another answer to this problem, since the emerging gases have a high calorific value, and a substantial proportion of the carbon in these gases originates from the plastics present in the original waste (Kaupert 1968).

A certain amount of plastics waste can be recycled at source within industry, however recycling of plastics in refuse presents other problems. The objectionable nature of handpicking would involve high labour costs, and since plastics still represent a small fraction of town waste this

whole operation would be uneconomical (Mack 1971). The plastics would have to be graded according to type and then cleaned of surface debris, since "dirty" plastics cannot be recycled. There would then be the problem of marketing such material. The HMSO (1970) report states that at present there is no demand from industry for re-use of plastics. The U.S.A. government will not accept any plastic product in the military or civil service that is not "100% virgin material" (Mack 1971). It is now possible however to grind up waste plastic with paper and press it into boards. The particular company involved say that it would be quite feasible to use plastics waste from town refuse if the problems associated with separation of the plastics could be overcome.

It is generally agreed that the majority of polymers in use today are resistant to biodegradation, but that the plasticisers, for example in plasticised P.V.C., can be degraded biologically. Since the majority of plastics ending up in town waste are of the unplasticised type, it might be anticipated that such plastics would be unchanged at the end of the composting period. Compost containing a high quantity of plastics poses aesthetic problems and few farmers are willing to spread this material onto their fields.

If existing plastics are not biodegradable, can they be produced in a biodegradable form in the future? It is still unknown why synthetic polymers are resistant to microbiological attack (Lewin 1971), and the idea of "microbial infallibility" has been called into question by the advent of these new materials (Hughes 1969). To produce a completely new "biodegradable polymer" would be inevitably more expensive than present materials due to the more limited scale of manufacture (Lewin 1971,

Eggins et al. 1972), however a new approach to this problem has recently been worked out by Scott and his team at Aston University, Birmingham. They have incorporated into existing plastics photoactivators, which will act as a "switch" mechanism to induce photodegradation of the polymer when the polymer becomes litter. The U/V component of sunlight splits these hydroperoxide groupings into free radicles:-

$$ROOH \xrightarrow{U/V} RO\cdot + \cdot OH,$$ and these radicles then initiate the autocatalytic oxidation process:-

$$RO\cdot + 2RH \longrightarrow ROOH + ROH + R$$
$$\cdot OH + 2RH \longrightarrow ROOH + H_2O + R$$

Initial laboratory experiments have produced oxidation products with molecular weights as low as 5000 (Dr. L. Gan - personal communication) and it is hoped that biological testing will indicate whether such material is subsequently biodegradable. This approach however, does not recognise the problems posed by plastics waste in town refuse, where only photo-degradable plastics in the outer layers of a compost heap will be exposed to the U/V component of sunlight. It would appear therefore that to produce an acceptable compost from town waste the plastics content must be reduced, or in some way eliminated, to overcome the reluctance of potential users to accept the product. The final stage of the composting process produces a stable, dark coloured end product, "humus".

Municipal compost, it is claimed, has little value as a fertiliser, being deficient in potash and inorganic nitrogen (Kershaw 1968, HMSO 1970). Typical figures for compost in this country show 0.67% nitrogen, 0.6%

phosphate (P_2O_5) and 0.06% potash (K_2O) [Chesterfield data], and figures for American compost show 0.58% nitrogen, 0.23% phosphorus (sic) and 0.21% potassium (sic) [Hortenstine 1971]. The value of this type of compost is based therefore on its soil conditioning properties; the humus when added to the land helps to improve the structure of the soil and its moisture retaining properties (Humbolt Information - undated -, Kershaw 1968, Stickelberger 1971).

Composting - the biological process

The composting of town waste involves a three stage biological process during which heat is generated within the mass of solid waste, organic material is degraded and the production of a stable humus-like material is achieved. The first, or prethermophilic stage, is usually a relatively short stage during which microbial thermogenesis raises the temperature within the mass of town waste to within the temperature range for growth of the thermophilic microorganisms. The second, or thermophilic stage, usually lasts for several weeks (in the windrow method of composting), during which time the activity of the thermophilic microorganisms may raise the temperature within the mass of town waste to 75°C and above. The third, or mesophilic stage, occurs when the temperature within the mass of town waste falls back to within the temperature limits for growth of the mesophilic microorganisms. During these three stages the complex organic material within the town waste is enzymatically degraded to simpler stable compounds.

Initial studies by mycologists at the beginning of the present century demonstrated the existence of a small group of fungi, the thermophilic

fungi, which could survive at the high temperatures recorded within compost heaps but could not grow at normal ambient temperatures. To date, about twenty-one species of thermophilic fungi have been described, and the majority of these fungi have been isolated from self heating composting systems. Although data is beginning to accumulate about the taxonomy, ecology andphysiology of these fungi, very little is known about their activity towards solid wastes and their possible role in the process of the composting of town waste. It is necessary therefore, at this stage, to define what is meant by a "thermophilic" fungus, and to review what is already known about this interesting group of microfungi.

Thermophilic Fungi

Thermophily - a love of heat - is a characteristic found amongst relatively few groups of organisms which have discovered a unique ecological niche where competition for nutrients, oxygen and other vital parameters will be limiting only to other members of the thermophilic population. Thermophily within the fungi was first described eighty-six years ago by Lindt (1886) who isolated Mucor pusillus from damp bread which had been incubated at 30°C. There are, of course, many mesophilic fungi which will grow actively at 30°C but Lindt went on to determine the temperature limits for growth of M. pusillus and found them to be approximately 20°C to 55°C. The fact that no thermophilic fungus had been isolated before 1886 was most likely due to the inability of mycologists of the time to incubate their substrates and cultures at temperatures above 25°C; and yet here was a fungus showing very little growth at ambient temperatures but having an optimum temperature for growth of about 50°C.

Tsiklinskaya (1889) described the second thermophilic fungus, Humicola lanuginosa*, which she had isolated from a potato which had been inoculated with crumbs of soil and incubated at "slightly elevated temperatures". She maintained the fungus on damp bread at 52 - 53°C.

It was not until 1907 that thermophily within the fungi came under the scholarly review of Hugo Miehe. In his study of self heating organic materials he isolated a thermophilic zygospore producing Mucor species he believed to be M. pusillus, and H. lanuginosa. He also isolated the third and fourth thermophilic fungi, Malbranchea pulchella var. sulphurea and Thermoascus aurantiacus (Miehe 1907 a, b). Miehe's isolate of T. aurantiacus would not grow at temperatures below 30°C and showed optimal growth between 40 and 50°C, whilst his isolate of M. pulchella had growth limits between 26 and 53°C.

Four years later in 1911 Griffon and Maublanc described the fifth thermophilic fungus Talaromyces (Penicillium) duponti which had been isolated from self heating manure and damp hay by a M. Dupont (Griffon and Maublanc 1911). This fungus had growth limits between 25 and 60°C.

No new thermophilic fungi were described between the years 1912 to 1950, and then in 1950 La Touche described Chaetomium thermophile. In the same year Cooney and Emerson isolated, and later described (1964) two varieties of C. thermophile viz: variety dissitum and variety coprophile.

*Footnote Taxonomic nomenclature from Cooney and Emerson (1964)

Growth limits for these varieties were between 27 and 58°C.

Cooney and Emerson described four new species and three new varieties of thermophilic fungi. The first new species was Humicola insolens, originally isolated by Allen et al. (1944) and designated as # 3HF. This fungus was isolated from self heating (retting) guayule and had growth limits between 23 and 55°C. Their second new species was also isolated from retting guayule and named Mucor miehei in honour of Miehe. This fungus was shown to be homothallic by single spore cultures and to regularly form large numbers of zygospores in culture, in contrast to the heterothallic M. pusillus. The temperature growth limits for M. miehei were 25 to 57°C and it seems likely that this fungus was in fact the zygospore producing "Mucor pusillus" isolated in 1907 by Miehe (see Cooney and Emerson 1964 p. 21). The third and fourth new species to be described by Cooney and Emerson were both isolated from straw nesting material; these were Myriococcum albomyces (temperature limits 26 to 57°C) and Torula thermophila (temperature limits 23 to 58°C).

As well as the four new species the authors also describe three new varieties - the previously noted varieties of Chaetomium thermophile, and Humicola grisea var. thermoidea which they isolated from elephant dung in 1948. This last variety had its growth range between 24 and 56°C.

Cooney and Emerson's study of the eleven thermophilic fungi known up to 1950 and the four others to be isolated by other authors between 1950 and 1964 led them to propose a working definition of a thermophilic fungus as "one which has a maximum temperature for growth at or above 50°C and a minimum temperature for growth at or above 20°C."

This definition is the one usually accepted today and excludes mesophilic and thermotolerant fungi, many of which can grow at temperatures above 40°C but which can also grow vigorously at temperatures below 20°C. The only thermotolerant fungus to be considered as a "thermophile" in this thesis will be Aspergillus fumigatus Fresenius, a species which will grow at 12°C but is usually isolated along with the true thermophilic fungi at 50°C from self heating systems. The growth range of A. fumigatus has been given as 12 to 52°C (Cooney and Emerson 1964), however the author has observed slight growth of this fungus with highly misshapen spore heads even at 58°C.

The fourth thermophilic Humicola species was isolated in 1959 from mouldy hay that had spontaneously heated during storage, and named Humicola stellata after its stellate aleuriospores. Its growth limits were determined as 24 to 50°C (Bunce 1961).

The first thermophilic species of Sporotrichum was isolated initially by Henssen (1957) from stable manure and later by Apinis from soil. He described it as Sporotrichum thermophile (Apinis 1963) with growth limits 24 to 55°C (Cooney and Emerson 1964).

The second thermophilic Talaromyces species was isolated from compost (Stolk 1965) and from palm oil kernels (Eggins and Coursey 1964) in 1961 and named T. emersonii in honour of Professor R. Emerson. In contrast to T. duponti the fungus readily produces ascocarps. Stolk sets the temperature limits of this fungus at 25 to 55°C, however the author's isolate from soil (IMI 155697) produced very little growth below 30°C, had an optimum temperature of about 50°C and still produced good growth at

55°C. It would not grow at 60°C and its upper temperature limit for growth appeared to be about 58°C (Mills et al 1971).

Allescheria terrestris was the next new thermophilic fungus to be described (Apinis 1963). It has a Cephalosporium imperfect stage and a range for growth of 22 to 55°C (Evans 1969). A thermophilic Cephalosporium species isolated by the author was found to produce only the asexual stage. This isolate will be referred to as Cephalosporium sp since the taxonomy of this group of fungi is still under review (W. Gams, Centraalbureau voor Schimmelcultures - personal communication).

The first coremioid thermophilic fungus was described by Fergus (1964) as Stilbella thermophila, isolated from mushroom compost, Its growth limits are between 35 and 55°C.

A thermophilic lipolytic fungus was described in 1966 by Apinis and Eggins. This fungus, Thermomyces ibadanensis, having growth limits between 31 and 60°C, was isolated in 1961 from self heating palm kernel stacks in Nigeria by Eggins.

Thielavia thermophila was described by Fergus and Sinden (1969). It has a Sporotrichum imperfect stage, but unlike S. thermophile which does not produce a sexual stage (Hedger and Hudson 1970), this new species produces small black cleistothecia as well as the imperfect stage. Its growth limits are given as 20 to 56°C.

A second coremioid fungus was described by Mirza and Qureshi (1970) as Didymostilbe coprophile. They mention that this fungus grew

only very slowly at 35°C and not at all at room temperature. They make no mention of its upper limit of growth but say that growth and sporulation was good at 40°C. This fungus, isolated from farm yard manure would appear to be a true thermophilic fungus, although, to date, little information is available about its physiology. Fergus (1971, a) isolated another new thermophile, Papulaspora thermophila, from mushroom compost. This fungus has the most limited range of temperature over which it can grow of any of the thermophilic fungi viz: 30 to 52°C.

The latest known thermophile, at the time of writing, is a thermophilic Mycelia Sterilia isolated by Craveri et al. (1972) from compost and soil. Its growth range is given as 26 to 53°C and the authors found that this fungus is able to produce an antifungal antibiotic, thermozymocidin. Table 3 shows the growth temperature ranges of the known thermophilic fungi.

The Occurrence of Thermophilic Fungi

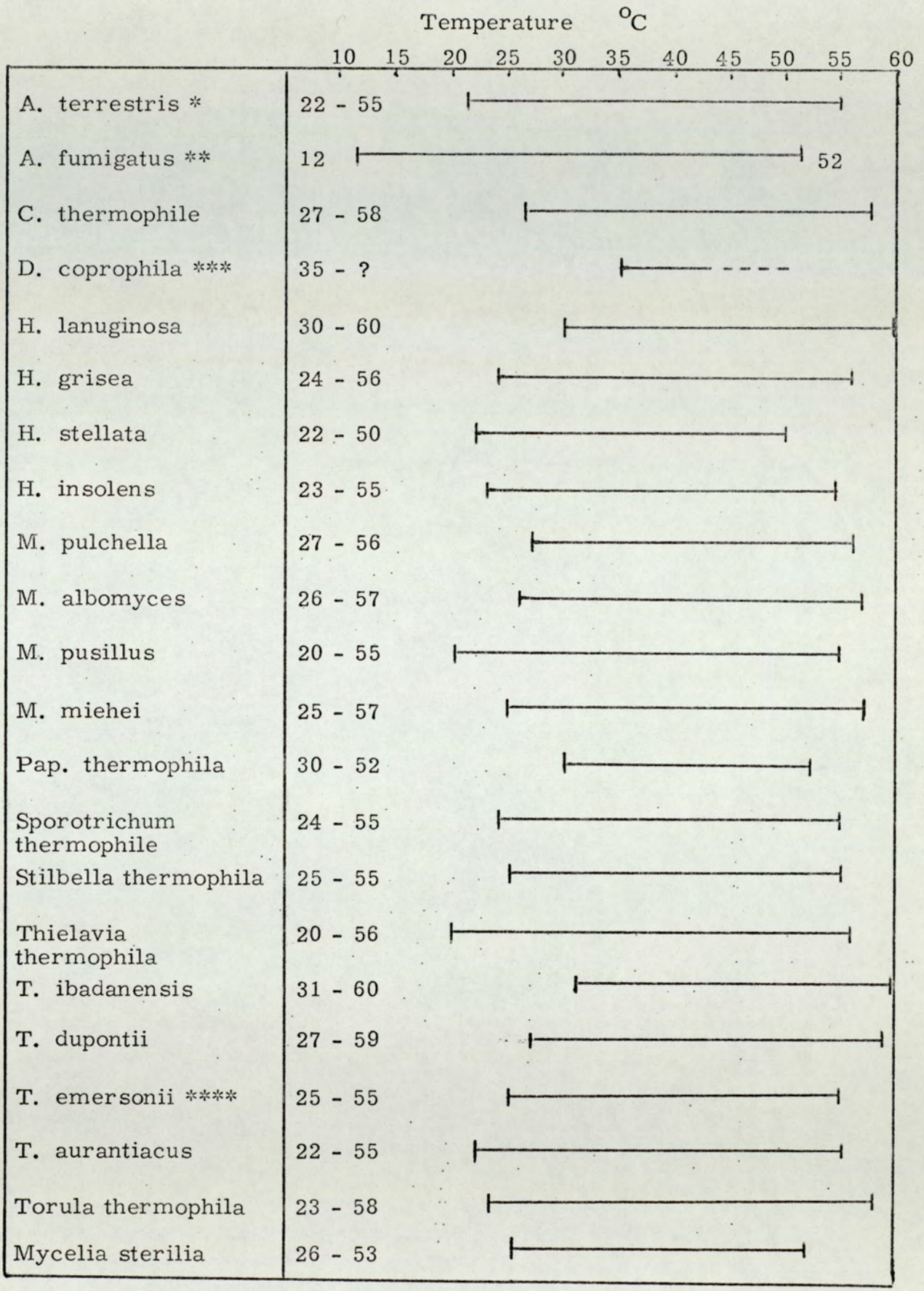
Table 4 adapted from Cooney and Emerson (1964) gives an indication of the natural and man made habitats of the thermophilic fungi. It can be seen from this table that the majority of the thermophiles have been isolated from self heating organic materials, including town waste.

The physiology of thermophilic fungi found in composting systems

Because composting is a dynamic process involving the interaction of many groups of microorganisms, the thermophilic microorganisms must be considered to play an important part in the first stages of the process

TABLE 3

Temperature relationships of the thermophilic fungi



* Data by Evans (1969)
** Data by Cooney and Emerson (1964)
*** Data by Mirza and Qureshi (1970)
**** Data by Stolk (1965)

Source / Fungus	Guayule Rets.	Hay, Straw, grass comp.	Garden compost	Town waste compost	Manure	Leaf mold	Mushroom compost	Peat	Animal dung	Plant debris	Nesting material	Living animals	Soil
A. terrestris (Ceph,)										+			+
Chaetomium thermophile		+		+		+	+		+	+	+		+
D. coprophile									+				+
Humicola lanuginosa	+	+	+	+	+	+	+	+	+	+		+	+
Humicola grisea			+	+			+		+				+
Humicola stellata		+						+					
Humicola insolens	+			+	+		+	+	+				+
M. pulchella	+	+		+	+	+			+	+			+
M. albomyces							+				+		+
Mucor pusillus	+	+		+		+			+	+		+	+
Mucor miehei	+	+							+			+	+
Papulaspora thermophila							+						
Sporotrichum thermophile		+							+	+			+
Stilbella thermophila							+						
Thielavia thermophila		+											
Therm. ibadanensis										+			
T. duponti	+	+	+	+	+					+			+
T. emersonii				+						+			
T. aurantiacus	+	+	+	+				+		+			+
Torula thermophila				+			+		+		+		+
Mycelia sterilia		+											+

TABLE 4

Natural and man made habitats of the thermophilic fungi

when the temperature within the mass of solid waste rises to above 40°C. Field observations on town waste compost windrows showed the importance of the fungi and the actinomycetes in the overall decomposition of cellulose and more resistant material, whilst the thermophilic bacteria played a major role in breaking down protein and other readily decomposable material (Berkely Report 1953).

James et al. (1928) discovered that A. fumigatus was capable of raising the temperature within cornmeal to a maximum of 51.5°C in the presence of a constant oxygen supply; and Norman (1930) found that the same fungus raised the temperature within sterilised straw to 43.5°C. At the same time Miehe (1930 a,b) showed that Malbranchea pulchella and Humicola lanuginosa could raise the temperature within sterilised bread to 60° or over; whilst Okafor (1966) investigating the self heating of damp maize isolated H. lanuginosa and M. pusillus. This work shows that at least some of the thermophilic fungi could be involved in the actual process of heat generation, or thermogenesis, within composting materials.

Waksman et al. (1939), studying the action of fungi in the breakdown of stable manure found that H. insolens was capable of destroying 40% of the dry weight of the manure after 42 days at 50°C. It doubled the water soluble organic matter, decreased the hemicelluloses from 22.8 to 17.2% and decreased the cellulose content from 19.7 to 12.6%. They achieved similar results in a later paper (Waksman and Cordon 1939) when the same organisms was very active in a mixture of 60% straw and 40% alfalfa. Reese (1946) found that H. insolens and A. fumigatus could cause a 40 - 50% loss of cellulose within 4 days at 45 °C using a shake flask technique (see Cooney & Emerson 1964 p. 130), whilst Kaila (1952)

working on the "humification of straw at various temperatures" isolated "possibly some species of Thermomyces" at 50°C from chopped straw. She notes that it was unable to decompose cellulose and it appears probable that the isolate was H. lanuginosa.

Stable manure proved to be a source of A. fumigatus, H. lanuginosa, H. insolens and Sporotrichum thermophile (Henssen 1957). She grew these thermophiles on a cellulose-dextrin agar and demonstrated cellulose decomposition by Sporotrichum and H. insolens but not by H. lanuginosa.

Self heating palm oil kernels were a source of T. emersonii, Chaetomium thermophile and T. ibadanensis (Eggins and Coursey 1964, Apinis and Eggins 1966). These investigators found all three thermophiles to possess a lipolytic activity but only Chaetomium thermophile to be cellulolytic when grown on ball milled cellulose agar medium.

Thermophiles were isolated from self heating grain enclosed in Dewar flasks by Festenstein et al. (1965). Among the fungi isolated they noted H. lanuginosa, M. pusillus and Malbranchea pulchella from hays of differing water content.

Species of thermophilic fungi were isolated from self heating straw by Fergus (1964) and later tested (Fergus 1966) for their ability to grow on paraffin. He found that Chaetomium thermophile, M. pusillus, and Stilbella thermophila grew abundantly on liquid paraffin at 55°C but only M. pusillus sporulated well. Fungi unable to grow on paraffin at 55°C were H. grisea, H. insolens, H. lanuginosa, M. pulchella, M. albomyces, T. duponti and

T. aurantiacus, although they could all produce growth in the nutrient salts medium alone at 55°C.

Thermophiles were shown to be involved in the composting of bagasse and horse dung in India by Ramabadran (1967). He isolated eleven thermophilic fungi from these materials.

Fletcher et al. (1967) isolated thermophilic species of Mucor, Humicola, Myriococcum and Aspergillus from cured tobacco leaves at 50°C and mention that these fungi were isolated from 10% of all leaf discs tested.

A detailed ecological study of the microflora of composting wheat straw was carried out by Chang and Hudson (1967). They classified their isolated fungi into three groups according to the temperature limits within the straw. Their "Group One" fungi included several mesophiles plus M. pusillus and A. fumigatus. These particular fungi were limited to the short period before the temperature reached its maximum. "Group Two" fungi consisted entirely of thermophilic fungi which developed in abundance during the "plateau" phase of heating immediately after the maximum heat phase. Fungi in this group were H. insolens, H. lanuginosa, Chaetomium thermophile, M. pulchella and T. duponti. Their "Group Three" fungi established themselves when the temperature began to drop and included Sporotrichum thermophile, a Mycelia Sterilia and several mesophilic fungi. Chang (1967) went on to characterise the cellulolytic activity of her isolates using the filter paper weight loss technique of Garrett (1962). She found that weight losses were produced by H. insolens, Chaetomium thermophile, Sporotrichum thermophile, M. pulchella and A. fumigatus; but no weight

losses were recorded for T. duponti, H. lanuginosa and M. pusillus. Chaetomium thermophile was also able to cause a 40.3% breakdown of straw within 3 weeks at 45°C.

This work was extended by Fergus (1969a) to include H. grisea, Torula thermophila and Myriococcum albomyces in Chang's list of thermophiles capable of degrading insoluble cellulose. His isolates of H.stellata, T. duponti, H. lanuginosa, M. pusillus, M. miehei, Stilbella thermophila, M. pulchella and T. aurantiacus would not degrade insoluble cellulose, but those fungi capable of degrading insoluble cellulose plus M. pulchella, Stilbella thermophila and T. duponti were capable of producing a cell free extract that would hydrolyse carboxymethyl cellulose (CMC) to reducing sugars. In another of his many studies Fergus (1969b) found that H. insolens, H. lanuginosa, H. stellata, Malbranchea pulchella, M. pusillus and T. duponti were capable of producing considerable amounts of amylase enzymes in both surface(agar) and submerged (liquid) cultures. Five thermophiles viz: the two varieties of Chaetomium thermophile, H. grisea, M. miehei and Torula thermophila produced small amounts of the enzyme whereas Myriococcum albomyces, Sporotrichum thermophile, Stilbella thermophila and Thermoascus aurantiacus did not produce the enzyme. He compared his results with those of Tendler et al. (1967) and showed that his isolate of Theromoascus aurantiacus did not produce amylase whereas Tendler's isolate did, and, in contrast, all four species of Humicola studied by Fergus produced amylase whereas the Humicola sp. of Tendler did not. These particular results, and those for the cellulolytic activity would tend to suggest that there might be several strains, some deficient in particular enzyme production, within any one species of thermophilic fungus.

Cellulolytic thermophilic fungi were isolated from a pastureland soil by Eggins and Malik (1969). These investigators enriched the soil with Whatman chromatography cellulose powder and incubated at 50°C in order to select out those thermophilic fungi capable of degrading cellulose. They found that thirteen species of thermophilic fungi could be isolated from the cellulose enriched soil, and that of these fungi only M. pusillus and T. duponti did not produce any clearing of ball milled cellulose agar. This work indicated the widespread occurrence of thermophilic cellulolytic fungi in nature, in contrast to the results of Crisan (1959) who was able to isolate only five thermophilic fungi from a large variety of composting materials and soils.

Wood chip piles in Novia Scotia proved to be a source of thermophilic fungi (Shields 1969) whilst self heating coal spoil tips, mushroom, grass and straw composts and animal manure yielded a whole range of fungi, including some thermophiles (Evans 1969). Evans went on to test the cellulolytic activity of the thermophiles using the filter paper weight loss technique and found that A. fumigatus, Cephalosporium sp., Chaetomium thermophile, H. insolens, M. albomyces, Sporotrichum thermophile, M. pulchella and Torula thermophila could produce small weight losses of the cellulose after 3 weeks incubation at 40 to 45°C.

Hedger and Hudson (1970) studied Thielavia thermophila and Sporotrichum thermophile isolated from their wheat straw composts. They found that Thielavia thermophila produced cleistothecia in the cooler, outer regions of the compost and conidia only in the central regions, whereas Sporotrichum thermophile failed to produce any cleistothecia. Malik (1970) used a perfusion technique at different pH levels to demonstrate the fungal

colonisation of a cellulose strip at 50°C. He found maximum degradation of the strip at pH 6.0 to pH 7.0 and that Chaetomium thermophile was largely responsible for the weight losses produced. He then went on to assay his thermophiles using Rautela and Cowling tubes (Rautela and Cowling 1966) and noted the depth of clearing of cellulose agar buffered at different pH levels. He found Chaetomium thermophile, Cephalosporium s and Sporotrichum thermophile to be the most cellulolytic thermophiles with maximum clearing at pH 6.4 and pH 4.0 and pH 5.0 respectively.

Fergus (1971) measured the growth and heat resistance characteristics of his newly isolated Papulaspora thermophila. It grew well on fragments of composting wheat straw and horse manure, but no data is yet available on its cellulolytic activity. He then went on to study the heat resistance of all the thermophiles when grown on wheat straw mushroom compost (Fergus and Amelung 1971) when exposed to a range of increasing temperatures. He found that H. grisea, H. lanuginosa and M. pusillus were the most heat resistant, surviving at 68°C for 30 minutes, 45 and 45 minutes respectively. No fungus was able to survive 72°C for 15 minutes and this would tend to confirm the generally accepted view that the centre of a compost heap becomes effectively sterile when the temperature reaches 70°C or above. Fergus found that H. lanuginosa and T. duponti could both survive a temperature of 59°C for at least 10 days and Fergus remarks that the spores of these fungi are more resistant than the mycelium - his Myriococcum albomyces (mycelium only) had very low heat resistance. Fergus's results for T. duponti correlate well with the results of Mills (1968) who found that the spores of this fungus were killed after 6 minutes at 62°C. The low heat resistance of Fergus's Stilbella thermophila could be explained by the fact that the spores are produced in a slime, and during heat treatments it

is a well known fact that wet heat is a better sterilising agent than dry heat. Celerin and Fergus (1971) went on to study the thermal death point of the ascospores of C. thermophile var. coprophile and found that they were killed after 10 minutes at 66°C.

Fewer species of thermophilic fungi were isolated from fresh wood chip piles than from piles which had self heated (Tansey 1971a). He correlated the increase in thermophilic fungi in heated chip piles with the ability of many to degrade cellulose. Tansey then went on (1971b) to characterise the cellulolytic activity of his isolates using cellulose agar in Rautela and Cowling tubes. He found that all the thermophiles tested, with the exception of H. lanuginosa, H. stellata, M. pusillus, M. miehei and T. duponti, would produce clearing of the cellulose. His isolate of T. emersonii also cleared the acid swollen cellulose although Eggins and Coursey (1964) found this fungus to be non-cellulolytic.

Thermophilic fungi were first noted in composting town refuse by Glath (1959) and shortly afterwards by Klopotek (1962). They isolated and studied H. lanuginosa, T. aurantiacus, Chaetomium thermophile, A. fumigatus, M. pusillus and T. duponti. These results were later reported by Shilesky and Maniotis (1969) in a new journal concerned with the composting and recyling of solid wastes called "Compost Science". Klopotek isolated mainly mesophilic fungi from her town waste windrows, and found that the thermophilic fungi represented only 0.02% of the total population. Of this, A. fumigatus represented 70%, M. pusillus 20% and the rest 10%. She concluded that the thermophilic fungi were very poorly represented in town waste. Stutzenberger et al. (1969) using the open

windrow method of municipal solid waste composting isolated three thermophilic microorganisms from town waste viz: A. fumigatus, a Bacillus sp. and a Thermoactinomyces sp. These authors went on to demonstrate the cellulolytic nature of these organisms.

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Plan of this thesis

We have seen that thermophilic fungi can be isolated from a range of composting materials but that little work has been carried out, and few species of thermophilic fungi have been isolated from composting town waste. Several of these fungi have been found to be cellulolytic, and so it would appear that they might possibly be actively involved in the biodegradation of cellulose within composting town waste. If town waste composting is to become an accepted method for the recycling of solid wastes we must be able to understand the basic biological principles underlying the whole process and this includes an understanding of the microorganisms, including the thermophilic fungi, which are actively involved in the process. Previous failure in composting ventures too often occurred because the approach to this method of recycling was focussed on engineering to the exclusion of biology (Stickelberger 1971). This last observation appears to describe well the present day situation. The approach to several of the problems

outlined in the Introduction will be:

(a) to investigate the process of town waste composting using the windrow method (since this method does not involve expensive, complex machinery), and to try and monitor some of the basic biological processes occurring throughout the composting period,

(b) to try and relate these parameters to the activity of individual thermophilic fungi growing on cellulose and "plastic" materials, two isolated "components" of town waste, and

(c) to try and outline the possible role played by the thermophilic fungi in the production of the stable humus-like end products of the composting process.

CHAPTER 2

CHAPTER 2

The Process of Composting

Composting is a biological process which involves the aerobic thermophilic and mesophilic decomposition of solid wastes to produce a relatively stable humus-like material. The waste must be heaped or piled up in some way, or enclosed within some sort of container, so that the heat produced by microbial metabolism is retained and encourages the development of the thermophilic microorganisms.

The earliest methods of composting consisted of forming heaps or windrows of waste materials which were watered and turned by hand to thoroughly mix the contents so that complete breakdown of the material within the heap could be achieved. Sir Albert Howard introduced such a system into India, in which domestic wastes were mixed with night soil and the material piled up into long windrows to compost. This was known as the Indore process (Howard 1935) and this method of windrowing (without the night soil - but sometimes with the addition of sewage sludge) is still practised widely throughout the world.

Many types of mechanical and semi-mechanical composting plants have been designed since Howard's day (Berkely Report 1953). Some have an anaerobic followed by an aerobic stage (the Beccari cell), others have a grate-type digestor through which air is forced (Bordas method). Multi floored arrangements are also used in which the refuse, contained in shallow trays, is tipped from the top floor downwards until composting is complete e.g. the John Thompson plant at Jersey and the Earp-Thomas plant. Newer types of plant involve fully mechanised, slowly revolving

drums into which pulverised refuse is fed under gravity. These include the Dano process, originally developed in Denmark, in which pulverised refuse passes down a long rotating drum and is gradually disintegrated by the rotational action of the drum and by the greatly accelerated composting process which is initiated at the same time. The whole process takes 5 or 6 days, however the emerging compost is not a true compost (see Wiley 1957) and has to be left to mature in large heaps 5 or 6 feet high in the open. Variations upon this type of method are the John Thompson "Fermascreen" consisting of a rotating drum with screen mesh sides, and the Simon-Lawden plant in which the refuse is stirred and conveyed from floor to floor by slowly rotating arms through which air and water are passed.

Composting plants of the type present in the U. K. are presented in a table by Gray (1966).

In the windrowing process, which will be used in this thesis, temperatures of 40°C and over are usually reached in a very short time and are maintained for up to 30 days. Thermophilic temperatures are usually reached within the first 24 to 48 hours of composting and such temperatures have been shown to exceed the thermal death points of a large number of pathogenic microorganisms (Berkeley Report 1953 , Jansen and Kunst 1953, Burman 1967, HMSO 1970, Gray et al. 1971). Recent work has shown however that Salmonella paratyphi B and E. coli were not killed at composting temperatures when introduced into summer and winter composts (Parrakova et al. 1970), and this sort of work must not be overlooked if public acceptance of the product is to be a future sales technique.

Many composting parameters have also been worked out (see Berkely Report 1953) but essentially windrowing as a process will be successful if (a) material with an initial carbon to nitrogen (C/N) ratio of 30 to 50 is used, (b) the material is watered to a 50 - 60% moisture level before heaping and (c) the height of the windrow does not exceed the width of the base. When considering the final product it has been found that a finished compost with a C/N ratio of greater than 20 will create a tendency for soil microorganisms to utilise the excess carbon in the compost by drawing upon soil nitrogen, thus depriving plants of valuable nitrogen (Berkely Report 1953, Jann et al. 1959, HMSO 1970, Hortensteine 1971).

The composting study carried out here was done using one ton of pulverised town refuse heaped up into a windrow.

Materials and Methods

The windrow was constructed using one ton of town waste from Newton Abbot, Devon and kindly supplied by Lucas Furnace Developments Ltd., Wednesbury, Staffordshire. The refuse had been through a primary pulverising machine but had not been screened and consequently contained large pieces of crushed metal, rags and slightly damaged plastics in the form of toys, polythene bags and holloware. Paper and vegetable matter had been pulverised into small pieces, one inch or less in diameter and it was this fraction that was studied biochemically in the laboratory. The degradation of plastics within the heap was not studied here, since these materials have been shown, in the past, to be non biodegradable.

The initial refuse was virtually dry upon receipt and contained approximately 80% paper, 5% vegetable matter, 2 - 3% plastics and the remaining 12% inert inorganic matter in the form of ashes, cinders and metals.

The refuse was mixed, watered to 50 - 60% moisture level and made into a windrow approximately 5' x 5' x 4' high and covered loosely with a sheet of black polythene. The polythene sheeting was used solely to aid sampling since the heap was constructed in late autumn and it was anticipated that composting would continue well into the winter months. The heap was not mixed or turned during composting as an aid to biochemical studies, and samples were taken periodically from the centre of the heap and from three preselected sites at a depth of one foot in from the midpoint of two sides of the heap. At each sampling time approximately 20g of material was taken from each site, dried overnight in a hot air oven at 80°C and homogenised in a high speed blender for 15 seconds. The fine powder resulting from this treatment was then placed in sealed, screw-top jars for subsequent biochemical analysis. Additional samples were taken for pH and moisture analysis. The temperature of the heap was measured with ordinary mercury thermometers at the sampling sites and the mean of ten to twelve readings was computed for each site. The pH of the refuse was measured using a Cambridge pH meter with glass electrode after shaking 10 - 12 g. of wet material in 100 ml distilled water for 15 seconds. Samples for microbiological analysis were picked directly out of the heap using sterile forceps and placed into sterile petri plates to be later covered with E & P cellulose agar (Eggins and Pugh 1962) according to the method of Warcup (1950). The plates were then incubated at 48°C in high humidity incubators for seven days when developing thermophiles were recorded. The

percentage carbon present within the heap throughout composting was measured by the "New Zealand" method (Anon 1951) and calculated according to the equation:-

$$\% \text{ Carbon} = \frac{100 - \% \text{ Ash}}{1.8}$$

This method has been shown to produce results within two to ten percent of more accurate determinations (Anon 1953) and appeared to give satisfactory results here. Percentage ash was calculated by ashing the "compost powder" at dull red heat in porcelain crucibles and the mean of six results was used to calculate the percentage of carbon of any particular sample.

The micro-Kjeldahl method was used to calculate the total nitrogen of the samples, again the mean of six readings being taken as the total nitrogen for any one sample.

The organic fractions of the composting refuse were assayed using methods based on those of Chang (1967). The "compost powder" was initially extracted in a Soxhlet apparatus for a period of four hours with an 80% ethanol/water mixture, replacing the mixture after 60 minutes with fresh ethanol/water. /Initial experiments using anthrone reagent to test for reducing sugars (Chang 1967) had shown extraction to be complete after 4 hours/. The ethanol/water soluble fraction contains reducing and non reducing sugars, glucosides, inorganic nitrogen, amide and amino nitrogen, basic, cyclic and alkaloidal nitrogen, oils and lipids, chlorophyll, carotin, xanthophyll, flavones and tannins (Loomis and Shull 1937), anthroquinones, gums and resins (Chang 1967). The extracted powder was then washed with

distilled water and dried in a hot air oven overnight at 80°C and the % EtOH/H_2O soluble substances extracted was calculated from the difference in dry weight of the initial and extracted samples. Duplicate samples were used for this test.

After extraction the samples were combined and extracted further with 24% KOH and for 4 hours to remove hemicelluloses. Again the difference in dry weight between initial and extracted samples was used to calculate the % hemicelluloses in the EtOH/H_2O extracted sample after neutralising the KOH with dilute HCl solution and then washing with distilled water. The residue, containing cellulose, was then further fractionated using 72% (w/v) sulphuric acid at room temperature for two hours followed by diluting to an acid level of 4% and autoclaving for one hour at 120°C to hydrolyse any remaining cellulose (Canevascini 1970). The insoluble residue containing ash and lignin was then filtered off, washed with distilled water to remove traces of acid and dried overnight at 80°C so that the weight of the extracted cellulose could be determined. The final lignin containing residue was ashed at dull red heat to calculate the percentage of organic matter remaining in the "lignin fraction".

Results

The temperature changes within the heap over the 76 days of composting are shown in Figure 1. During the first 48 hours a very rapid rise in temperature was recorded with the sides of the windrow reaching 62°C and the centre of the heap 58°C. The temperature at the centre of the heap remained above 50°C for eight days whilst the sides of the heap remained above 50°C for 15 days. A gradual decline in temperature was

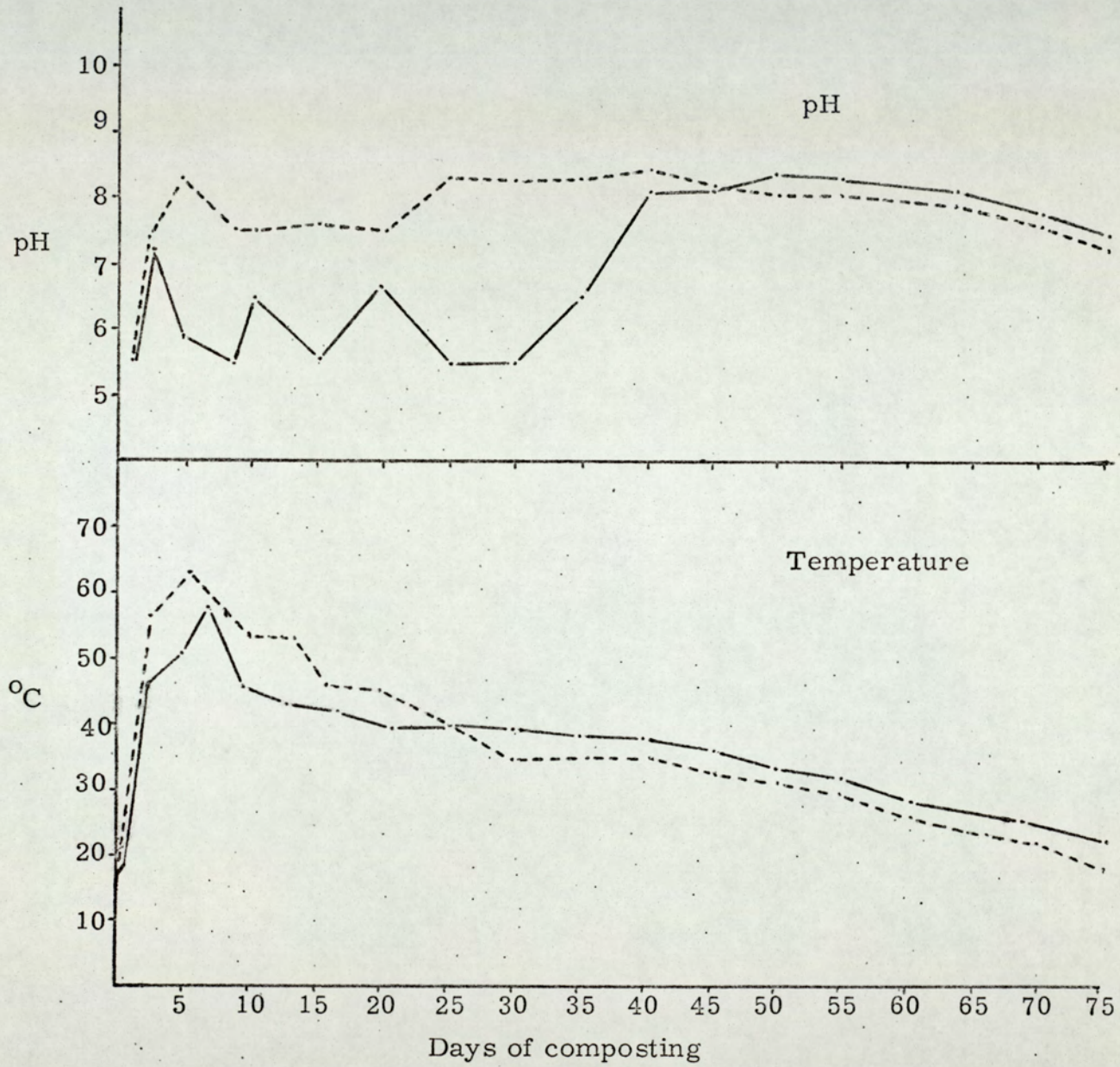


FIGURE 1

Showing changes in temperature and pH within the heap over a period of 76 days

—— · —— Temperature (pH) at centre of heap

------ · ---- Temperature (pH) at sides of heap

then noted until day 25 when both the sides and centre of the heap were at 42°C. This point of time and temperature will be taken as the "thermophilic phase" of the composting process. The temperature then remained fairly constant between 35 and 40°C until about day 54 of composting when a fall to ambient temperatures began and was reached on day 76. These latter phases will be referred to as the "mesophilic phase" of the process.

Changes in pH within the heap are shown in Figure 1. The initial refuse had a pH of 5.5 but during the thermophilic phase the sides of the heap rapidly showed a change towards neutrality and a slight alkalinity, whilst the material in the centre of the heap, which appeared to be tightly compacted and therefore probably slightly anaerobic, remained acidic for 32 days before changing towards neutrality and eventually rising to pH 8.0 after 48 days. The pH of the compost after 76 days was very nearly neutral (pH 7.3).

Table 5 shows the % Carbon, % Total Nitrogen, % Moisture and % Ash within the windrow during the 76 days of composting. It must be noted here that slight discrepancies in these results were probably due to the non homogeneity of the material under test. The % carbon fell from about 35 to 22 % in the sides and from about 40 to 33% in the centre of the windrow during the thermophilic phase. As the windrow cooled down further after 25 days the % carbon fell to 16% in the sides and to about 24% in the centre of the windrow after 76 days, and if one studies the C/N ratio of the finished product it can be seen that a very reasonable compost indeed was produced, with a C/N ratio of about 19.

TABLE 5

Days of Composting	% Carbon	% Total Nitrogen	C:N	%Moisture	%Ash
Initial	32.2	0.6505	50:I	60	42.4
7	36.7 (40.9)	0.5950 (0.6860)	62:I (60:I)	62.5 (62.2)	33.7 (26.3)
I2	37.6 (35.9)	0.8000 (0.7000)	47:I (5I:I)		32.2 (35.3)
I6	30.0 (34.5)	0.7000 (0.6950)	43:I (50:I)		45.9 (37.9)
20	25.4 (32.4)	0.7920 (0.7I80)	32:I (45:I)	55.5 (56.0)	59.I (4I.6)
25	22.5 (32.9)	0.8340 (0.5880)	27:I (56:I)	58.0 (56.0)	6I.3 (40.7)
3I	26.6 (32.I)	0.9070 (0.8530)	29:I (37:I)	55.5 (58.2)	52.0 (42.2)
36	24.8 (25.4)	0.8260 (0.6900)	30:I (37:I)		55.3 (54.3)
43	I9.8 (2I.9)	I.II20 (0.8800)	I8:I (25:I)	54.5 (58.5)	64.I (60.4)
56	I9.2 (27.0)	I.4600 (0.6900)	I3:I (39:I)		65.3 (5I.3)
76	I6.7 (24.7)	0.9760 (I.I790)	I7:I (2I:I)	53.0 (55.0)	69.8 (55.4)

TABLE 5 Numbers in parentheses denote readings for centre of heap
Numbers without parentheses denote readings for sides of heap

The percentage moisture remained fairly constant throughout the composting period. The windrow was at no time exposed to the rain or snow, thus avoiding severe waterlogging. Slight waterlogging was noted at the centre of the windrow where the material was tightly compacted. This could explain the fact that the pH in this region remained slightly acidic for 32 days and why the temperature in this region did not rise above that in the sides of the heap as one would have expected. With a decrease in the percentage of organic matter within the windrow there was a correspondin rise noted in the percentage Ash over the composting period.

When the results for the ethanol/water soluble substances were computed it was noted that after an initial fall in the concentration of these substances there then appeared to be a rise in the amounts of these products from about day 20 to about day 25 (Figure 2). After day 25 the amounts of these compounds again fell off gradually, with a slight rise towards the end of the composting period. It then became obvious that the initial fall off in these materials corresponded to the thermophilic phase of composting. At temperatures below 40^{o}C the mesophilic organisms would be taking over from the thermophiles, and this could explain the sharp rise in the amounts of these substances from day 20 to day 25, when mesophilic release and subsequent utilisation of these compounds was taking place.

Table 6 shows the overall decrease in the amounts of hemicelluloses in the windrow over the period of composting. In practice the method of hemicellulose fractionation (for this particular material) proved to be a messy procedure, and difficult to control in general. The residue, after extraction, required large amounts of dilute HCl to neutralise the 24% KOH and very much washing to arrive at a neutral residue

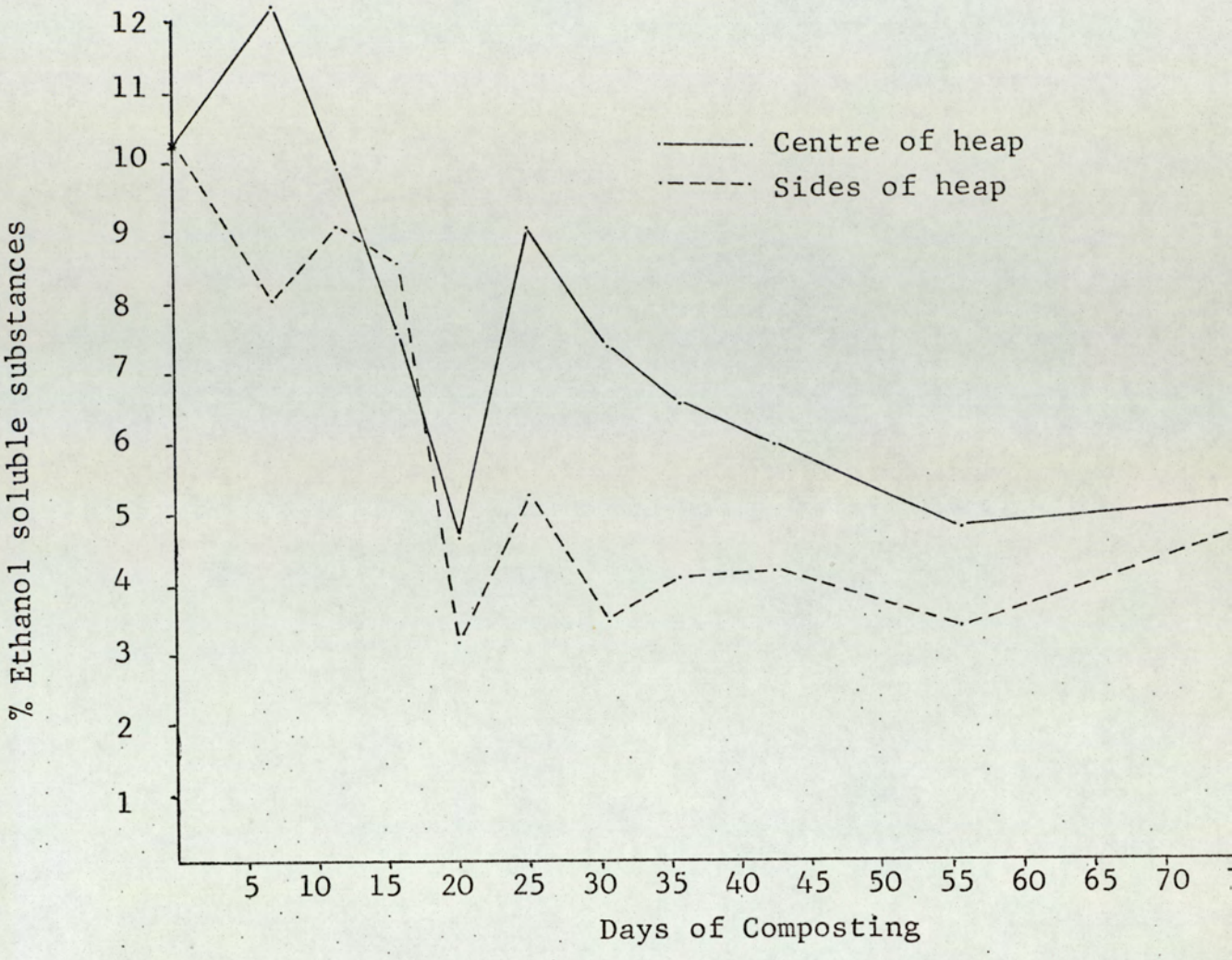


FIGURE 2

Changes in % Ethanol/water soluble substances throughout the composting period.

TABLE 6

Showing decrease in hemicelluloses

over 76 days of composting

DAYS	SIDES	CENTRE
INITIAL	14.5%	
7	-	22.0%
12	15.0%	8.7%
25	20.0%	18.3%
36	5.7%	-
56	12.7%	-
76	9.65%	13.4%

containing cellulose and lignin. The figures in Table 6 represent the decrease in the hemicelluloses present within the ethanol/water extracted material, and blank results indicate where final dry weight increases were found - no satisfactory explanation can be given here for such results. If the results are studied objectively however, it can be seen that there was an overall decrease in the percentage of hemicelluloses over the 76 days of composting with the sides of the heap showing a decrease from about 15% to about 10% and the centre from 20% to about 13%.

The decrease in the percentage cellulose present within the ethanol/water/hemicellulose extracted material is shown in Figure 3. The cellulose, originally present at about 61% fell extremely rapidly in the sides of the heap within the thermophilic phase, reaching about 27% after 25 days. A less rapid fall was noted in the centre of the heap, for the reasons previously explained, and a figure of 53% was reached within this initial phase. The large fall in cellulose in the sides of the heap seems to be clear evidence for the high cellulolytic activity of the thermophilic population present during this phase. The downward trend of the graph continued throughout the mesophilic phase and the final compost had cellulose containing content of about 18%.

The percentage lignin-containing fraction, after all the above procedures had been carried out, was calculated by subtracting the % cellulose containing fraction from 100. It must be remembered however that this fraction also contains inorganic material, and so it was then ashed at dull red heat and the percentage of ash present was subtracted from the above figure to give a true representation of the amount of organic material within the "lignin" fraction. The results are presented in Table 7

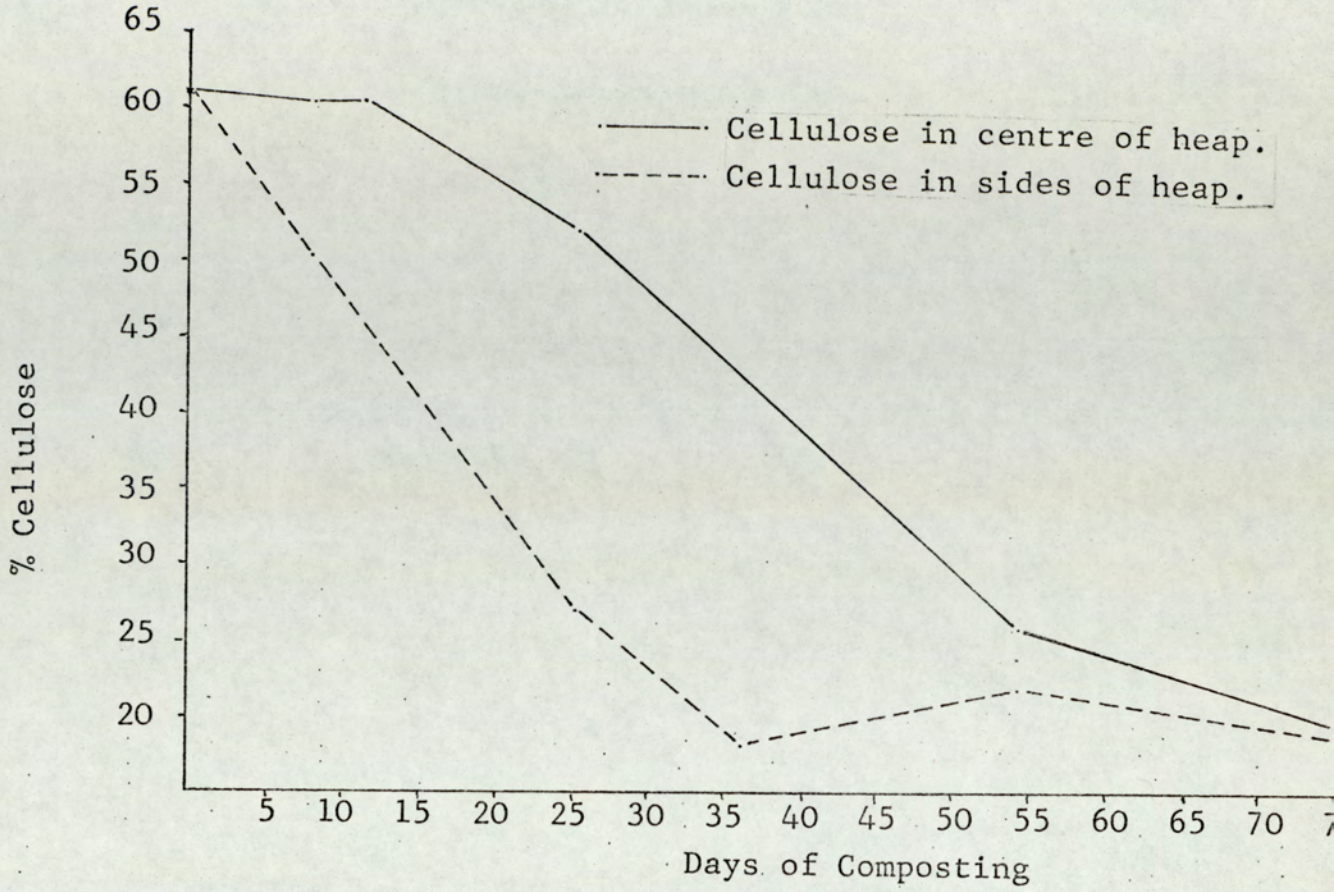


FIGURE 3

Showing decrease in % cellulose-containing fraction over period of composting

TABLE 7

Showing the decrease in the % lignin containing fraction

DAYS	SIDES	CENTRE
INITIAL	14.8%	
7	27.3%	12.75%
12	25.1%	15.8%
25	20.0%	19.1%
36	9.6%	19.3%
56	6.5%	10.3%
76	8.7%	11.1%

and show an overall slight decrease in this fraction over the 76 days of composting. It must be noted here however that inclusion of any woody material such as pieces of twig, matches etc. could result in any one reading being unnecessarily high.

The dominant thermophilic fungi present within the composting town refuse were Chaetomium thermophile and H. lanuginosa. Thermophilic actinomycetes were also isolated in large numbers at all stages of composting and were identified by Dr. T. Cross at Bradford University as Thermoactinomyces vulgaris, Streptomyces thermoviolaceus var. pingens, Streptomyces rectus, and Thermoactinomyces glaucus. (Table 8).

It should be noted here that a full scale ecological survey of the thermophilic fungi present within the town waste was not carried out, since such surveys are being made by other research workers at the B.I.C. The three thermophilic fungi with a high frequency of isolation during the thermophilic phase were Chaetomium thermophile, H. lanuginosa and M. pusillus. H. grisea and Torula thermophila became evident during the initial and mesophilic stages of composting, whilst A. fumigatus did not appear until the later, cooler stages of the process. Only 6 thermophilic fungi were isolated throughout the composting period which would tend to indicate that only these six fungi had been present in the original material, or had subsequently invaded the windrow from the atmosphere. However, only one isolation medium was used - but the majority of the thermophilic fungi are able to grow upon E & P cellulose agar, whether they clear the cellulose or not.

TABLE 8

Thermophilic fungi and Actinomycetes isolated during composting period using Warcup's technique

Days of testing	Initial	2	14	37	76
Fungi isolated on E & P Cellulose Agar	% Frequency of isolation				
C. thermophile	100	100 (100)	100 (100)	100 (100)	100 (100)
H. lanuginosa	100	100	100 (100)		100 (100)
M. pusillus	75	75	(20)	(33)	20 (60)
H. grisea		(100)		100 (66)	
Torula thermophila		(25)		33	
A. fumigatus				(33)	80 (20)
Actinomycetes	25	75 (100)	100 (100)	100 (100)	100 (100)

Figures in parentheses refer to fungi isolated from centre of heap.

Figures without parentheses refer to fungi isolated from sides of heap.

Discussion

From the above results it was seen that the composting process was largely indeed a two stage process involving a thermophilic and a mesophilic stage. The thermophilic phase lasted 25 days and was reached within the first 48 hours of composting. These results are in line with those of other authors (Berkely Report 1953, Stutzenberger et al. 1969). Stutzenberger appears to be the only worker to have carried out a biochemical study of cellulolytic activity within composting town refuse and his windrows, involving many tons of refuse, reached their maximum temperature of 60 - 74°C in 21 days. Maximum temperatures within the Berkely windrows (73 - 76°C) were reached within 8 to 14 days.

Both these studies however involved many tons of town refuse in large windrows. In contrast, the highest temperature reached in this study was 62°C in the sides of the heap after 6 days. The mesophilic stage lasted approximately 50 days and a dark, earthy smelling humus, full of earthworms, was produced.

The pH of the heap, in general, very quickly reached alkaline values and stayed in the alkaline region for the remainder of the composting period. The windrows of Stutzenberger (1969) attained alkaline pH after about 28 days, but in this study the material within the sides of the heap became alkaline within 7 days. It has been observed that an alkaline pH was necessary to obtain rapid composting (Jann et al. 1959); but what is not known is whether the cellulolytic thermophilic fungi are able to elaborate their cellulase systems at these sort of pH values.

This then is one line of research that might be worth following i.e. how cellulolytic are the cellulolytic thermophilic fungi at composting temperatures, and what is the activity of their cellulase enzymes at the pH levels found within composting town refuse? Since the C/N ratio of the finished compost was around 20 it appears that as a system, the small windrow was quite successful, and that apart from an initial amount of anaerobiosis in the centre of the heap, composting processes occurred throughout in a normal manner. The amount of cellulose in the sides of the heap fell very rapidly during the thermophilic phase from about 60% to 27 %. Stutzenberger (1969) measuring loss of total cellulose in his windrows found that cellulose decreased by 50% during the 7 to 8 weeks of composting and the greatest loss of cellulose occurred within the first four weeks of composting. These particular results correlate quite reasonably with the results presented here, and would tend to suggest that cellulose degradation within the thermophilic phase is being carried out by Chaetomium thermophile alone as a representative of the thermophilic fungi, or by thermophilic organisms other than the thermophilic fungi. Since only Chaetomium thermophile and two other non cellulolytic thermophilic fungi were isolated during the thermophilic phase, and since thermophilic cellulolytic actinomycetes were isolated in abundance during this phase, it appears that the latter hypothesis might stand. If this is so - and this must be confirmed by ecological work as well - is it possible to study the cellulolytic activities of the thermophilic fungi in isolation, and in combination, with regard to parameters such as pH and interaction of these organisms and to relate these results back to parameters occurring within the town waste composting environment? If this could be done the answers to the above questions might be forthcoming.

CHAPTER 3

Isolation of Thermophilic Fungi

The thermophilic fungi used in the practical work for this thesis were either isolated by the author or obtained from the culture collection of the Commonwealth Mycological Society, Kew, Surrey, England. Fungi were isolated from soil known to contain thermophiles (Eggins and Malik 1969), from samples of composting straw chaff being used in other studies at the B.I.C. and from two sources of town waste - the author's windrow and from a municipal refuse dump near Birmingham. The latter site was visited during the dustmen's strike of 1968 when town refuse had been temporarily dumped, (Birmingham incinerates its refuse), and was found to be spontaneously heating. Small pieces of each of these samples were plated out using the methods of Waksman (1916) and Warcup (1950) onto Eggins and Pugh (E & P) cellulose and glucose agars (Eggins and Pugh 1962). The plates were incubated in high humidity incubators at 48^{o}C and developing thermophiles were subsequently isolated and maintained on E & P cellulose agar and YpSs agar (Cooney and Emerson 1964). It was found necessary to slightly modify the YpSs agar to include half the quantity of yeast extract. This had the effect of eliminating the bacterial contamination experienced when using the stated amounts of yeast extract. The thermophilic fungi were identified according to Cooney and Emerson (1964) and subsequent authors.

Results

In all, 13 thermophilic fungi were isolated from the four

sources (Table 9). The several isolates of Chaetomium thermophile were later differentiated into variety dissitum and variety coprophile using the morphological characteristics outlined by Cooney and Emerson (1964). It was found, for example, that variety coprophile always produced masses of perithecia in concentric rings, whilst variety dissitum produced a smaller number of perithecia and these were scattered all over the plate.

In addition to the six thermophilic fungi isolated from the author's windrow (see Chapter 2), three additional thermophiles, H. insolens, M. pulchella and Talaromyces duponti were also isolated from the Birmingham town refuse. Thermophilic fungi not isolated were obtained from C.M.I. and included Humicola stellata (I.M.I. 77,024); Mucor miehei (I.M.I. 126,334); Myriococcum albomyces (I. M.I. 126,326); Stilbella thermophila (I.M.I. 121,659); and Thielavia thermophila (I.M.I. 145,136).

The nineteen organisms were plated out onto E & P cellulose agar and, although most of the organisms were able to produce some growth on this medium, it was found that twelve of the thermophiles were able to grow profusely and to produce clearing of the cellulose particles. These fungi viz:- A. fumigatus, Cephalosporium sp., Chaetomium thermophile vars. coprophile and dissitum, H. grisea, H. insolens, M. pulchella, M. albomyces, Sporotrichum thermophile, Stilbella thermophila, Thielavia thermophila and Torula thermophila were subsequently maintained on E & P cellulose agar throughout the three years of research. It is interesting to note here that Myriococcum albomyces and Thielavia thermophila never produced cleistothecia on this

TABLE 9

Thermophilic fungi isolated from a pastureland soil, composting straw chaff, the author's town refuse windrow and self heating town refuse from Birmingham. Plus signs + refer to fungi isolated from the particular source.

Thermophilic Fungus	Soil	Straw	Windrow	Town Waste
Aspergillus fumigatus	+	+	+	+
Chaetomium thermophile	+	+	+	+
Humicola grisea	+	+	+	+
Humicola insolens	+	+		+
Humicola lanuginosa	+	+	+	+
Malbranchea pulchella	+			+
Mucor pusillus	+	+	+	+
Sporotrichum thermophile	+			
Talaromyces duponti	+	+		+
Talaromyces emersonii	+			
Thermoascus aurantiacus	+			
Torula thermophila	+		+	
Cephalosporium sp.	+			

medium. The remaining seven fungi were maintained throughout on the modified YpSs agar.

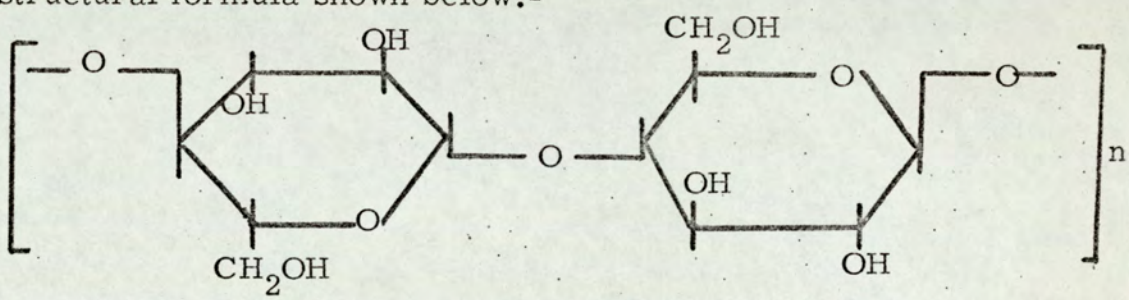
CHAPTER 4

CHAPTER 4

The Enzymatic degradation of cellulose by thermophilic fungi

Introduction

The cellulose molecule consists of a linear polymer of β1 - 4 linked glucose units, having the empirical formula $(C_6H_{10}O_5)_n$ and the structural formula shown below:-



In nature, cellulose molecules are aggregated into microfibrils each with a width of 50 - 200 Å (Rånby and Rydholm 1956; Hess et. al. 1957; Preston and Cronshaw 1958; Cowling 1959, 1963; Rollins and Tripp 1961; Mühlethaler 1967; Rånby 1969; Cowling and Brown 1969; Isherwood 1970). Highly ordered regions of microfibrils are known as crystallites or micelles, whilst more randomly ordered regions are known as amorphous or paracrystalline regions (Figure 4). The crystalline nature of cellulose was first discovered by Gibson (1893) using polarised light, and first confirmed by Nishikawa and Ono (1913) using X-ray diffraction methods. The highly crystalline regions of microfibrils may reach lengths of 600 Å (Isherwood 1970) and parallel chains are held together by intra chain hydrogen bonds between the -OH on carbon atom 3 of one "glucose unit" and the $-O\diagdown$ of an adjacent "glucose unit" (Rånby 1969). The average degree of polymerisation is at least 3000 glucose units (Isherwood 1970)

but may range from as low as 15 to as high as 10,000 (Cowling 1963) giving cellulose a molecular weight of more than one million at the higher figure.

Cotton seed hair cellulose is the purest form of cellulose to be found in nature with greater than 90% cellulose and minor amounts of waxes and other material. This type of cellulose, "native cellulose", contains a high proportion of crystalline regions. Wood, the second most prevalent source of natural cellulose contains 40% or less cellulose and a lower proportion of crystalline regions. The cellulose in wood is closely associated with the complex, three dimensional polymer, lignin.

The resistance of cellulose to enzymatic attack is a function of the degree of crystallinity of the cellulose (Abrams 1950, Walseth 1952, Thomas 1956, Norkrans and Ranby 1956, Sison et al. 1958, Rollins and Tripp 1961, Kaplan et al. 1970), and whilst many fungi are capable of degrading the more amorphous regions of the microfibrils, relatively few species have the ability to degrade native cotton containing highly crystalline cellulose.

Enzymatic attack

Cellulose is broken down into lower molecular weight oligomers, trimers, dimers and glucose units by a complex of enzymes collectively known as cellulase. This complex, as currently understood contains (a) a C_1 component capable of separating the laterally bonded cellulose chains in highly ordered (crystalline) forms of cellulose, (b) C_x components - a series of hydrolytic β 1 - 4 glucanases capable of attacking the more amorphous regions of the chains and (c) β-Glucosidases which hydrolyse the smaller oligomers.

C_1 component

The C_1 component, whose action is required for the ultimate hydrolysis by the C_x components of highly ordered forms of cellulose, has all the characteristics of an enzyme (Selby 1969). It is probably a glycoprotein (for P. funiculosum and T. viride) with an apparent molecular weight of about 57,000 (Selby 1969). Its mode of action on crystalline cellulose may be the rupture of the intra chain hydrogen bonds (King and Vessal 1969) producing a more amorphous form of cellulose upon which the hydrolytic C_x complex can act. That the C_1 is not hydrolytic itself has been postulated by King (1965) and King and Vessal (1969) who state that "the reaction rate is essentially unaffected by substituting D_2O for H_2O in the reaction system - an observation that is highly improbable for a hydrolytic reaction". Also, the activation energy of the C_1 reaction has been observed to be as low as 3000 calories per mole - a value more in line with hydrogen bond cleavage than hydrolysis (Rautela and King 1968). Highly purified C_1 enzyme has been shown to be virtually inactive in the absence of the C_x components. It has been observed that there was no synergism between components of the C_x fraction, and that synergism between C_1 and individual components of C_x is limited; only when all the components were present together could crystalline (cotton) cellulose be attacked and broken down (Selby 1969, Wood 1970). Selby (1969) goes on to present a new hypothesis for the action of the C_1 enzyme in which he assumes that chain endings within crystalline regions will disturb the intra chain hydrogen bonding. This may be insufficient to enable C_x acting alone to split off soluble sugars, but when both components are present a single bond rupture by C_x may allow further disturbance of the hydrogen bonding by C_1 with subsequent loosening of a short length of surface chain which could then be attacked by C_x (Figure 5).

FIGURE 4

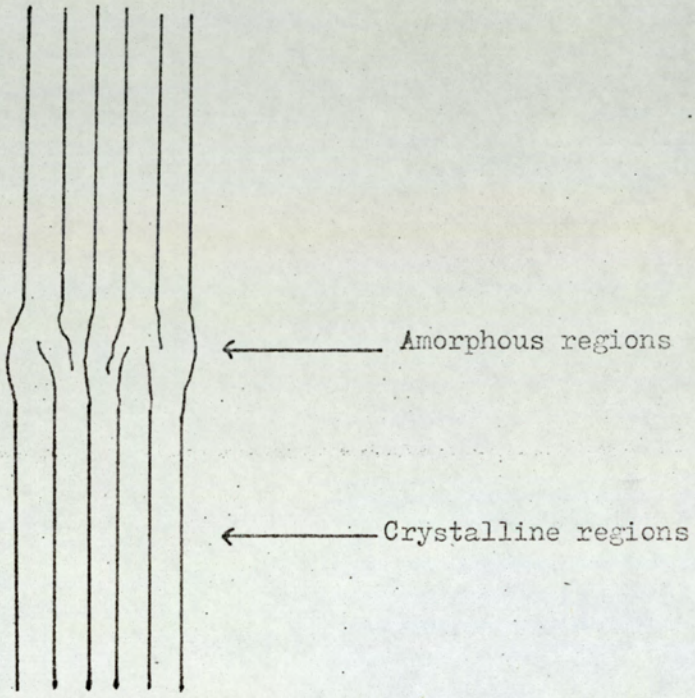
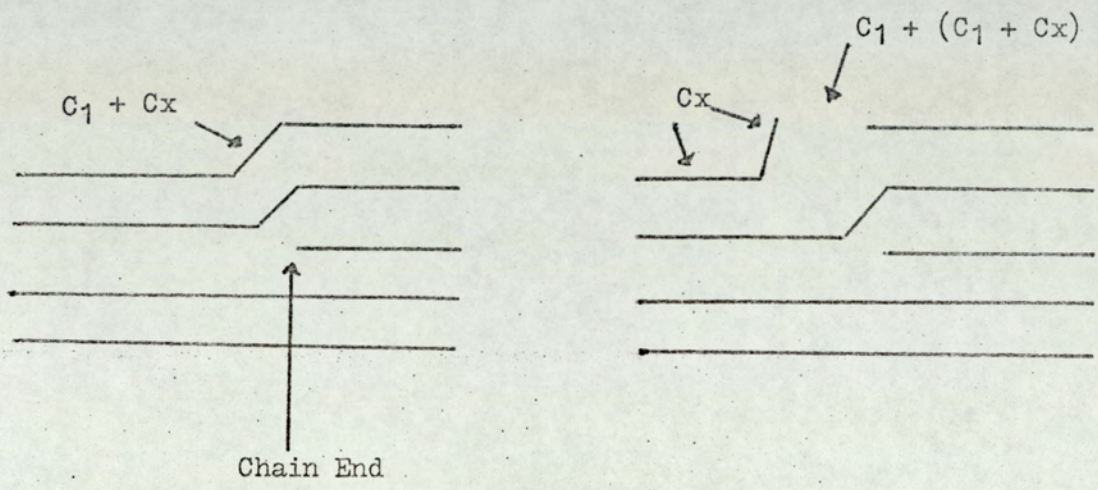


FIGURE 5



C_x Components

The enzymes are of two distinct types:- (a) the exo β - 1 - 4 glucanases, which split mono and disaccharide units from exposed, non reducing chain ends (Youatt and Jermyn 1959), Nisizawa et al. 1963, Li et al. 1965, Okada et al. 1968, Wood 1970), and (b) the endo β 1 - 4 glucanases which have a more random nature and act upon internal β 1 - 4 linkages (Jermyn 1952, Reese and Mandels 1963, King and Vessal 1969, Bailey et al. 1969, Keilich et al. 1969, 1970, Wood 1970).

The endo enzyme has a greater affinity for substrates of longer chain length and initiates the attack on amorphous forms of cellulose. The exo enzyme acts by inversion i. e. it produces α - glucose units from the β 1 - 4 linked breakdown products of the endoglucanase. Respective molecular weights of the exo and endo enzymes are given as 76,000 and 49,000 (Datta et al. 1963, King and Vessal 1969)

β - Glucosidases

These enzymes vary in their specificities and have been given the colloquial name "Cellobiase". They hydrolyse the smaller oligomers produced by the C_x complex to glucose. In contrast to the exoglucanases the β - glucosidases retain configuration i. e. they produce β -glucose.

Methods for Studying Cellulase Action

The Commission on Enzymes of the International Union of

Biochemistry (1961) defined a unit of cellulase activity as "that amount of enzyme which under defined conditions hydrolyses β 1 - 4 glucosidic linkages at the initial rate of one micro equivalent per minute". This definition is obviously very difficult to apply to the enzymes of the cellulase complex, either individually or collectively. One must also be able to define a standard substrate and standard conditions to be used throughout for the assay of these enzymes. A search through the literature will reveal the multiplicity of substrates, conditions and organisms used in assaying the microbial enzymatic degradation of cellulose (see Siu 1951 and Gascoigne and Gascoigne 1960 for accounts of methods up to 1960). Halliwell (1963) and Whitaker (1963) discuss problems involved in the assaying of cellulolytic organisms and Halliwell states that "the key to the problem lies in the basic form of cellulose used by workers". Cowling (1963) feels that the results of tests for cellulose degradation are certainly more meaningful when degradation "as it is carried out by the organism" is considered, rather than by enzymologists testing cell free extracts on highly modified forms of cellulose under artificial environmental conditions.

If pure cotton cellulose is used as a standard substrate then, as we have seen, its degradation is limited to those relatively few specialised fungi capable of producing both C_1 and C_x enzymes. The substrates most commonly used therefore are insoluble and soluble forms of cellulose which, because of preparatory methods, are usually highly amorphous. Such substrates are used to test for the presence of the C_x complex and form the basis of several test methods.

Insoluble forms of cellulose

In the first series of methods cellulose in the form of a very

finely divided powder is incorporated into an agar medium and enzymatic "clearing" of the cellulose particles is taken as proof of the cellulolytic activity of the particular test organism. This method was used as early as 1915 by Scales who tested the ability of 39 fungi to clear cellulose agar in plates and tubes. Hungate (1950) used ball milled, acid treated cotton cellulose, whilst Hazra et al. (1958) used acid disintegrated Whatman filter paper after ball milling the treated paper for 72 hours. After fungal digestion the remaining cellulose was visualised by staining with chloro-iodide of zinc. Hazra stated that this method was particularly suited for rapid survey work. Eggins and Pugh (1962) improved upon the above methods to prepare a medium for the isolation of cellulose decomposing fungi from the soil. They ball milled Whatman chromatographic cotton cellulose powder without acid treatment and incorporated it into a mineral salts agar containing asparagine ; the finely divided particles of cellulose remain in suspension when the medium is poured and cellulose breakdown is indicated by clearing of the medium. Their method was modified by Savory et al. (1967) who placed a plug of growing fungal mycelium into a recess in a plate of the same medium which was then further incubated. They also incorporated 0.005 mole per litre of sodium azide into the agar to supress fungal growth. Using this method they obtained "well defined" clearance zones around the fungal inoculum produced by the enzymes diffusing out from the inoculum into the fresh, ball milled cellulose agar. This method was subsequently used by Evans (1969) who found that thermophilic fungi were completely (sic) inhibited by the sodium azide and no cellulase activity was recorded for any fungus tested. To overcome factors such as diffusibility and stability of cellulases Walsh and Stewart (1969) prepared plates containing monolayers of E & P ball milled cellulose agar supported upon a layer

of water agar. Using this method they measured the intensity of clearing underneath a fungal colony by counting the remaining particles of cellulose under a microscope. They found good correlation between this method and results obtained using the same fungi on cotton yarn which was subsequently tested for loss in tensile strength, however it must be noted here that as a method it appears to be extremely tedious and time consuming. Thin layers of cellulose agar were used by Stranks and Bieniada (1971) to test the cellulolytic activity of enzyme solutions. Using their method, as little as 1µl of enzyme solution could be applied to the plate which was then incubated at 50°C for several hours, and clearing zones were noted if cellulase was present in the enzyme preparation.

To overcome the problem of lateral fungal growth Rautela and Cowling (1966) used cellulose agar in tubes to provide anaerobic conditions to check fungal growth and penetration but not cellulase activity. This method has been successfully used by Sharp (1969), Sharp and Eggins (1970), Malik (1970) and Malik and Eggins (1970) to study the ecology of cellulose decomposing fungi present in, and isolated from the soil. Tansey (1971 b.) used Walseth cellulose in Rautela-Cowling tubes to study the cellulolytic activity of the thermophilic fungi.

The second series of methods make use of "macro" forms of insoluble cellulose in a form suitable for visual evaluation of degradation, weight or strength loss determinations or for the production or reducing sugars released during the hydrolysis of the cellulose.

Reese (1946) used filter paper cellulose in shake flasks to study

cellulose degradation by A. fumigatus and H. insolens, these fungi causing a 40 - 50% loss of the cellulose within four days. Grey cotton duck was used by White et al. (1948) who enclosed the strips within boiling tubes with the lower end of the strip immersed in nutrient salts. They also inoculated fungal species onto filter paper discs, ground filter paper and cotton contained in nutrient salts solution and estimated weight loss of the cellulose. Abrams (1950) measured tensile strength losses of cotton duck placed on the surface of agar in bottles and inoculated with fungi, during the first 72 hours of degradation. He then went on to examine microscopically the number of "notches" and "holes" produced on individual fibres by Chaetomium globosum. He treated the fibres, after fungal attack, with NaOH to remove degraded, alkali soluble regions, and counted the number of pits produced over a period of 72 hours. He found a large increase in holes within 48 hours and this, he explains, could account for the large loss in strength of cotton in a short time with relatively little loss in weight. He then went on to study the I/R spectra of cellulose, before and after attack by Chaetomium globosum, using films of regenerated cellulose.

Siu and Sinden (1951) used bleached cotton sheeting placed on U-shaped glass rods in petri dishes. One end of the cotton dipped into a nutrient salts solution and the strips were exposed to fungal attack. Using this method the authors were able to vary the pH of the medium and to measure the effects of additional nutrients on the subsequent tensile strengths of the decayed strips. Cotton linters were treated with 85% phosphoric acid by Walseth (1952) to swell the fibres and reduce the degree of crystallinity of the cellulose. He then went on to calculate the percentage solubilisation of this "Walseth cellulose" using an enzyme

preparation from Aspergillus niger (Walseth 1952). This form of cellulose was used by Gilligan and Reese (1954) to estimate a "Walseth value" (W) equivalent to the amount of enzyme in 2ml assay medium needed to produce 0.5 mg/ml. reducing sugar in 2 hours at 50°C. They also measured the "swelling factor" (SF) defined as the amount of enzyme per ml of filtrate needed to produce a swollen weight of cotton fibre 50 mg above the control value in 1 hour at 50°C and pH 5.4, under the conditions of the alkali swelling test of Marsh et al. (1953). Thomas (1956) grew Stachybotrys atra in a liquid medium with 1% acid swollen cellulose. He used the resulting cell free extract to measure the production of reducing sugars (RS) from ground filter paper, Walseth cellulose, sodium carboxymethyl cellulose and hydroxyethyl cellulose. The enzyme preparation was then used to produce changes in the birefringence of cellulose (cellophane) film. These changes can be followed using a polarising microscope and Thomas found them to be extremely slow with his S. atra cellulase. He said that this method could provide a measure of one of the earliest stages in the digestion of cellulose i.e. the disorientation of the cellulose chains in crystalline cellulose. Cellophane discs were used by Talboys (1958) to study penetration by a root pathogen of hops, Verticillium albo-atrum. He sectioned the discs and hyphal penetration, a measure of cellulase activity, was noted. He also used a cell free extract of this fungus after growth on filter paper to measure the production of RS from filter paper pulp by converting the carbohydrates produced to their osazones. He found that the osazone produced resembled that of cellobiose. Golley (1960) buried regenerated sheets of cellulose, attached to an aluminium frame, in soils and then computed the "remaining area" of the original sheet. Jute, jute holocellulose and filter paper cellulose were used as substrates by Basu and Ghose (1960) who tested the cellulolytic activity of 124 fungi; whilst

cellulose powder and dewaxed cotton fibres were used by Halliwell (1961) to estimate the amount of cellulose made soluble by Myrothecium verrucaria. Selby (1961) used the same organism and measured weight losses of mercurised and non mercurised cellulose. He also used repeated treatments of the enzyme on cotton yarns and found that with repeated treatments strength losses of the yarn reached 100%. Bose (1962) studied strength and weight losses caused by Paecilomyces elegans on cotton, filter paper and jute fibres. This fungus preferred jute to cotton and production of RS was very feeble on filter paper. Filter papers were incorporated into a nutrient salts medium and inoculated with fungi by Garrett (1962). His method is still used as a standard test method. Mandels and Reese (1963) used cotton slivers, filter paper and Walseth cellulose as substrates for T. viride cellulase, whilst Toyama (1963) used small L-shaped tubes containing buffered enzyme solution and small strips of filter paper. The tubes were shaken at 40°C and degradation of the cellulose was estimated from the production of RS and from a decrease in size of the strips. Norkrans (1963) used a liquid medium containing ground filter paper to prepare cell free extracts of T. viride and Collybia velutipes. He used his own turbidimetric method (Norkrans 1950 a., b.) to measure cellulase activity. Aspergillus japonicus was used by Agarwal and Sahgal (1964) who measured cellulase activity by titrating the RS formed from filter paper cellulose with N/200 sodium thiosulphate. Chang (1967) used Garrett's (1962) filter paper technique to study the cellulolytic activity of several thermophilic fungi. She found that weight losses were produced by Chaetomium thermophile, H. insolens, Sporotrichum thermophile, M. pulchella and A. fumigatus, but not by T. duponti , H. lanuginosa or M. pusillus. The enzyme powder from an unidentified Basidiomycete was used by le Grand and Thivend (1967)

on powdered cotton, ∝-cellulose and cotton linters. They studied weight loss of the substrates against pH and they also measured the products of hydrolysis using the anthrone reagent of Loewus (1952) and a glucose oxidase test. Ulezlo (1968) used 2% filter paper with salts and yeast extract to study the growth of M. verrucaria. He found optimum pH for cellulase production to be pH 5.3. Mycelial biomass was one parameter used by Costa and Pacheco (1968) who grew T. viride on Solca floc cellulose. They also measured production of glucose from the cellulose. Fergus (1969 a.) used Garrett's filter paper method to assay the cellulolytic activity of the thermophilic fungi. He extended Chang's (1967) list of cellulolytic thermophilic fungi to include H. grisea, Torula thermophila and M. albomyces. Ball milled, spruce pulp cellulose was used as a substrate for T. viride by Ghose (1969) who measured production of RS using a 3'5' Dinitrosalicylic acid reagent, whilst Betrabet and Patel (1969) grew Aspergillus terreus on cellulose powder and a sawdust-wheatbran mixture. They measured RS by the alkaline copper reagent method of Somogni (1945). Garrett's technique was again used by Evans (1969) to study the weight losses produced by several thermophilic fungi. He found quite small weight losses produced by his thermophiles after three weeks incubation at 40 or 45°C. A whole range of techniques were used by Kaplan et al. (1970) to show that the resistance of weathered cotton to cellulase increased with increased exposure to U/V light over a period of 14 months.

They tested the strips by tensile methods, viscometry, I/R data, G.L.C. and x-ray diffraction. Wood (1971) used Walseth cellulose and dewaxed cotton fibres to study the products of hydrolysis by T.L.C. produced by Fusarium solani cellulase, whilst Walsh and Stewart (1971) used the type

of cotton yarn used in conveyor belting in mines to study the fungal degradation of the belting under a variety of environmental gaseous conditions. Garrett (1971) used his own filter paper technique to study weight loss and colony diameter of cereal root pathogenic fungi; and similar studies were carried out by Bhargava (1972) using 16 isolates of cereal foot rot fungi.

Newer techniques have just become available for studying the fungal degradation of cellulose. These "Perfusion Techniques" of Eggins et al. (1968), Malik (1970), Malik and Eggins (1971) involve the use of strips of chromatography paper along which nutrient salts can be perfused. Organisms are inoculated onto the cellulose and the perfusion current of nutrient salts ensures a constantly available supply of nutrients to the organism and at the same time removes its waste and staling products. A particular advantage of this technique is the ability of a solid small-scale system to function in a Petri dish for a considerable time. The technique was used originally to screen out cellulolytic organisms from the soil, but it can easily be modified to test the cellulolytic activity of individual fungi.

Soluble forms of cellulose.

These forms of cellulose have the advantage of being water soluble and can be used in tests such as viscometry and tests designed to show the production of reducing sugars. The substituent groupings, such as carboxymethyl, serve to space the cellulose chains apart and permit solvation of the unsubstituted hydroxyls (Bass et al. 1943). Carboxymethyl cellulose (CMC) was first described in 1921 (Jansen 1921), and forms smooth, viscous solutions in water. Enzymatic degradation will cause a decrease in the viscosity of such solutions and this fact

is the basis for the first type of test method using soluble cellulose derivatives.

Viscometry

The procedure employed is subject to several limitations: (a) the degree of substitution (DS) of the derivative i. e. the average number of carboxymethyl groups per anhydroglucose unit. The rate of enzymatic hydrolysis of CMC is inversely proportional to the DS (Reese et al. 1950, Levinson and Reese 1950, Halliwell 1963, Joos et al. 1969), probably due to the fact that if too many group ings are introduced, the number of hydroxyl groups available for hydration is greatly reduced and the product is incompletely soluble in water. CMC solutions with a DS of about 0. 5 are normally used in enzyme studies, (b) the buffer employed must be carefully checked since it too can influence the viscosity of a cellulose derivative, (c) the effect of other contaminating substances such as trace metals, proteins and electrolytes on the viscosity of the solution and (d) the concentration of the derivative itself upon the relative viscosity of the solution (Gascoigne and Gascoigne 1960)

The viscosity of CMC in particular, is dependent upon the ionic strength of the solution and a major difficulty with this type of method is the preparation of solutions of CMC which have reproducible concentrations and initial specific viscosities (Halliwell 1963). Joos et al. (1969) mention that there is a "complexity of viscosity factors" involved in such systems and therefore themethod must be considered as a relative one since cellulase preparations are mostly mixtures of

enzymes. They also mention that the number of subunits split per unit of time, or the reducing groups released,are still difficult to determine with accuracy.

Nevertheless, this method has been used by many research workers, since initial degradation of these high molecular weight products leads first to other products of high M. Wt. (which can be detected by changes in viscosity), and only later to low M. Wt. reducing sugars (Mandels and Reese 1963, Keilich et al. 1970). Typical viscometric determinations have been carried out by Lindenfors (1962) who tested the cellulases of Aspergillus niger. Agarwal et al. (1963) measured the fall in viscosity of CMC using enzymes from Chaetomium globosum, whilst Göransson and Fahraeus (1963) used M. verrucaria, S. atra, A. niger, Cladosporium sp. and T. viride as cellulolytic test organisms on ethyl hydroxyethyl cellulose (EHEC). Horton (1964) used a temperature of 40°C to assay the enzymes from a root pathogen, Pyrenochaeta terrestris, using 1% CMC solutions. Viscometry was used by Bailey et al. (1969) to characterise the cellulase of Polyporus schweinitzii and he then went on with Keilich et al. (1969) to further characterise the enzymes pH and temperature stabilities. Domsch and Gams (1969) used the reduction in viscosity of CMC to determine the cellulolytic activity of 300 soil fungi, whilst Whitney et al. (1969) demonstrated a double peak for enzyme activity from Verticillium albo-atrum when incubated with solutions of CMC buffered at various pH values. An enzyme powder of "fungal origin" was used by Amsallem (1970) who used viscometry to assay the activity of the different fractions of the enzyme after elution through tubes of Sephadex. Wood (1971) assayed Fusarium solani cellulase using viscometry, whilst Ogunda et al. (1971)

used this technique to compare the relative cellulolytic activities of four fungi causing soft rot of yams.

Production of Reducing Sugars (RS)

CMC has been used in cellulase studies to provide reducing sugars which can be further determined colorimetrically or volumetrically. Many methods have been evolved to assay RS released after enzymatic hydrolysis. Folin's alkaline ferricyanide method (Folin 1929) was modified by Park and Johnson (1949) so that they could read off the colour produced by the reduction of the ferricyanide at 690 nm in a spectrophotometer. They then compared values obtained with each known amounts of D-glucose as standard. A similar method to the above, but using a phenol/sulphuric acid reagent, was used by Levvy (1946) to obtain a stable orange colour which could be read at 490 nm.

The reduction of cupric to cuprous oxide by aldehyde groupings formed the basis of a copper reduction test (Nelson 1944). This method was used by Stutzenberger et al. (1970) to measure RS production from CMC by clarified extracts of town waste compost; and by Boothby (1970) to assay the cellulases of T. viride. Nelson's method was subsequently revised by Somogyi (1952) and the Somogyi-Nelson method is capable of detecting as little as 5µg of glucose. Nisizawa et al. (1963) used this revised method to test highly purified cellulases of Irpex lacteus, whilst Whitney et al. (1969) used it to test "cellobiase" activity of Verticillium albo-atrum. A similar method was used by Betrabet and Patel (1969), Toda et al. (1970) and Flannigan (1970).

The enzymes produced by Aspergillus oryzae were assayed by Jermyn (1952) who used iodine consumption by reducing groups as a basis for his test method. This method gave an index of the number of reducing groups liberated from CMC by the enzyme.

Agarwal et al. (1963) measured the RS liberated from CMC by Chaetomium globosum by titrating with N/200 sodium thiosulphate, whilst Wood (1970) measured the production of RS by Fusarium solani cellulase using dichromate/sulphuric acid mixtures.

Glucose oxidase, an enzyme, was used by Levinson et al. (1951) and by Boothby (1970) to determine the amount of enzymatically produced glucose; whilst Amsallem (1970) used an anthrone reagent to achieve similar results.

A widely accepted test method for determination of RS was introduced by Sumner (1924) and Sumner and Howell (1935). It involves the use of a reagent containing 3', 5' - dinitrosalicylic acid (DNSA) which produces a dark brown colour when boiled with RS. The colour can be read at 550 nm (Fergus 1969) and compared to the results obtained using known concentrations of D-glucose as standard. This method has been used by Reese and Mandels (1963) to study the effect of temperature and pH on the cellulase activity of T. viride, and by Fergus (1969) who assayed for C_x production by the thermophilic fungi. He found that 9 out of the 15 thermophiles tested were capable of producing hydrolysis of the CMC solution he used.

These, and other, evaluation techniques have been used by

many workers to assay the cellulolytic activity of a wide range of microorganisms. When choosing test methods for these enzymes it is important to remember the comments of Halliwell (1963), Whitaker (1963) and Cowling (1963) [see Introduction to this Chapter]. The majority of test methods in use today measure the C_x components of the cellulase system and these systems will in fact be used by the author in these studies.. It was decided to use a weight loss and a strength loss technique to study the cellulolytic activity of the thermophilic fungi, since these techniques are true measures of cellulolysis. Other workers have used cellulose agar methods (Evans 1970, Malik 1970, Tansey 1971), and CMC - reducing sugar methods (Fergus 1969a.) to prove that several thermophilic fungi can produce cellulase, but these methods are largely qualitative and bear little relationship to the problems involved in composting where weight losses of cellulose are more meaningful. Weight loss methods have been used for the thermophiles by Reese (1946), Chang (1967), Fergus (1969a.) and Evans (1969); however these investigators, with the exception of Reese, found very small overall weight losses produced by their cellulolytic, thermophilic fungi when incubated in the presence of mineral salts on filter paper cellulose at temperatures of 40 to 45°C. In this study therefore two techniques will be used:- (a) inoculating the thermophilic cellulolytic fungi directly onto cellulose strips supported on nutrient salts agar - this avoids problems of anaerobiosis in liquid culture and (b) inoculating these fungi onto cellulose strips which are being continuously perfused with nutrient salts, in the Perfusion systems of Eggins et al. (1968) and Malik (1970). It was thought that by using these methods, and an incubation temperature of 48°C, conditions more conducive to the growth and cellulase production of the thermophilic fungi might be achieved.

Materials and Methods

(1) Agar-strip technique

Strips of Whatman 3MM chromatography paper (4 x 0.5 cm) were cut out using a paper guillotine and four strips (after dry autoclaving) were placed on the surface of E & P nutrient salts agar. The agar medium was made up according to the formula of Eggins and Pugh (1962), however 30 g/l of agar was employed to produce a hard, smooth surfaced medium upon setting. It was found that the strips did not stick to the surface of this agar and could be very easily removed, after fungal degradation, for subsequent testing. The test strips were moistened with sterile distilled water and inoculated at their centre with a 5mm plug of actively growing fungus taken from a culture growing on E & P cellulose agar. The plates were incubated at 48°C for up to 14 days and four strips per plate for each fungus were evaluated daily for strength loss and weight loss in comparison to uninoculated test strips on agar at 48°C as controls.

(2) Perfusion Technique

Strips of Whatman 3MM chromatography paper (9 x 0.5 cm) were used in the kits of Eggins et al. (1968), Malik (1970), Malik and Eggins (1970). It was found necessary to slightly modify the perfusion technique, outlined by the above authors, to overcome the tendency of the system to build up a back pressure at thermophilic temperatures. This was very easily achieved by discarding the silicone rubber bungs in the necks of the reservoir flasks, and substituting non absorbent

cotton wool plugs. Another slight handicap, exacerbated by the high rate of evaporation of moisture from the tail wicks, was also experienced. Malik in his thesis (1970) mentions that the build up of waste metabolites and salts that occurs as water evaporates from the tail wick can be removed by cutting off the end of the tail wick and its solidified encrustations. It was found here that the salts tended to crystallise out at the top end of the evaporation wick next to the protective silicone rubber, and block the flow of the perfusing nutrients. This deposit, however, could be easily removed by periodically washing the tail wicks in distilled water to dissolve away the salts and waste metabolites.

The strips were inoculated with 5m.m. agar plugs of actively growing thermophilic fungus on E & P cellulose agar. Eight strips per fungus were removed daily for up to 6 days and tested for loss in wet strength in comparison to four uninoculated, but perfused and incubated strips as controls. All the kits were incubated at 48^{o}C. It was thought unwise to cut the strips up into 4 cm pieces, or in fact to take the 9 cm. strip as such for subsequent weight loss testing since (a) the growth characteristics of individual fungi on theperfused strips were, in many cases, so unlike those on the agar supported strips e.g. growth in all cases on the agar supported cellulose strips was symmetrical, from the centre outwards whereas on the perfused strips some fungi grew towards the flow of the nutrient salts and some away, whilst others tended to produce a greater biomass on one side of the inoculum but greater cellulolysis on the other side. For these reasons a representative strip could not be cut out and (b) prior observations had suggested that growth and biomass were very much greater on the agar supported strips where there was build up of waste products and eventual lack of nutrients,

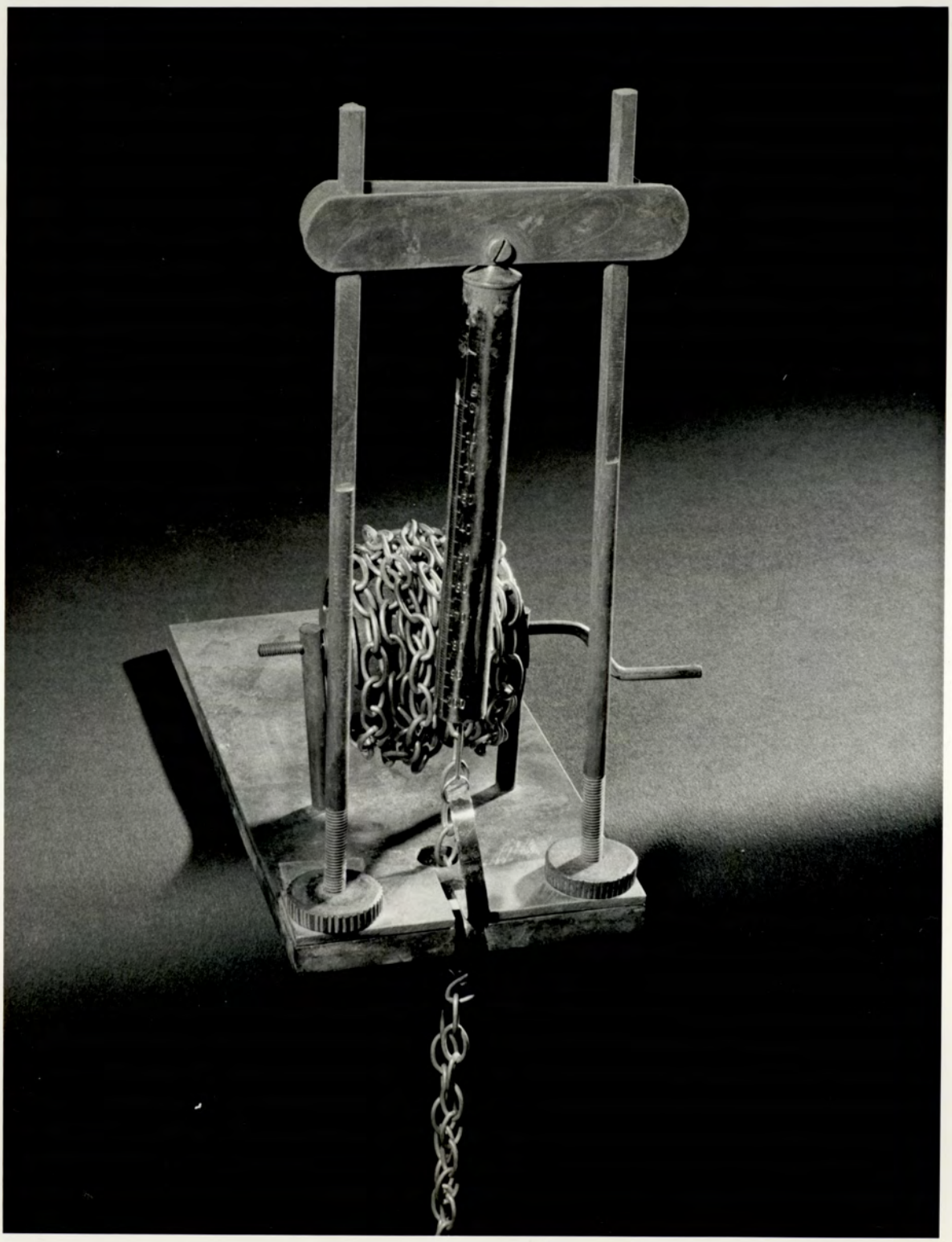
than on the perfused strips where ideal conditions produced a more concentrated growth around the area of the inoculum.

Strength Testing

A Problem arose over strength testing of the strips as there was not access to a tensometer which could be used daily in the laboratory. However, a small machine, designed by Dr. R. F. Sharp of the B.I.C., was used which was capable of measuring the wet strength of the paper strips to an accuracy of 1 gm. The machine (Plate 1) consisted of an accurate spring balance calibrated from zero to 100 g, attached at its upper end to two metal plates which were free to move and could be clamped firmly to the flattened upper ends of two metal supports by the wing nut and screw holding the top of the balance. The lower ends of the supports passed through a heavy steel baseplate and each support had a screw thread at the lower end on which ran a locking device to firmly clamp the brass jaws, also attached to the lower ends of the supports, to the baseplate. The baseplate had a cut out section across which the paper specimen strip was clamped by the brass jaws, and a small hole through which a brass chain passed and was attached to a revolving drum mounted on the back of the baseplate. The other end of the chain was attached to the underside of a light, stainless steel breaking bar, the upper part of which was attached to the bottom of the spring balance.

In operation the paper strip was wetted (see below) by dipping for 5 seconds in distilled water - this ensured a constant level of water in each replicate strip - and each strip was carefully clamped

PLATE 1



by the brass jaws over the cut out section in the baseplate. With the brass chain fully wound onto the drum at one end and attached to the lower part of the breaking bar at the other end, the breaking bar was hooked onto the lower end of the spring balance. By slowly revolving the drum, brass chain was payed out until the breaking bar rested on top of the strip. A weight of 4g. was recorded on the balance at this point. The brass chain was then slowly run out using the fingers around the edge of the revolving drum (the handle served only to return the chain onto the drum). When the paper strip broke the weight recorded on the balance was taken as the weight necessary to break that strip of paper. The results were calculated as the percentage loss in breaking strength of the inoculated strips compared to values obtained for uninoculated strips under identical conditions as controls.

The strips were placed with the point of inoculation - after carefully removing the agar plug - over the hole in the baseplate. With greatly decayed specimens the wetting procedure, which would have caused breakup of the strip, was omitted and in this way it was possible to measure breaking strengths down to 4g i. e. the breaking bar just resting on the strip. It was found that constant and reproducible results could be obtained by one operator using this machine. The run out time of 50 cm. of chain over 50 consecutive operational runs was recorded. The author operated the machine throughout for the work reported in this thesis, and found that the run out times varied from 10 to 13 seconds with a mean of 11. 5 seconds.

Weight loss testing

The four broken strips per fungus from the agar plate technique

were dried to constant weight in a hot air oven at 80°C and weighed directly from the oven to prevent the strips picking up atmospheric water. The dry weights of the strips were compared to those of the broken uninoculated control strips and the percentage weight loss of the inoculated strips calculated against time.

Results

(a) Agar-strip technique

The results of the strength and weight loss testing are shown in Figures 6 to 8. From these results it can be seen that most of the twelve thermophiles tested could produce a 100% loss in wet strength of the strips within 7 days and a weight loss of at least 75% within 14 days. Fungi causing a 100% strength loss within 4 days were the two varieties of Chaetomium thermophile, Sporotrichum thermophile and Cephalosporium sp.. Thielavia thermophila and H. grisea produced 100% strength loss within 5 days and H. insolens within 7 days. Although strength loss results for the two varieties of Chaetomium were almost identical, slight differences were noted for the weight loss technique with variety dissitum producing a slightly higher weight loss than variety coprophile. Total weight losses could not be recorded for these and for some of the other fungi because of very heavy mycelial production. In respect to strength and weight loss, Sporotrichum thermophile must be considered as undoubtedly most cellulolytic of the twelve fungi tested, producing 100% strength losses of the strips within 72 hours and almost total weight loss within 9 days. Thielavia thermophila, which only produced the asexual state on the strips, caused total strength loss of the cellulose within 5 days, and 80% weight loss within 14 days.

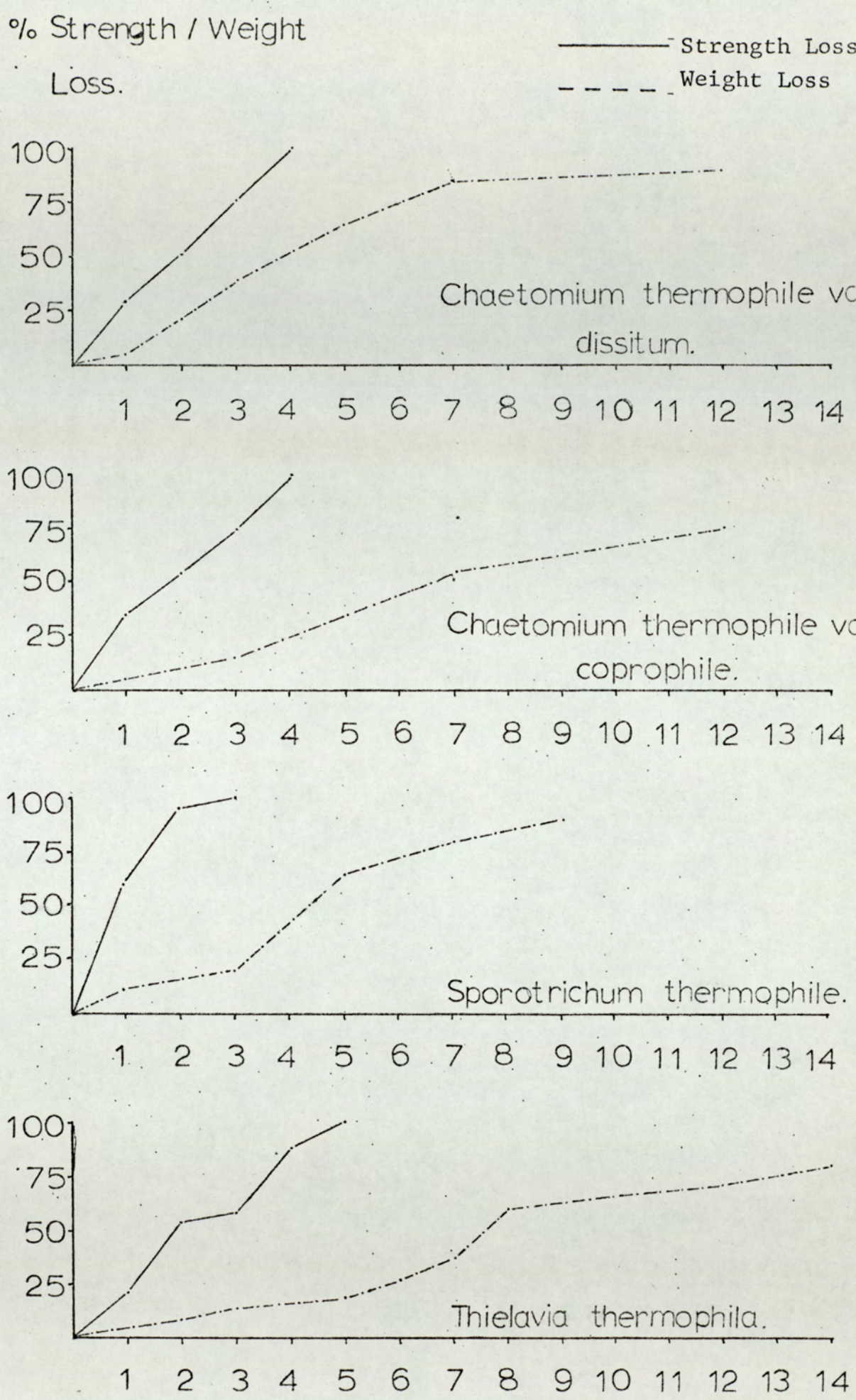

% Strength / Weight Loss.
Strength Loss
Weight Loss
100
75
50
25
Chaetomium thermophile var. dissitum.
1 2 3 4 5 6 7 8 9 10 11 12 13 14
100
75
50
25
Chaetomium thermophile var. coprophile.
1 2 3 4 5 6 7 8 9 10 11 12 13 14
100
75
50
25
Sporotrichum thermophile.
1 2 3 4 5 6 7 8 9 10 11 12 13 14
100
75
50
25
Thielavia thermophila.
1 2 3 4 5 6 7 8 9 10 11 12 13 14
Days of incubation

FIGURE 6

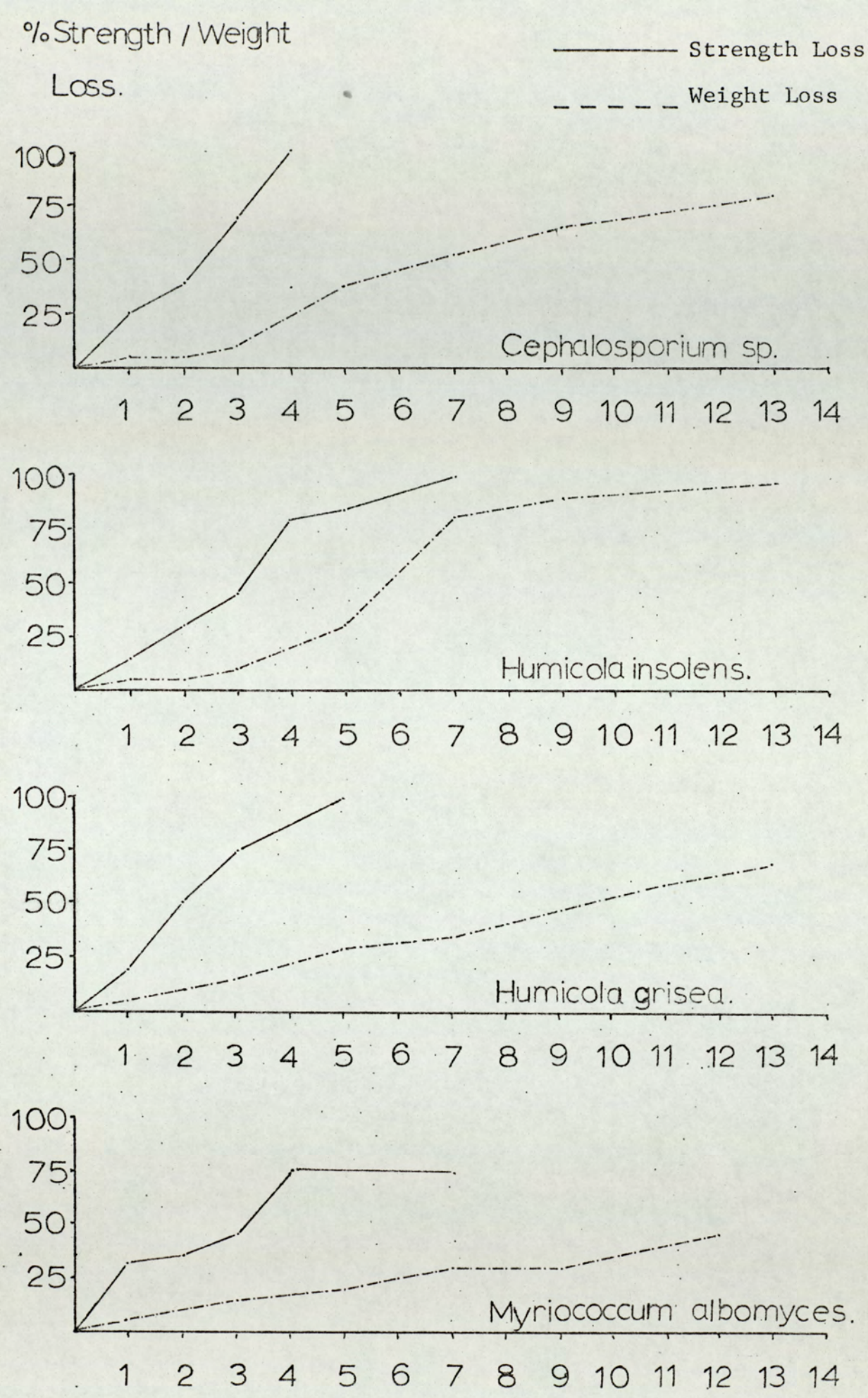

% Strength / Weight Loss.
Strength Loss
Weight Loss
100
75
50
25
Cephalosporium sp.
1 2 3 4 5 6 7 8 9 10 11 12 13 14
Humicola insolens.
Humicola grisea.
Myriococcum albomyces.
Days of incubation

FIGURE 7

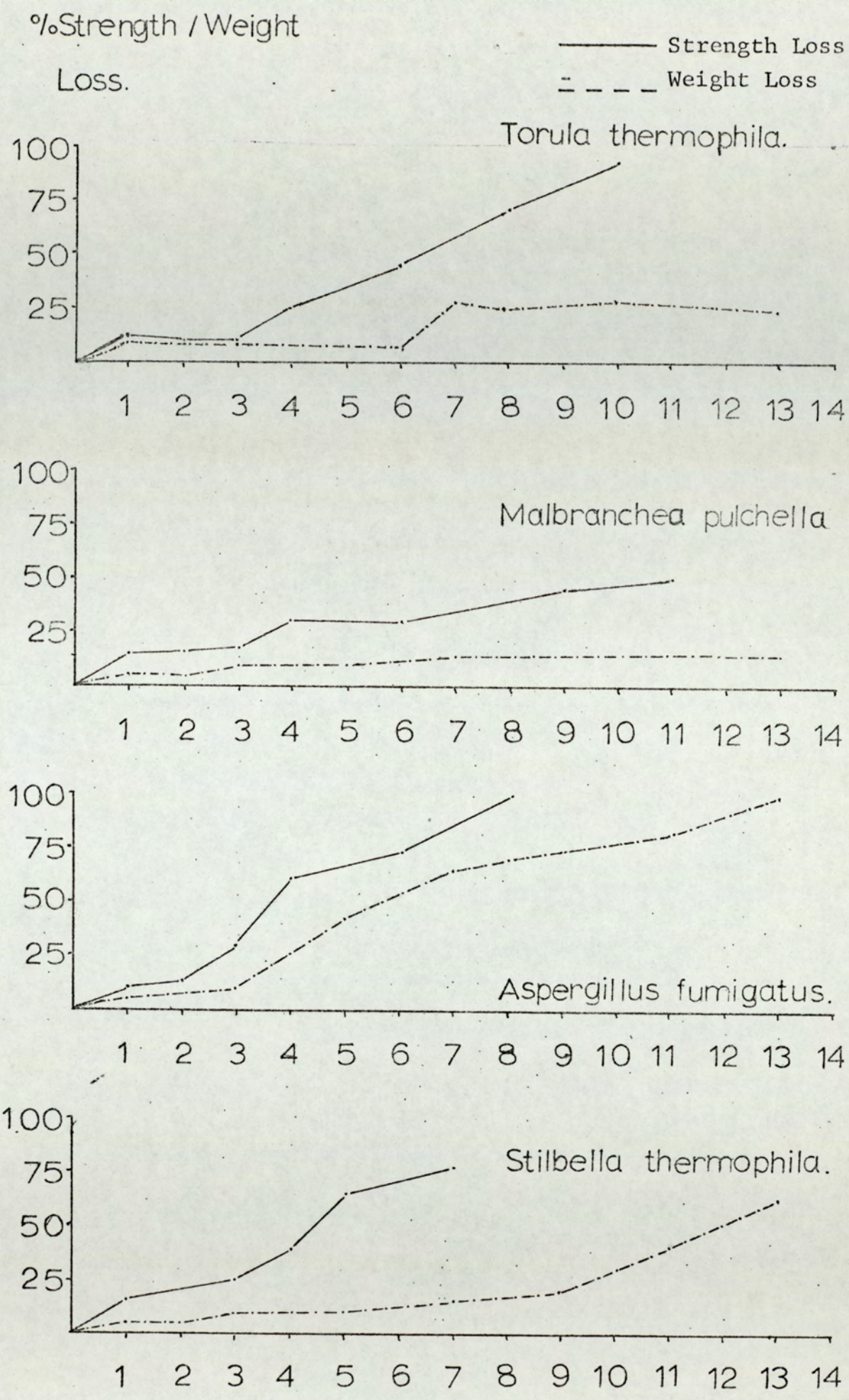

%Strength / Weight Loss.
Strength Loss
Weight Loss
Torula thermophila.
Malbranchea pulchella
Aspergillus fumigatus.
Stilbella thermophila.
100
75
50
25
1 2 3 4 5 6 7 8 9 10 11 12 13 14
Days of incubation

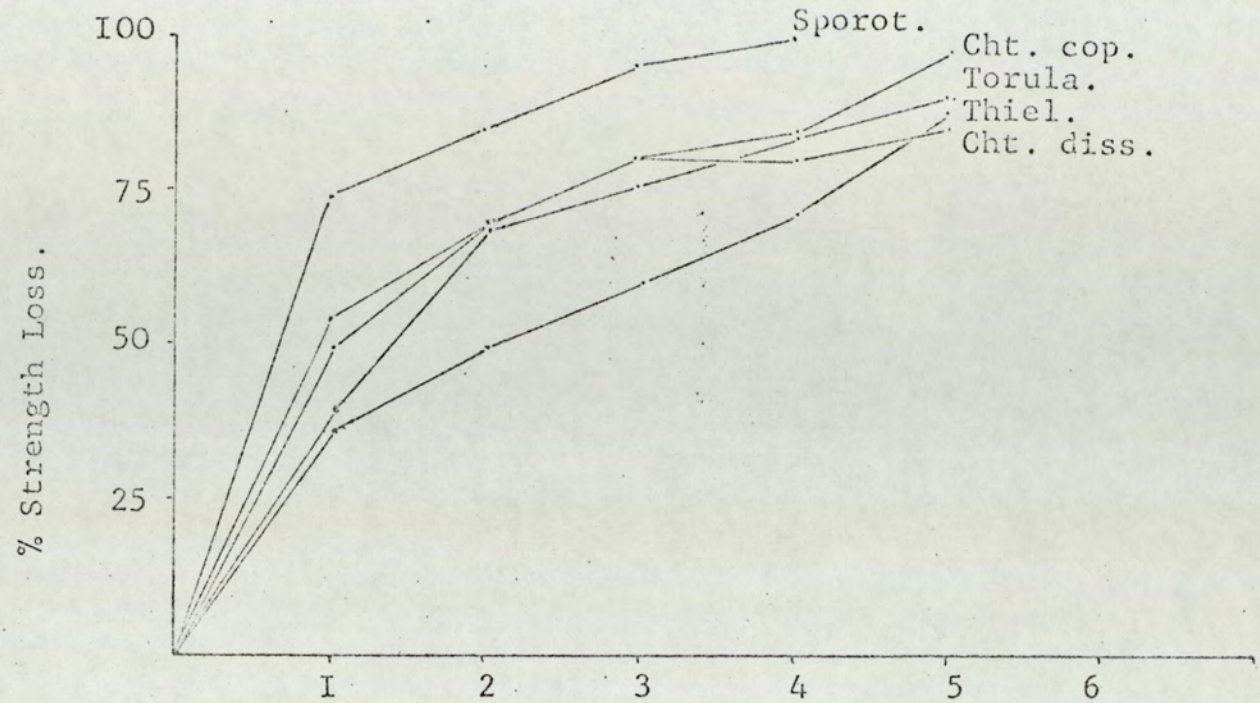
FIGURE 8

Cephalosporium sp., again producing only the asexual stage, brought about total strength loss within 4 days and 80% weight loss of the strips after 13 days. Growth of H. insolens over the strips was very rapid indeed and an 80% weight loss was recorded within 7 days. Total weight loss was produced by this fungus in 13 days, whilst in contrast, H. grisea grew more slowly over the cellulose, producing a 70% weight loss after 13 days. Myriococcum albomyces grew slowly over the strips and was only able to cause a 45% weight loss after 12 days, and similar observations applied to Torula thermophila and Malbranchea pulchella which produced weight losses of 25 and 15% respectively after 13 days growth. Trouble was experienced with Aspergillus fumigatus, since the strips were covered in a dense mat of non wettable spores. In this case the plates were flooded with a dilute solution of "Teepol" and the spores were gently scraped off the strips using the blade of a scalpel. Subsequent tensile and weight loss testing proved that A. fumigatus was extremely cellulolytic under these conditions, producing total strength and weight losses in 8 and 13 days respectively. Stilbella thermophila, in contrast to the data of Fergus (1969a), produced a weight loss of 63% after 13 days.

(b) Perfusion Technique

Results for the strength loss of the perfused strips are shown in Figures 9 and 10. The results indicate that, with the exception of Myriococcum albomyces and Malbranchea pulchella, all the fungi tested were able to produce strength losses of greater than 75% within 6 days. Sporotrichum thermophile again proved to be the most cellulolytic of the fungi tested, causing total strength loss of the strips within 4 days. The two varieties of Chaetomium produced good strength losses after 5 days,

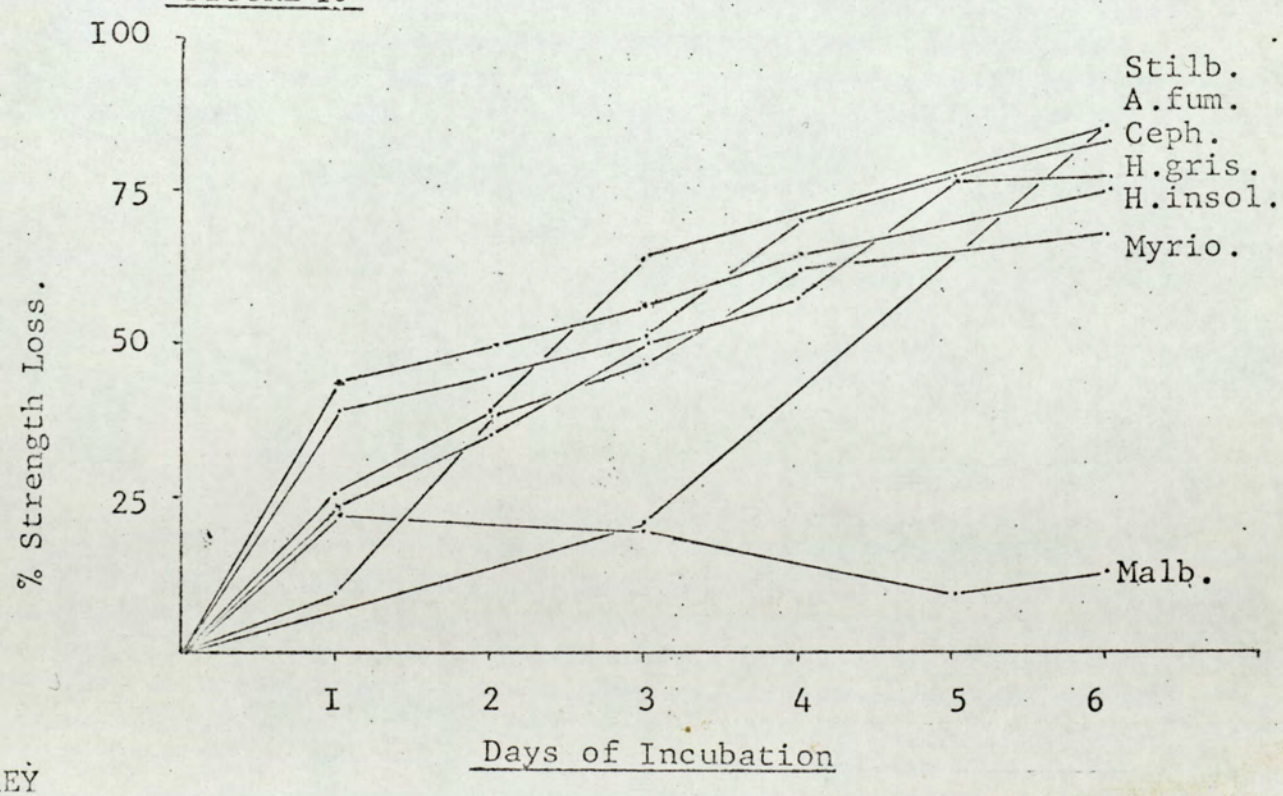
FIGURE 9



KEY

Sporot. (Sporotrichum thermophile)
Cht. cop. (Chaetomium thermophile var. coprophile.)
Torula. (Torula thermophila)
Thiel. (Thielavia thermophila)
Cht. diss. (Chaetomium thermophile var. dissitum)

FIGURE 10



KEY

Stilb. (Stilbella thermophila)
A.fum. (Aspergillus fumigatus)
Ceph. (Cephalosporium sp.)
H.gris (Humicola grisea var. thermoidea)
H.insol. (Humicola insolens)
Myrio. (Myriococcum albomyces)
Malb. (Malbranchea pulchella)

with variety coprophile producing the better results.

Growth of all fungi on the perfused strips was good, and maximum strength losses were recorded in the area of the inoculum without exception. This technique proved itself to be an extremely useful test method for studying the enzymatic degradation of cellulose at thermophilic temperatures. In all cases minimal trouble was experienced with the perfusion kits, and they proved extremely simple to "take down", clean and re-use.

Discussion

There are many accounts of thermophilic fungi growing on, and being isolated from cellulosic substrates (see Chapter 1), however, relatively few workers have carried out a detailed investigation into the quantitative and qualitative aspects of cellulolysis within this group of organisms. Reese (1946) showed that H. insolens was capable of degrading 40 to 50% of the cellulose in a shake flask culture at 45°C within 4 days, whilst the work of Chang (1967), Evans (1969) and Fergus (1969) using the filter paper technique of Garrett (1962) showed very small overall weight losses for the cellulolytic thermophiles they tested. On the basis of clearing rates in plates of E & P cellulose agar, Evans classified his thermophiles into four groups viz:- Group (1) (non cellulolytic), Group (2) (producing slight clearing) - Malbranchea pulchella, Group (3) (medium cellulolytic activity) - Allescheria terrestris, Cephalosporium spp. Chaetomium thermophile, Sporotrichum thermophile and Aspergillus fumigatus and Group (4) (strongly cellulolytic) - A. fumigatus (different strain), H. insolens, Myriococcum albomyces and Torula thermophila.

When he went on to determine weight losses of cellulose produced by these fungi he found that after 3 weeks at 40 to 45°C his Group (2) fungus (M. pulchella) produced only 1.2% weight loss; his Group (3) fungi produced 3.3 - 4.6% (Cephalosporium spp.), 4.6, 4.4, 4.7% (Chaetomium varieties), 3.3 - 3.5% (Sporotrichum thermophile), 3.7% (A. fumigatus); and his strongly cellulolytic thermophiles produced 3.1 - 5.7% (H. insolens), 2.2 - 2.4% (M. albomyces) and 4.7% (Torula thermophila). These latter results would tend to obviate Evans' earlier system of classification since, apart from M. pulchella, his other cellulolytic thermophiles could all be grouped together as being (comparatively speaking) equally cellulolytic.

The results presented here for percentage strength loss of cellulose by the cellulolytic thermophilic fungi at 48°C show that nearly all could produce a 100% strength loss of the cellulose. The most cellulolytic thermophiles in this respect were Sporotrichum thermophile, Cephalosporium sp. and the two varieties of Chaetomium. These results are in agreement with those of Malik (1970) who found that these three fungi produced greatest clearing of ball milled cellulose in Rautela-Cowling tubes. In the perfusion kits, where conditions appeared to be optimal for growth and cellulolysis, all fungi tested, with the exception of Malbranchea, were able to produce strength losses of greater than 60% within 4 to 5 days at 48°C. These particular results extend previously published results, to include all of the twelve thermophiles tested as being capable of producing loss in wet strength of insoluble cellulose.

When the results for percentage weight loss of cellulose are compared to the previous results it can be seen that this group of fungi,

on the whole, were able to significantly degrade insoluble cellulose at 48°C within 12 days. Weight losses correlated reasonably well with strength losses, although the smallest weight losses were produced by M. albomyces, Torula thermophila and M. pulchella.

From what has been said previously, it appears that M. pulchella is certainly the least cellulolytic fungus within this group of organisms. These weight loss results extend Fergus' (1969a.) list of thermophiles capable of degrading insoluble cellulose to include Stilbella thermophila, Cephalosporium sp. and Malbranchea - although Evans (1969) found that very small weight losses could be produced by M. pulchella.

If we compare the results for the percentage weight loss of insoluble cellulose (a primary measure of cellulolytic activity) at 48°C, then the twelve cellulolytic thermophilic fungi can be classified into three new arbitrary groupings:- Group (a) - those thermophilic fungi capable of producing at least 75 - 100% weight loss of insoluble cellulose within 12 days at 48°C (strongly cellulolytic) - Chaetomium thermophile var. dissitum, Sporotrichum thermophile, A. fumigatus and H. insolens; Group (b) - those thermophilic fungi capable of producing 50 - 75% weight loss within 12 days at 48°C (reasonably cellulolytic) - Chaetomium thermophile var. coprophile, Thielavia thermophila, Cephalosporium sp. H. grisea and Stilbella thermophila and Group (c) - those thermophilic fungi capable of producing less than 50% weight loss within 12 days at 48°C (weakly cellulolytic) - M. albomyces, M. pulchella and Torula thermophila.

If one studies the lists of thermophilic fungi that have been

isolated from composting town refuse (Glath 1959, 1964, Klopotek 1962, Stutzenberger 1969) previous to this study it can be seen that only three cellulolytic thermophiles had been isolated, viz:- Chaetomium thermophile, M. pulchella and A. fumigatus. Of these fungi, Chaetomium and Aspergillus have been found to be strongly cellulolytic organisms at temperatures found within composting town refuse. Malbranchea, on the other hand, has been found to be a very poor decomposer of cellulose. Additional studies (see Chapter 3) have shown the presence of three more cellulolytic thermophiles within town refuse - H. grisea and H. insolens and Torula thermophila. The first two can cause considerable decomposition of cellulose at 48°C, whilst Torula is only weakly cellulolytic.

This study, therefore, has demonstrated that the majority of the twelve cellulolytic thermophilic fungi are considerably cellulolytic in ability, and that six of them - four reasonably to strongly cellulolytic and two weakly cellulolytic - are found within composting town refuse. Are these six fungi specially adapted to withstand the parameters imposed by the composting of town refuse; and why are not the remaining six cellulolytic thermophiles found in this environment? The possible answers to these questions can only be given by a further study of this group of organisms on cellulose under several of the environmental conditions found within composting town refuse. There are obviously many possible parameters worth studying here, and it was decided to study two in particular i.e. the effects of pH on the cellulolytic activity of these organisms, since pH is an important environmental factor in composting; and then to go on and study the interactions of the cellulolytic thermophilic fungi themselves, since very little is known about this important factor.

CHAPTER 5

CHAPTER 5

The effects of pH on the cellulolytic activity of the thermophilic fungi

Introduction

We have seen from the previous chapter that at least twelve of the thermophilic fungi are able to produce the cellulase enzymes necessary for the degradation of insoluble cellulose. Since all enzymes are proteinaceous in nature, they can be affected by changes in temperature, pH and by denaturing agents. Hydrogen ion concentration plays a key role in microbiological systems involving growth, enzyme synthesis and product formation (Ghose 1969) and each enzyme will have its own optimum pH value at which it will function with maximal efficiency. The enzyme may undergo irreversible change if held at a pH value above or below its optimum, or it may change its affinity for its substrate i.e. the enzyme will not be fully saturated with substrate at pH values outside the optimum value. For cellulase enzymes, Reese and Mandels (1963) state that Fungal glucanases have an optimum activity at about pH 4.5 and are rapidly inactivated below pH 2 and above pH 8. This statement holds for the extreme values in pH but, from a study of tables presented by Siu (1951) and Gascoigne and Gascoigne (1960) it can be seen that fungal cellulases have a wide pH tolerance range, with optima from pH 3.0 (Hydnum henningsii) to pH 9.0 (Gliomastix convoluta).

A glance through any elementary textbook on microbiology will inform the reader that "bacteria prefer alkaline conditions whilst

fungi prefer acidic conditions for growth". Research on town waste composting suggests that this form of material very quickly reaches alkaline pH values and either stays alkaline, or falls slowly to neutrality, during the composting period. Schulze (1962) measured the pH of town refuse entering his rotating steel composting drum as pH 5.6 to pH 6.7, and that of the composted material as pH 6.6 to pH 8.1. In similar experiments Brenner (1957) found respective "before" and "after" values of pH 5.4 and pH 8.0; Wiley (1957) found respective values of pH 5.5 and pH 8.5; and Jann et al. (1959) found respective values of pH 6.4 and pH 9.3, pH 6.8 and pH 8.5, and pH 4.7 and pH 8.0 depending upon initial material used. These latter authors in fact used pH as a test to show completion of the composting process. The results of the work carried out in Chapter 2 also show that the pH of composting town refuse quickly rises above neutrality in the more aerobic regions of the heap. Is it possible, then, that colonising thermophilic fungi are unable to grow, sporulate and produce cellulase enzymes at the alkaline pH values within such a system?

Many authors have emphasised the importance of pH when studying cellulolytic fungi, and investigations have shown that the pH optima of many fungi lies on the acid side of neutrality. Siu and Sinden (1951) found the optimal pH's for cellulase enzymes of Myrothecium verrucaria, Aspergillus flavipes, Curvularia lunata and Gliomastix convoluta to be pH 6.0, 6.5, 7.0 and 9.0 respectively, whilst Jermyn (1952) found the pH optimum of Aspergillus oryzae cellulase to be pH 3.5. The cellulase of Stachybotrys atra was shown to have a wide optimum, from pH 6.5 to pH 8.0, depending upon the substrate and method of assay used (Thomas 1955); and in contrast, that of Poria vaillantii was found to have a low

pH optimum of pH 3.2 (Sison et al. 1958). Talboys (1958) found the optimum pH level for a crude cellulase preparation from Verticillium albo-atrum to be pH 5.6 to pH 5.8, whilst Halliwell (1961), in contrast to Siu and Sinden (1951), found M. verrucaria cellulase to have an optimum pH of pH 5.0. In this latter respect cotton sheeting was used by one set of authors (Siu and Sinden 1951) and powdered cellulose as an initial substrate by Halliwell. This would tend to suggest that fungal cellulase has different pH optima depending upon the type of cellulose used as substrate. Other authors, Verma and Verma (1962), have determined the optimum pH of Curvularia lunata cellulase to be pH 4.6 to pH 5.0; whilst Reese et al. (1962) measured the β 1 - 6 glucanase activity of six fungi, and found all optima to lie at approximately pH 4.0. Agarwal et al. (1963) measured the cellulolytic activity of Chaetomium globosum cellulase and found optimal activity in the range pH 4.8 to 5.2, whilst Agarwal and Sahgal (1964) found that Aspergillus japonicus produced an intra- and an extra- cellular cellulase system with optima at pH 4.0 to pH 4.5 and at pH 5.5 to pH 6.0 respectively. Trichoderma viride cellulase gave pH optima which varied with substrate used. On amorphous cellulose it had a broad optima, around pH 4.2, whilst on CMC a sharp optimum at pH 5.3 was given (Li et al. 1965). Garber et al. (1965) testing cellulases from three Penicillium species found pH optima at approximately 4.2 (P. digitatum), approximately 5.5 (P. italicum) and approximately 4.3 (P. expansum); whilst cell free extracts from corn stalk rot fungi produced broad pH optima at pH 5.0 to 7.0 (Gibberella zeae) and one broad and one sharp peak at pH 4.0 to pH 6.0 and pH 7.0 in Fusarium moniliforme (Cappellini and Peterson 1966). Trichoderma konigii cellulase has an optimum pH value of pH 5.0 (Halliwell 1966) whilst that of Coniophora cerebella has its optimum at pH 3.5 to pH 4.0

(King 1966). Other determinations are pH 5.3 for M. verrucaria (Ulezlo 1968); pH 4.0 for Polyporus schweinitzii (Keilich et al. 1969); ph 5.5 and pH 7.0 for cellulase of Aspergillus terreus (Betrabet and Patel 1969); pH 4.0 to 4.5 and pH 7.0 to pH 8.0+ for purified fractions of Verticillium albo-atrum cellulase (Whitney et al. 1969) and pH 4.0 to pH 5.0 for T. viride (Ghose 1969). The perfusion technique was first used for studying the ecology of soft rot fungi at different pH levels by Sharp (1969) and Sharp and Eggins (1970). They perfused beechwood veneers with salts buffered at various pH levels and inoculated with individual fungi, and compared strength losses of the wood at these pH levels with depth of clearing of ball milled cellulose, buffered at the same levels, in Rautela-Cowling tubes. They found that eighteen out of twenty-three fungi gave maximum decay of the wood on the acid side of neutrality. Malik (1970) used similar techniques to perfuse cellulose strips with buffered salts at thermophilic temperatures. He inoculated his screened substrate with soil and noted colonisation and weight loss of the strips. Initial colonisation was by Chaetomium thermophile at pH 6.0 with H. insolens appearing shortly afterwards at pH 6.0, pH 6.4 and pH 7.0. H. grisea appeared after 3 days at pH 7.0; but Sporotrichum thermophile (pH 6.0 to pH 7.0), Torula thermophila (pH 5.0 to pH 7.0) and Cephalosporum sp. (pH 4.0, 6.4 and pH 8.0) only appeared after 12 to 16 days when the strips were well deteriorated. Maximum deterioration of the strips was caused by C. thermophile at pH 6.0 and pH 7.0, and this fungus, together with Cephalosporium and Sporotrichum were the most cellulolytic on the pH adjusted agars at pH 6.4 (Chaetomium), pH 4.0 (Cephalosporium) and pH 5.0 (Sporotrichum). The other cellulolytic thermophiles tested by Malik showed maximum depths of clearing at pH 5.0 and pH 7.0 (H. grisea), pH 6.4 (H. insolens) and pH 7.0

(Torula thermophila). Using ball milled newspaper, Updegraff (1971) found a wide pH optimum for M. verrucaria cellulase from pH 3.9 to pH 6.5; whilst Stutzenberger (1971) found the optimum pH of cellulase from a thermophilic actinomycete isolated from town waste to be pH 6.0, with optimal enzyme production at pH 8.0.

These and many other investigations have shown that the pH optima for many fungal cellulases lies on the acid side of neutrality, but that many fungal cellulases exhibit different pH optima depending upon the initial cellulosic substrate. From the work of Malik (1970) it would appear that the cellulases of thermophilic fungi prefer neutral to slightly acidic environments. The work to be carried out in this section, therefore, will be to assess the pH optima of the twelve cellulolytic thermophiles so that an attempt can be made to indicate the potential of each fungus to degrade cellulose at the pH levels found within composting town waste.

The majority of methods used in such work are based upon the production of reducing sugars from, or the change in viscosity of, CMC solutions buffered at various pH levels. However, Wood (1969) has warned that "the use of CMC alone does not appear to be a sufficient criterion for the assay of C_x activity"; and so other authors have used cotton sheeting (Siu and Sinden 1951), cellopentaose (Björndal and Eriksson 1968), cotton fibres (Halliwell 1966, le Grand and Thivend 1967), chromatography paper (Malik 1970), ball milled newspaper (Updegraff 1971), β 1 - 6 glucan (Reese et al. 1962), Solka floc cellulose (Ghose 1969) and cellulose present within composting town waste (Stutzenberger 1970) as substrates for their studies.

The methods to be used in this thesis will be the production of reducing sugars from CMC, buffered at various pH levels, and based upon the system of Fergus (1969a.); and the perfusion system, in which parameters of pH can be carefully controlled and where insoluble cellulose in the form of chromatography paper can be used to provide a more "natural" substrate for fungal cellulase enzymes.

Materials and Methods

(1) Perfusion Technique

The twelve cellulolytic thermophiles were grown at 48°C on strips of Whatman 3MM chromatography paper (9 x 0.5 cm) enclosed in the (previously modified) perfusion systems. The strips were continuously perfused with E & P nutrient salts solution which was buffered from pH 3.0 to pH 8.0 using the citric acid/phosphate buffer of McIlvaine (1921). It must be noted here that the salts solution was not adjusted to pH 6.0, since the salts solution of Eggins and Pugh was already buffered at pH 5.8; and so this value was used instead of pH 6.0. The strips were inoculated centrally with a 0.5 cm disc of each particular fungus taken from an actively growing culture on E & P cellulose agar. After incubation, eight strips were removed periodically from the kits for each fungus tested and at each pH value. The strips were visually evaluated to determine the growth characteristics of each fungus over the pH range, and then tested for loss in wet strength using the previously described strength testing machine (Plate 1). Controls consisted of uninoculated strips which had been perfused and incubated under the same conditions as the test strips, and four control strips were broken to each of the eight sets of test strips.

(2) The Enzymatic study of cellulase activity

The twelve cellulolytic fungi were grown initially in two media for the induction of cellulase enzymes. The media consisted of 50 ml E & P nutrient salts solution containing (a) one percent carboxymethyl-cellulose (Hercules CMC - 7HF) with an average D.S. of 0.7, and (b) one percent ball milled cellulose (w/v). The flasks were inoculated with a 12 m.m. disc of actively growing fungus on E & P cellulose agar and the flasks were incubated at 48°C for 15 days to induce maximal enzyme production (Fergus 1969a). The mycelium and spores were then removed from the flasks by filtration and high speed centrifugation to produce cell free extracts of each fungus grown initially on ball milled cellulose (BMC)and CMC. Sterile technique was used throughout these preparations, and the cell free extracts were kept at 4°C until tested. A 0.55% solution of CMC (Fergus 1969a) was prepared and buffered from pH 3.0 to pH 8.0 with the citric acid/phosphate buffer of McIlvaine (1921); and to 9.0 ml of this solution was added 1.0 ml of each cell free extract. The tubes containing the mixtures were incubated for exactly one hour at 48°C in a thermostatically controlled water bath, with occasional shaking. Again, sterile conditions were used throughout to avoid the use of bacteriocidal agents (Gascoigne and Gascoigne 1960). At the end of the incubation period the tubes were removed and the contents of each tube were thoroughly mixed - prior experimentation had shown that thorough mixing obviated the need for duplication. One millilitre of the reaction mixture was then removed from each tube and pipetted into another small test tube containing 1.0 ml distilled water and 1.0 ml (DNSA) reagent (Reese and Mandels 1963). It has been found by previous testing that the amount of DNSA (3ml) used by Fergus (1969a) was far in

excess of the amounts required to give meaningful readings in the Pye Unicam SP 500 spectrophotometer used in this study. The liberated reducing sugar was then determined by heating and each tube of DNSA - reaction mixture in a water bath at 100°C for 5 minutes, cooling quickly to room temperature, and reading the optical density of the solution at 550 nm. The reference control, in each case consisted of one millilitre of the enzyme/CMC reaction mixture taken at zero time i.e. no incubation at 48°C; and pipetted into 1.0 ml distilled water and 1.0 ml DNSA reagent. This control, used as a reference in the spectrophotometer, takes into account any reducing sugar produced from, and present in, the initial B.M.C. and CMC cell-free extracts. It is interesting to note here that this fact was not taken into account by Fergus (1969)[a.], who used 1.0 ml of uninoculated 0.55% CMC incubated for one hour as his reference control.

Solutions of D-glucose and DNSA reagent were used to construct a graph (Figure 11) of optical density against mg. glucose (RS) per ml. The total amount of RS produced by 1.0 ml of the cell free extract acting on the 9 ml of 0.55% CMC buffered at various pH values was then calculated from the graph and from the initial dilution factor (i.e. 1 in 10) of the cell free extract. After incubation for one hour at 48°C the pH of each reaction mixture was taken and this value was used as the actual pH value in the construction of graphs to show the amounts of RS liberated by the B.M.C. and CMC cell free extracts for each fungus (see Figure 12).

Results

(1) Perfusion Technique

The results for the percentage strength loss of the perfused strip

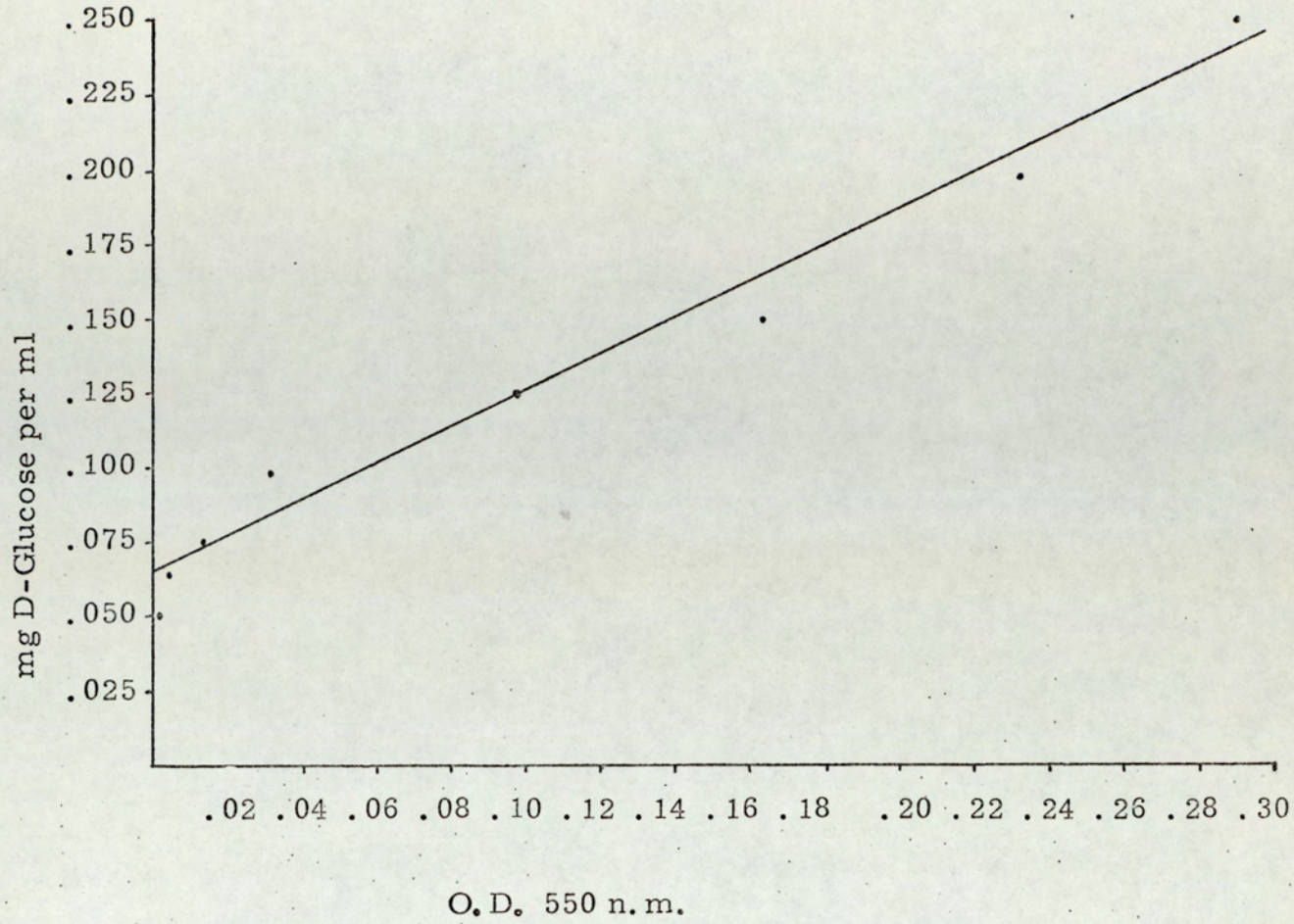


FIGURE 11

Graph showing optical density against milligrams of D-glucose per millilitre

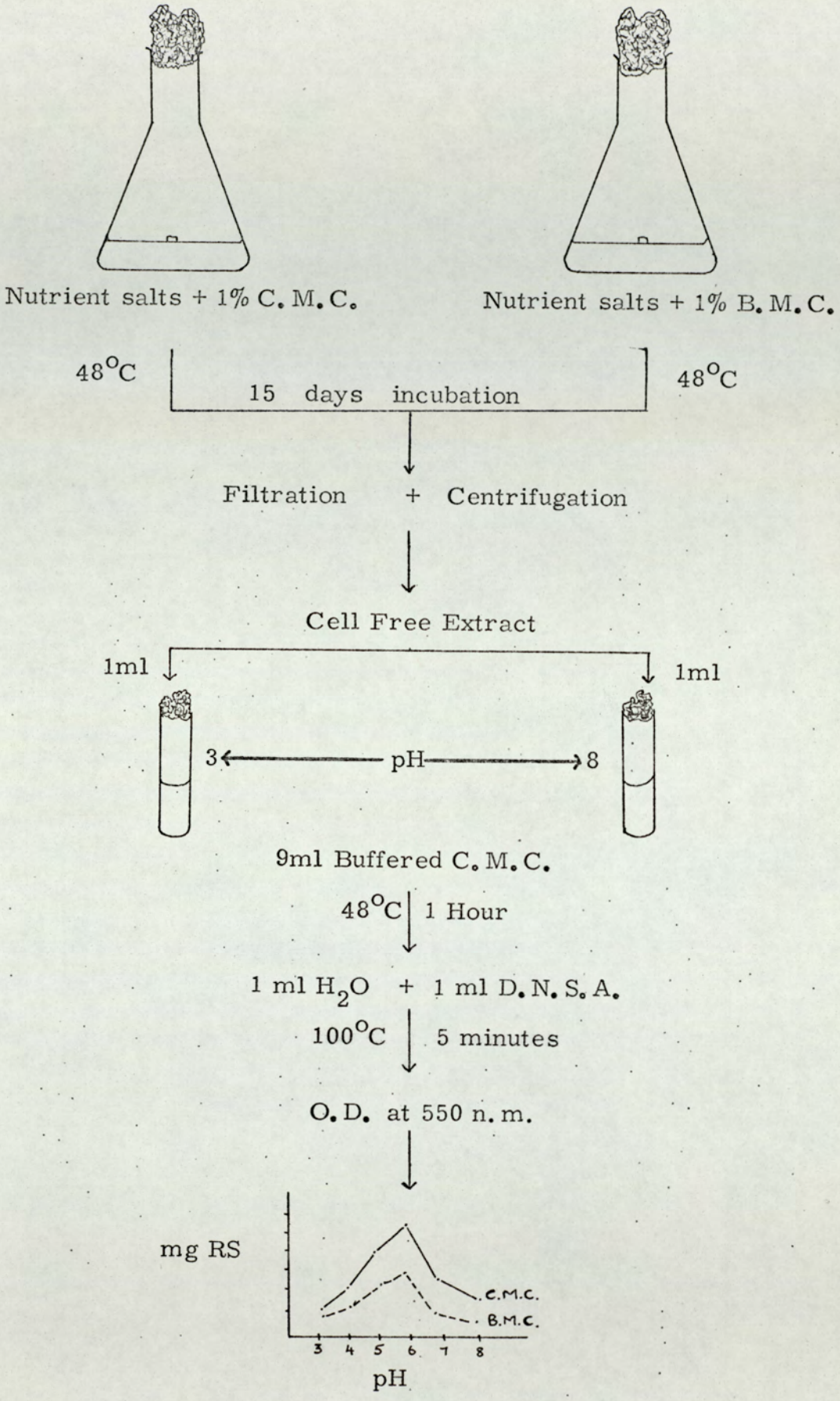


FIGURE 12

Procedure adopted to determine cellulase activity of cellulolytic thermophilic fungi grown initially on Carboxymethyl cellulose (C. M. C.) and Ball Milled cellulose (B. M. C.)

are presented in Figures 13 to 18. Again, little trouble was experienced with the perfusion technique and it proved to be a good test method for this particular parameter. Chaetomium thermophile var. coprophile produced good growth and perithecia at all pH values tested and a 100% loss in wet strength of the strips within 5 days. In contrast, variety dissitum produced good growth and perithecia at pH 3.0, 4.0 and 5.8, but less growth and few perithecia at pH 5.0, 7.0 and 8.0. Cellulase production, however, was good at all pH values and strength losses of 65 to 83% were noted within 5 days. A violet coloured pigmentation of the strips was produced by Sporotrichum thermophile, with very heavy growth and sporulation at pH 3.0, 4.0, 5.0 and 5.8. At pH 7.0 and 8.0, however, growth was poor with few spores, and a light green colouration of the strips was observed. Loss of strength of the strips, however, was total at all pH values within 5 days. Thielavia thermophila produced good strength losses of the cellulose within 4 to 5 days at all pH values tested, but as with Sporotrichum, it only produced heavy mycelial growth and sporulation at the lower, acidic pH values. Very little aerial mycelium was produced by M. albomyces at any pH value and no cleistothecia were observed. A 44 - 60% strength loss of the strips was recorded however within 5 days with the greater strength losses being recorded at the lower pH values. Stilbella thermophila produced good strength losses of the cellulose after 6 days within the range pH 4.0 to 5.8. Very little growth of this fungus was observed at pH 3.0, 5.8, 7.0 and 8.0, but good growth and coremial production was observed at pH 4.0 and pH 5.0. A very thick mycelial mat, which hampered strength testing, was produced by M. pulchella at pH 3.0, 4.0 and 5.0 together with a pink pigmentation of the cellulose. Very heavy sporulation was also observed at these lower pH values. Slight growth was produced at pH 5.8 and pH 7.0 but

FIGURE 13

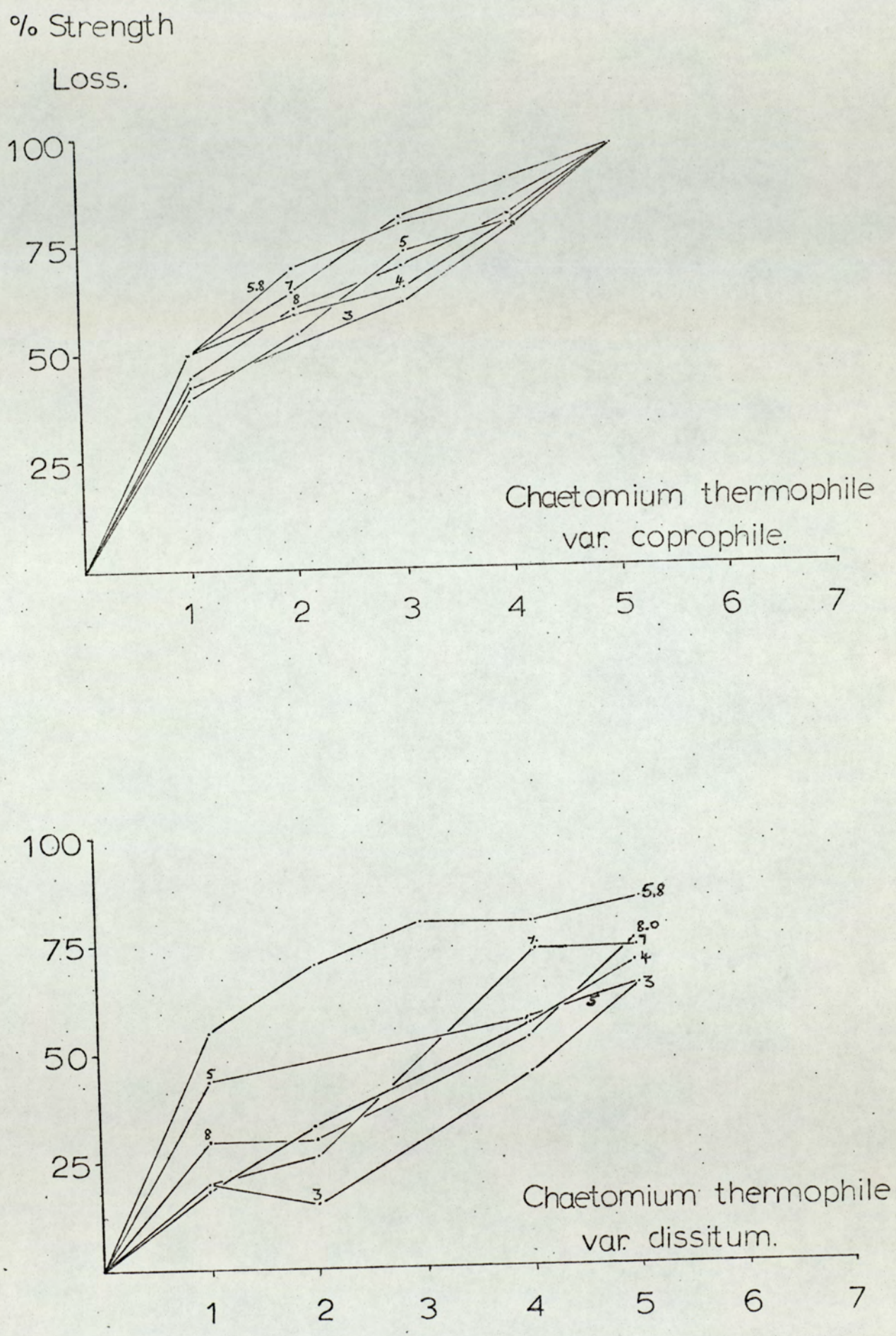

% Strength
Loss.
100
75
50
25
5.8
7
8
5
4
3
Chaetomium thermophile
var. coprophile.
1
2
3
4
5
6
7
100
75
50
25
5.8
8.0
7
4
3
5
8
Chaetomium thermophile
var. dissitum.
1
2
3
4
5
6
7
Days of incubation.

FIGURE 14

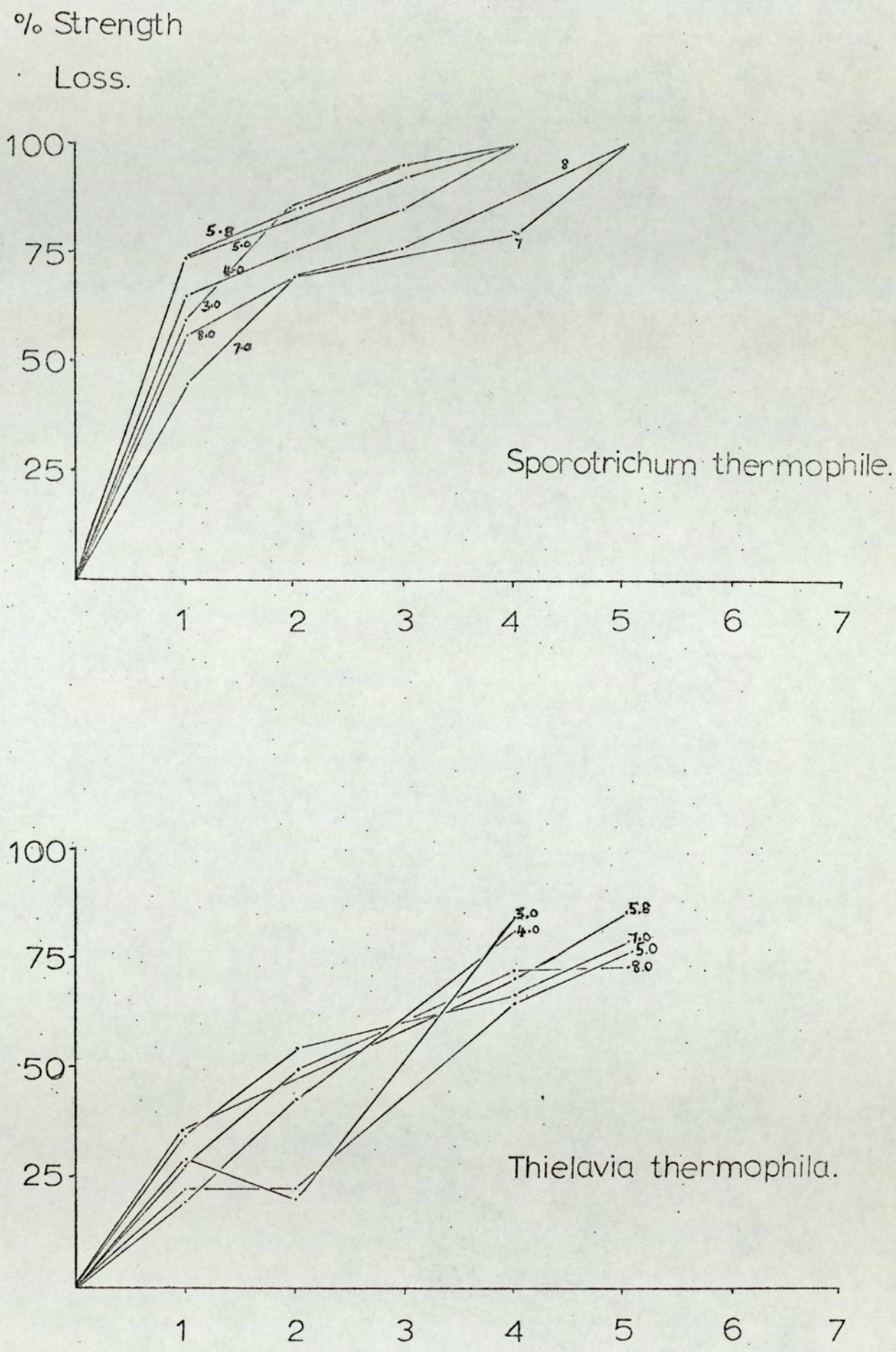

% Strength
Loss.
100
75
50
25
5.8
5.0
4.0
3.0
8.0
7.0
8
7
Sporotrichum thermophile.
1
2
3
4
5
6
7
100
75
50
25
3.0
4.0
5.8
7.0
5.0
8.0
Thielavia thermophila.
1
2
3
4
5
6
7
Days of incubation.

FIGURE 15

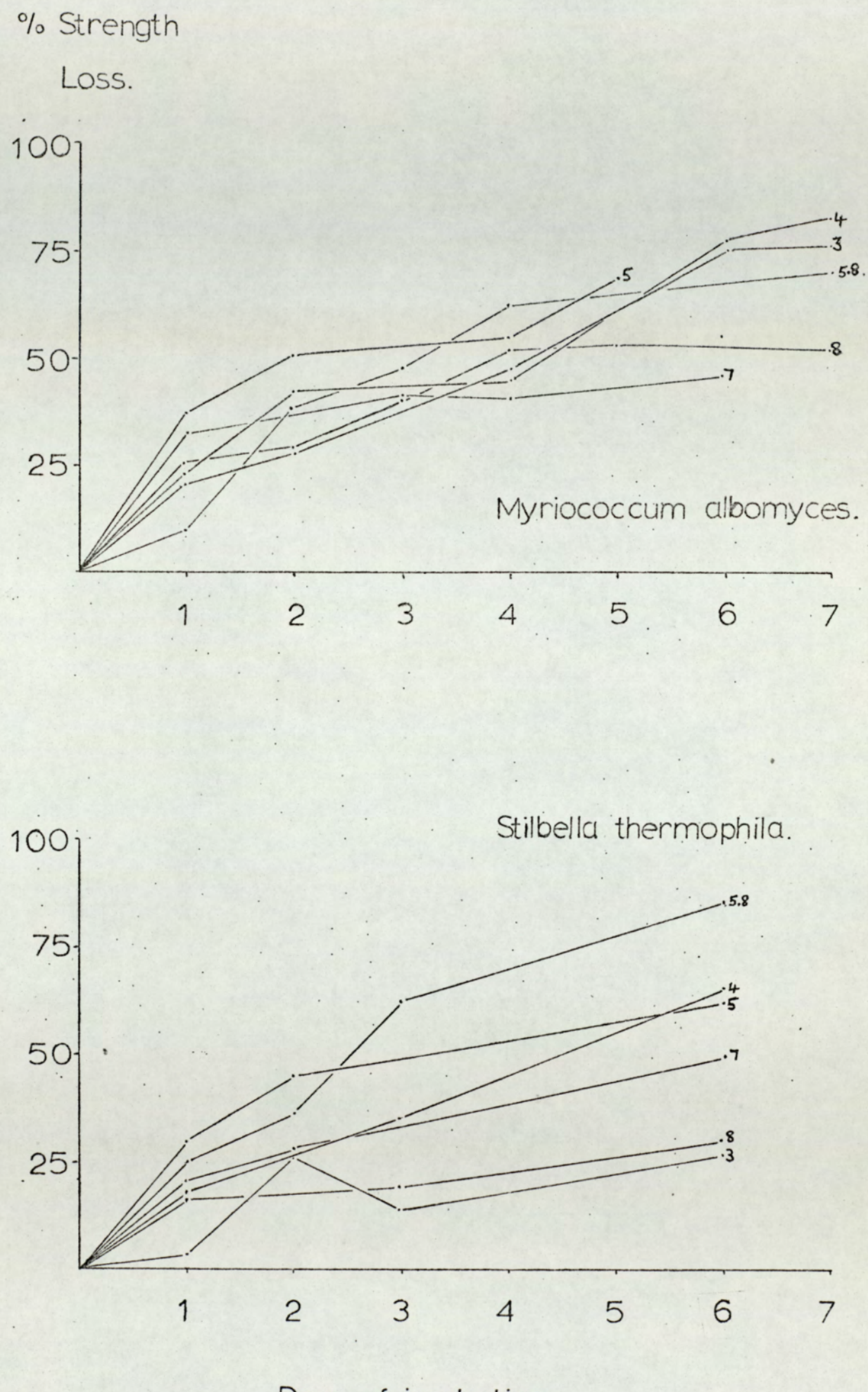

% Strength
Loss.
100
75
50
25
1
2
3
4
5
6
7
4
3
5.8
5
8
7
Myriococcum albomyces.
Stilbella thermophila.
5.8
4
5
7
8
3
Days of incubation.

FIGURE 16

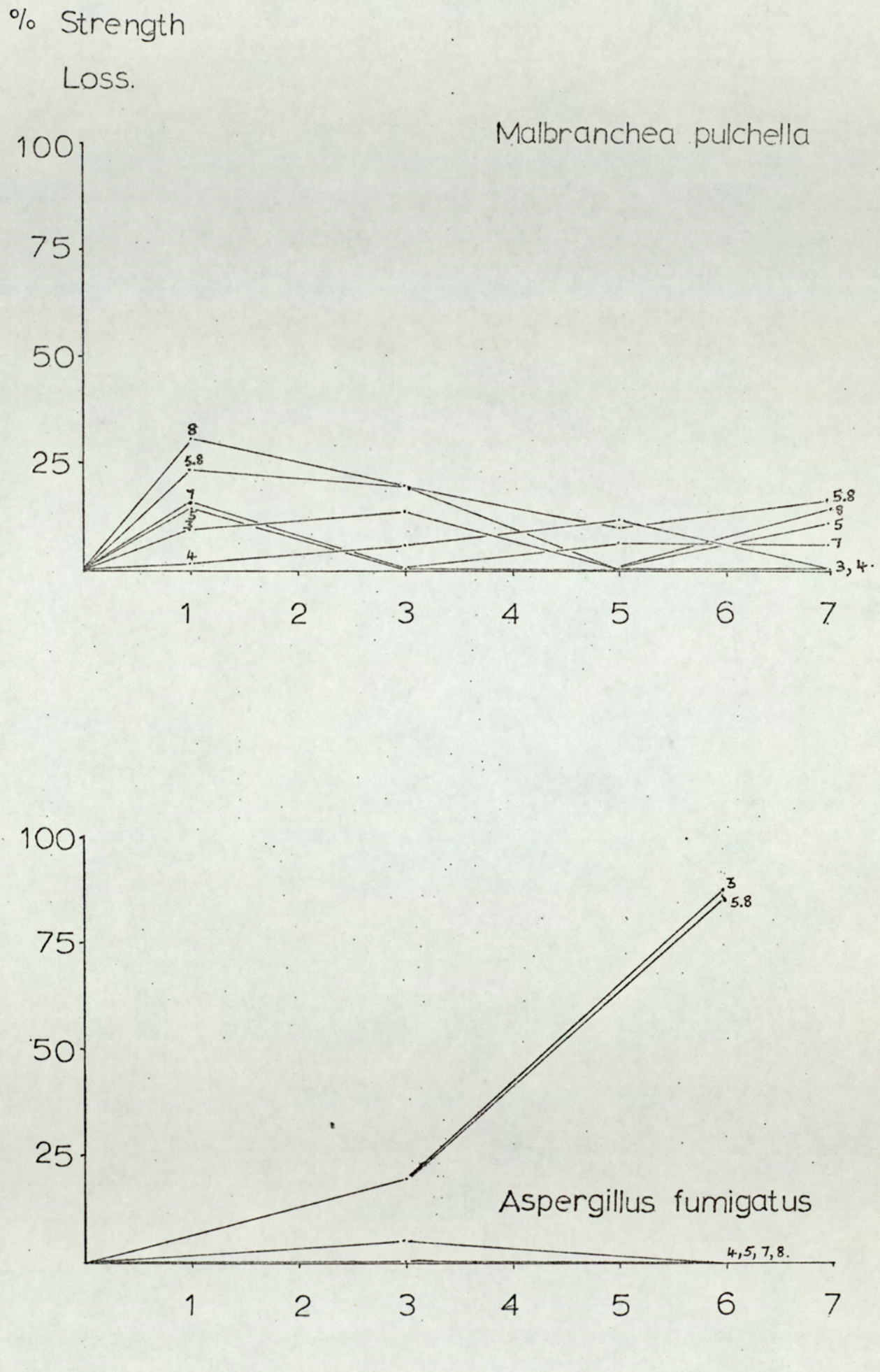

% Strength Loss.
Malbranchea pulchella
100
75
50
25
8
5.8
7
5
3
4
5.8
8
5
7
3, 4
1
2
3
4
5
6
7
100
75
50
25
3
5.8
Aspergillus fumigatus
4, 5, 7, 8.
1
2
3
4
5
6
7
Days of incubation.

FIGURE 17

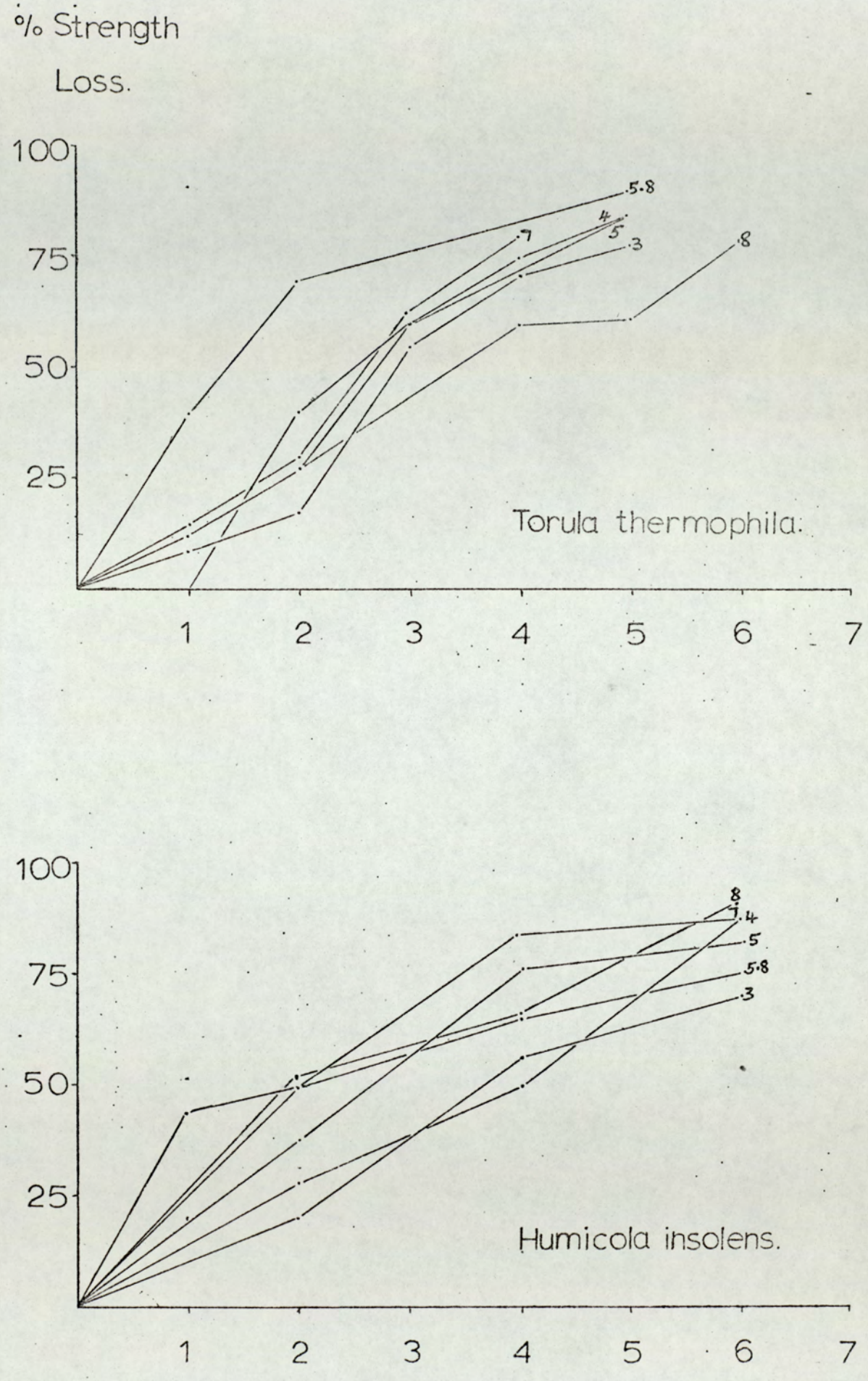
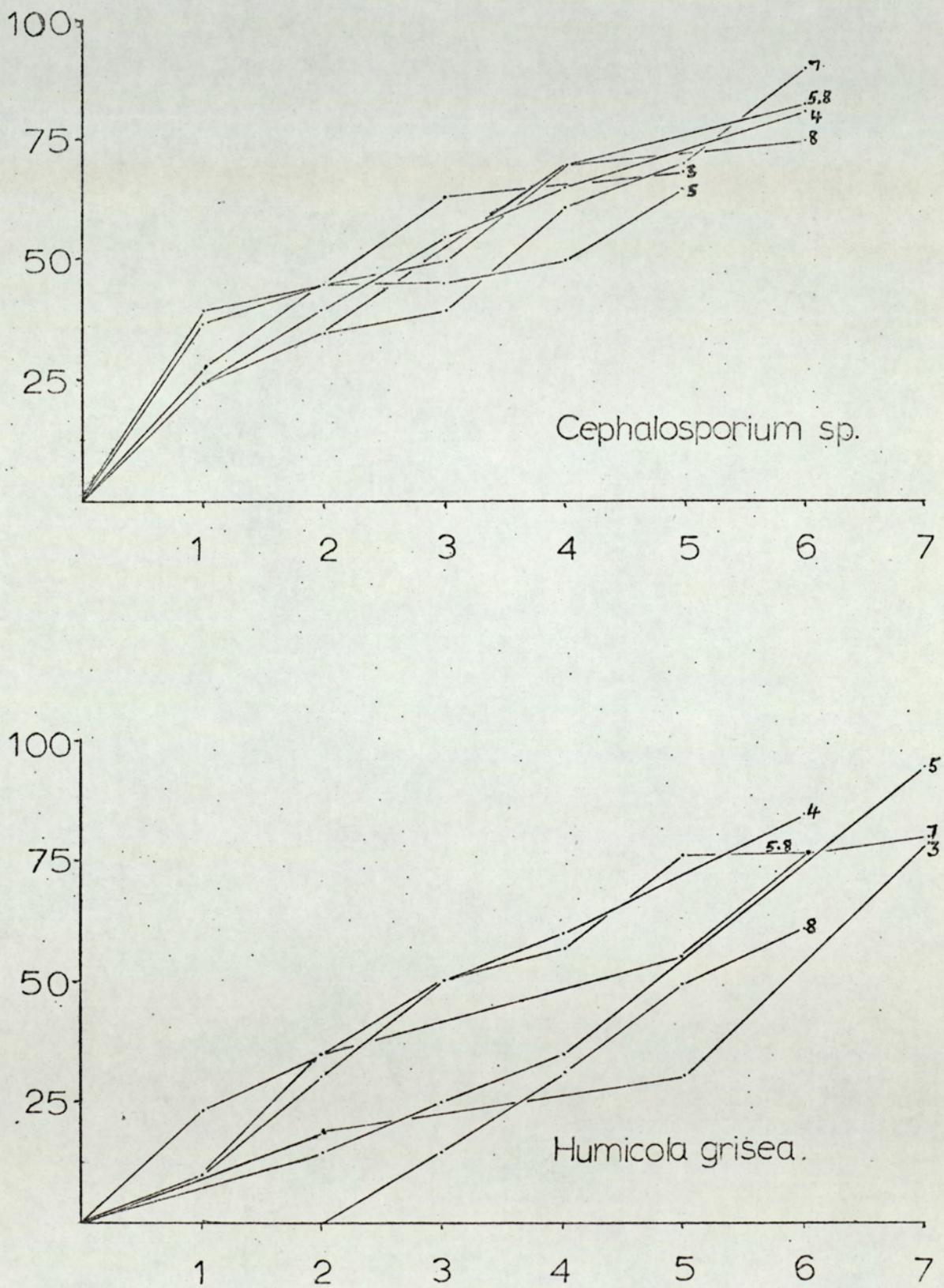

% Strength
Loss.
100
75
50
25
5·8
4
5
7
3
8
Torula thermophila.
1
2
3
4
5
6
7
100
75
50
25
8
7
4
5
5·8
3
Humicola insolens.
1
2
3
4
5
6
7
Days of incubation.

FIGURE 18


% Strength
Loss.
100
75
50
25
7
5.8
4
8
3
5
Cephalosporium sp.
1
2
3
4
5
6
7
100
75
50
25
5
4
5.8
7
3
8
Humicola grisea.
1
2
3
4
5
6
7
Days of incubation.

strength losses of the strips were very poor at all pH values tested, even after 7 days. Aspergillus fumigatus, rather surprisingly, only produced its characteristic thick mycelial mat with masses of dark green spore heads at pH 3.0 and pH 5.8, with strength losses being recorded only at these two pH values. White mycelial growth with few spore heads was produced at pH 4.0 and pH 5.0, and very little growth was produced at pH 7 and pH 8. Torula thermophila produced better growth at neutral to alkaline pH values (previously observed by Malik 1970) but loss in strength of the strips was good at all pH values tested, after 4 days. Good growth and sporulation was produced at all pH values by H. insolens and maximal strength losses were recorded at pH 4.0, 7.0 and 8.0 after 6 days. Cephalosporium sp. produced little aerial mycelium at any pH value, but good strength losses throughout the pH range after 5 days; whilst H. grisea produced good growth, sporulation and strength losses at pH 4.0 to pH 7.0 within 6 days.

(2) Enzyme Study

The results of the cellulase activity of the twelve cellulolytic thermophilic fungi are shown in Figures 19 and 20. The two figures also include, for comparison, the results obtained from the perfused cellulose strips. In this case, the maximal strength loss produced by each fungus after 5 to 7 days (see below) was designated as 100%, the lowest recorded strength loss as 0%, and intermediate values were calculated accordingly. This method has the advantage of "stretching out" the strength loss results so that any peaks in the resulting graphs become more apparent. The values for strength loss against pH were plotted from Figures 13 to 18 after 4 days (Sporotrichum thermophile, Thielavia thermophila,

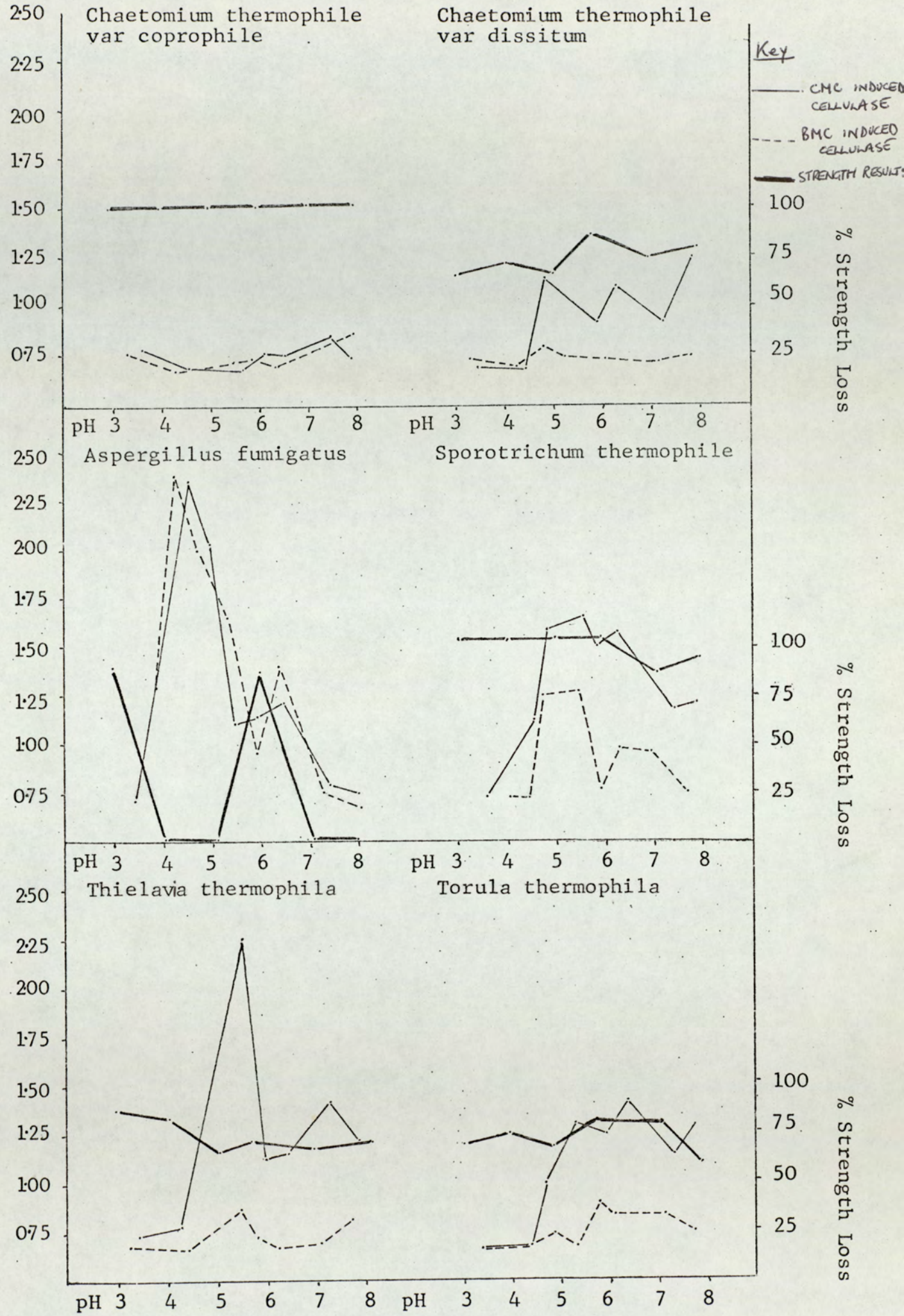

Chaetomium thermophile var coprophile
Chaetomium thermophile var dissitum
Aspergillus fumigatus
Sporotrichum thermophile
Thielavia thermophila
Torula thermophila
Key
CMC INDUCED CELLULASE
BMC INDUCED CELLULASE
STRENGTH RESULTS
% Strength Loss
pH 3 4 5 6 7 8
2·50
2·25
2·00
1·75
1·50
1·25
1·00
0·75
100
75
50
25

FIGURE 19

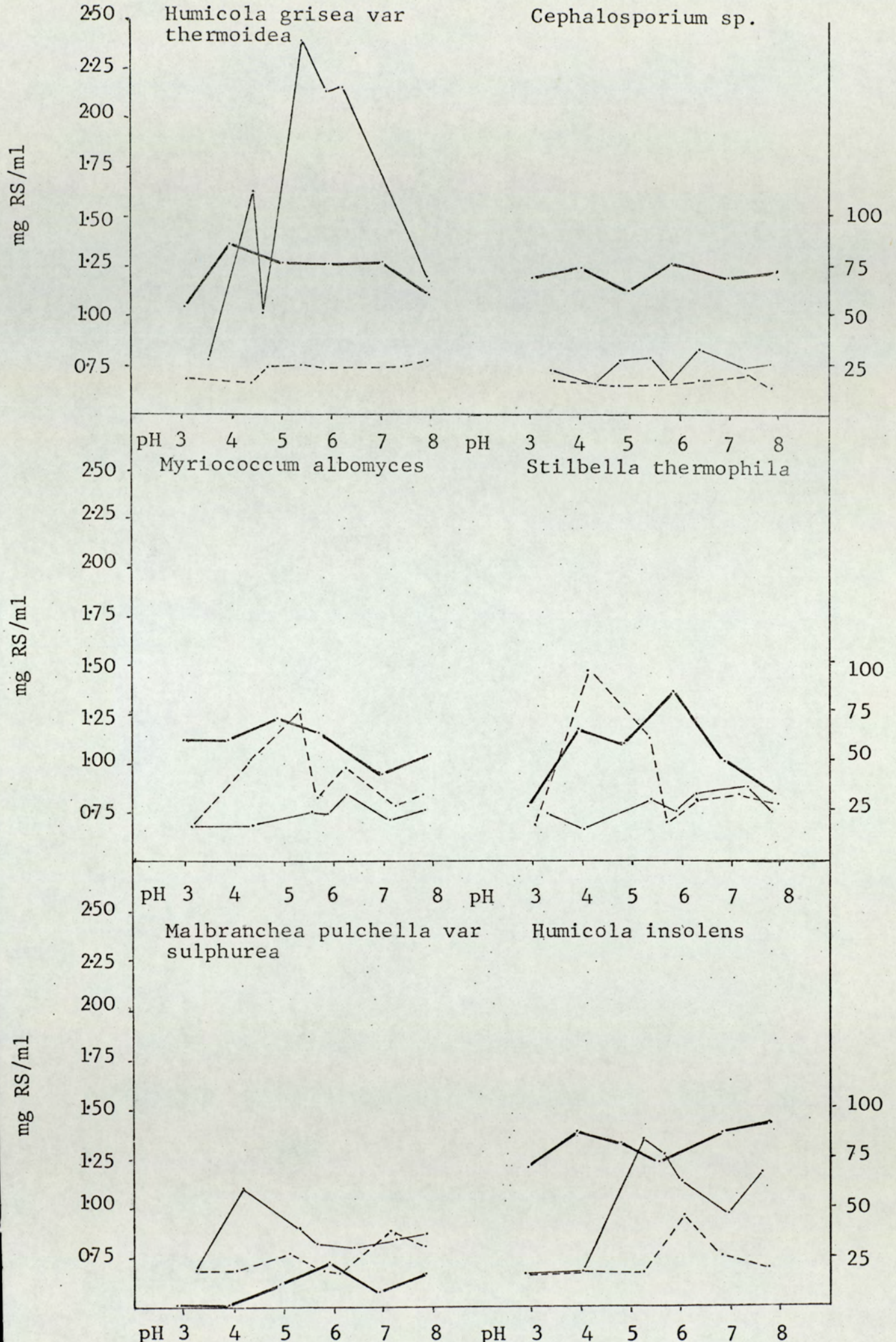

Humicola grisea var thermoidea
Cephalosporium sp.
Myriococcum albomyces
Stilbella thermophila
Malbranchea pulchella var sulphurea
Humicola insolens
mg RS/ml
% Strength Loss
2·50
2·25
2·00
1·75
1·50
1·25
1·00
0·75
100
75
50
25
pH 3 4 5 6 7 8

FIGURE 20

Torula thermophila,); 5 days (Cephalosporium sp., the two Chaetomium varieties, Myriococcum albomyces); 6 days (Humicola grisea, Humicola insolens, Aspergillus fumigatus, Stilbella thermophila) and 7 days (Malbranchea pulchella).

The majority of the fungi tested grew well on the CMC and produced considerable mycelial biomass. Cell free extracts from Chaetomium thermophile var. coprophile produced little solubilisation of the 0.55% CMC after one hour at 48°C, with the initial CMC extract producing more RS at pH 3.6 and pH 7.4 and the initial BMC extract producing more RS at pH 3.2 and pH 7.9. The results of the strength testing showed complete strength losses of insoluble cellulose at all pH values tested. In contrast, variety dissitum showed several pH optima with the CMC extract, but very little RS was detected using the BMC extract. Optima for this variety were given at pH 4.8, 6.2 and 7.8 with a broad optimum being given between pH 5.2 and 6.8 for the strength loss results. The cellulase produced by A. fumigatus in response to CMC and BMC showed two sharp pH optima, at pH 4.2 to pH 4.4 and at pH 6.2 to pH 6.4. The former values agree with those found by Levinson and Reese (1950) for (mesophilic) A. fumigatus cellulase. The strength loss results for this fungus however show one pH optimum at pH 3.0 and the other optimum at pH 5.8. This latter result agrees with the optimum value of pH 5.6 listed by Siu (1951) for A. fumigatus cellulase. The results for A. fumigatus again show the wide pH tolerance of the cellulase produced by this fungus and the fact that different pH optima are produced upon different substrates. The two cellulase "systems" of Sporotrichum thermophile produced wide pH optima from pH 4.8 to pH 6.2 for the CMC-induced cellulase, and from pH 4.6 to pH 5.4 and

from pH 6.2 to pH 6.9 for the BMC-induced cellulase. Good strength losses were produced at all pH values tested for this fungus with no clearly defined optima. Sharp optima were given by the CMC- and BMC-induced cellulases of Thielavia thermophila at pH 5.5, and a second peak was given by the CMC cell free extract at pH 7.3 with a rise in the BMC extract in the alkaline region of the graph. Strength loss results for this fungus were good at all pH values tested, but slightly higher losses were observed below pH 5.0. Torula thermophila cellulases showed a broad optimum from pH 4.8 to pH 7.8 and these results appeared to be followed by the strength loss results. The CMC cell free extract of H. grisea showed a wide pH tolerance with an optimum at pH 5.4. In contrast, little RS was produced by the BMC cell free extract at any pH value; whilst the results for the strength testing showed good strength losses at all pH values. Cephalosporium cell free extracts produced very little RS at any pH value; however, two small peaks were observed with the CMC-induced cellulase at pH 5.4 and pH 6.4. Good strength losses were produced at all pH values tested for this fungus. The BMC-induced cellulase of M. albomyces produced two pH optima at pH 5.4 and pH 6.3, whilst that induced by CMC produced a single small peak at pH 6.3. Better strength losses were produced by this fungus at pH values below pH 6.0. Similar results were produced by the cellulases of Stilbella thermophila. In this case the BMC-induced cellulase produced a broad peak from pH 3.0 to pH 5.8 with an optimum at pH 4.2. A smaller, broad peak was also produced in the range pH 5.8 to pH 8.0. Strength losses were produced by this fungus over the full pH range tested, and maximal losses were recorded at pH 5.8. Malbranchea pulchella produced small strength losses of insoluble cellulose at all pH values tested, but slightly better losses were produced

at pH 5.8. In contrast to these results, Malbranchea produced a pH optimum at pH 4.2 for the CMC-induced cellulase and at pH 7.3 for the BMC-induced cellulase. A single optimum was produced by the BMC-induced cellulase of H. insolens at pH 6.2, whilst the CMC-induced cellulase produced an optimum at pH 5.4 and a rise in the graph in the alkaline region. The results for the strength losses produced by this fungus show good losses at all pH values tested but slightly better losses at pH 4.0 and pH 7.8.

Discussion

It appears from the experimental evidence presented above that the cell free extracts from most of the cellulolytic, thermophilic fungi show a wide pH tolerance with one, or more pH optima. Double pH optima for cellulase preparations have been observed by other authors (Garber et. al. 1965; Cappellini and Peterson 1966, Björndal and Eriksson 1968; Betrabet and Patel 1969; Whitney et al. 1969) and probably indicate the presence of separate enzymes, each with its own pH optimum. It is interesting to note here that the enzymes produced in response to the initial BMC substrate show similar pH optima (Chaetomium thermophile var. coprophile, A. fumigatus, Sporotrichum thermophile, Thielavia thermophila, Torula thermophila and Myriococcum albomyces) and different pH optima (M. albomyces, Stilbella thermophila, M. pulchella, H. insolens) to the enzymes produced in response to the initial CMC substrate. Similar results have been observed by Whitney et al. (1969) who found similar pH optima for the cellulase of Verticillium albo-atrum grown initially on CMC and on cellulose powder; and by Ghose (1969) who found optimal substrate (Solka floc) utilisation by T. viride cellulase at pH 4.0

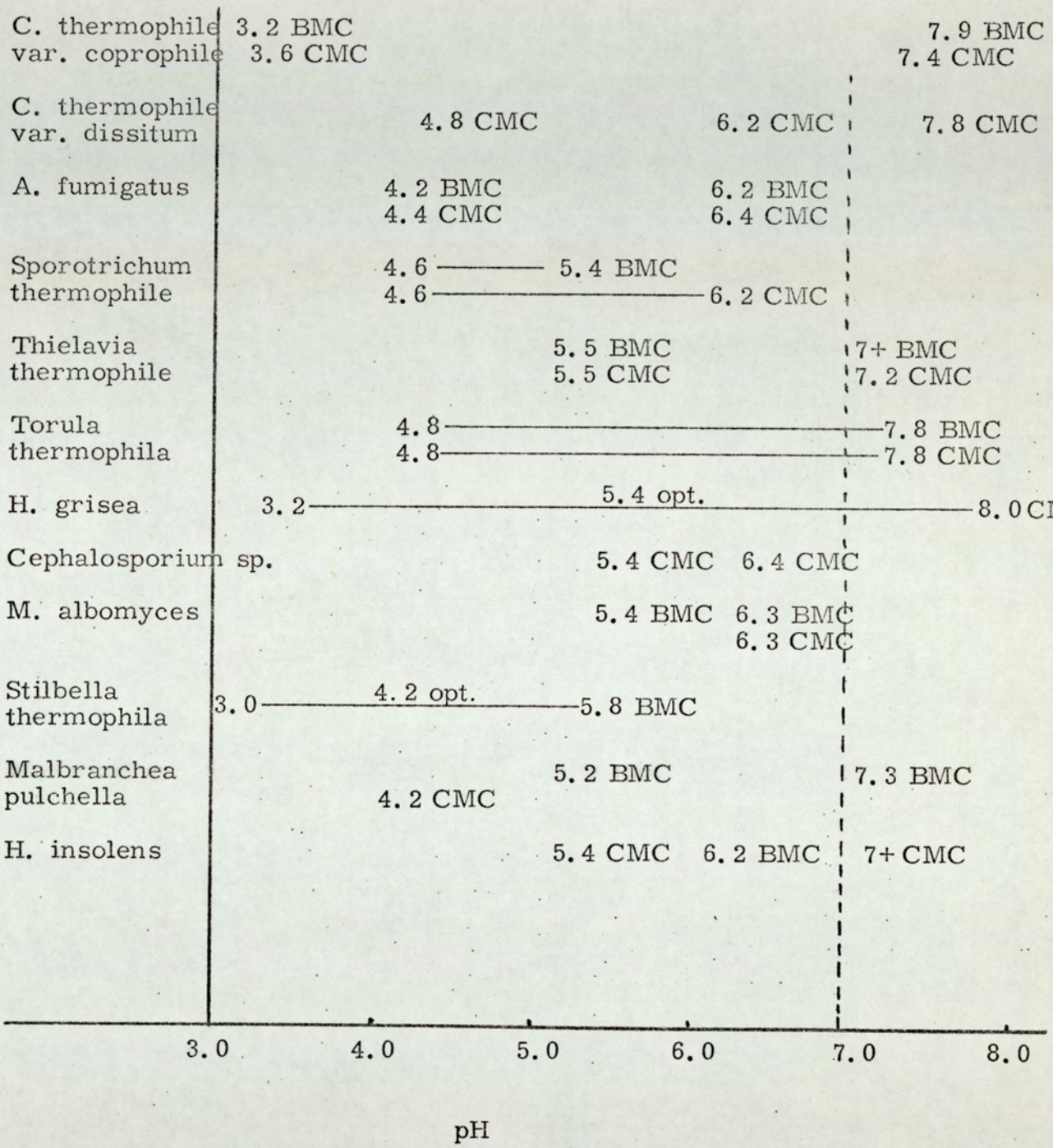
to pH 5.0 and greatest RS yield on CMC at pH 4.8 to pH 5.0. Other workers found pH optima to vary, depending upon the test substrate used (Thomas 1955; Li et al. 1965; le Grand and Thivend 1967).

If we summarise the two sets of results presented in this chapter, we might be able to build up a clearer picture of the possible nature of cellulolysis produced by the thermophilic fungi.

The pH optima of the cellulolytic thermophilic fungi are presented in Table 10, and from this table it can be seen that whilst the majority of the fungi had pH optima in the acidic region, six fungi also showed cellulase activity in the alkaline region. Five of these fungi, viz:- the two varieties of Chaetomium thermophile, Torula thermophila, M. pulchella and H. insolens have previously been isolated from composting town refuse (Glath 1959, 1964; Klopotek 1962, Chapter 3 of this thesis) and the fact that they do show definite cellulolysis in the alkaline region lends weight to the supposition that only fungi which can elaborate their cellulase systems at alkaline pH values are involved in cellulose degradation within composting town waste. This supposition does not preclude other thermophilic fungi from such systems, it merely supports the idea that certain fungi will be successful in colonising a substrate if they can adapt to the ecological and physiological limitations imposed by that substrate.

The two varieties of Chaetomium thermophile both produced good strength losses of the insoluble cellulose at all pH values tested, and whilst variety coprophile grew well under acid and alkaline conditions, variety dissitum produced slightly less growth under alkaline conditions. It should be noted here that variety coprophile produced very little

TABLE 10



Showing pH optima for cellulase preparations from thermophilic fungi grown initially on ball milled cellulose (BMC) and carboxymethyl cellulose (CMC)

extracellular cellulase when tested on CMC, although maximal strength losses on insoluble cellulose were reached within a very short time. Similar observations were made by Agarwal et al. (1963) for other members of the Chaetomiaceae. Malik (1970) observed maximal depths of clearing of ball milled cellulose agar in Rautela-Cowling tubes at pH 6.4 for Chaetomium thermophile, and it appears that this particular genus of thermophilic fungi plays an important role in degrading cellulose in the first phase of the composting of town waste. The extracellular cellulase of Torula thermophila showed a wide pH optimum, from pH 4.8 to pH 7.8. Growth of this fungus was certainly better at neutral to alkaline pH values on insoluble cellulose, and Malik (1970) found optimum depths of clearing of ball milled cellulose by Torula thermophila at pH 7.0. M. pulchella produced small strength losses on insoluble cellulose at neutral to alkaline pH values but growth of this fungus was better at the very low acidic pH values. It seems likely that M. pulchella plays only a very small part in the overall degradation of cellulose in composting town waste, although it may be that Malbranchea is a "secondary" invader, degrading the lower molecular weight oligomers produced by the more cellulolytic of the thermophiles. The cell free extract from H. insolens showed a rise in production of RS in the alkaline region, and growth of this fungus was good at all pH values tested. The optimum pH for clearing of ball milled cellulose by H. insolens was found to be pH 6.4 (Malik 1970).

All the above mentioned fungi, together with the remaining test fungi also produced better pH optima, growth and sporulation at pH values below pH 7.0, and this fact could account for the observations of Klopotek (1962) that the thermophilic fungi are very poorly represented in composting town refuse.

The sixth fungus to show a pH optimum in the alkaline region was Thielavia thermophila; however, this fungus produced little growth on insoluble cellulose at these pH values and there is no record of it having been isolated from composting town waste.

The results for the other cellulolytic thermophiles show that Sporotrichum thermophile produced a wide pH tolerance in the acidic region, with better growth at these lower pH values. Malik (1970) again found maximal depths of clearing by this fungus at pH 5.0. A. fumigatus showed clear pH optima and better growth at low pH values, and from the results presented here, doubts arise as to the cellulolytic activity of this fungus in composting town waste. In earlier studies (Chapter 2) it was not isolated until after the thermophilic stage of composting, whilst Chang and Hudson (1967) isolated this fungus in the brief period before temperatures reached a maximum in their wheat straw composts. Malik (1970) was able to isolate A. fumigatus only on deteriorated cellulose strips at pH 6.0 and pH 7.0 and the fungus had a very low frequency of isolation at pH 4.0 and pH 5.0. It seems likely, therefore, that A. fumigatus, whilst not playing so active a part in thermophilic cellulolysis, is successful in colonising town refuse compost at a cooler stage in the process and very quickly spreading by virtue of its prolific spore production. Growth of Cephalosporium sp. on insoluble cellulose was slight at all pH values tested, with very little aerial mycelium and spores being produced. Optimum pH values for this fungus were given at pH 5.4 and pH 6.4; and maximum depths of clearing were observed at pH 4.0 (Malik 1970). H. grisea cellulase showed a wide pH tolerance, with RS being produced from CMC at values up to pH 8.0. Growth of this fungus, which has been isolated from town waste (Chapter 3), was better at pH values between pH 4.0 and

and pH 7.0. Malik (1970) found maximum depth of clearing by this fungus at pH 5.0 and pH 7.0 and it seems likely that H. grisea could be actively concerned in cellulolysis within composting town waste. The remaining two fungi, Myriococcum albomyces and Stilbella thermophila, both demonstrated optimal cellulase activity at pH values between pH 3.5 and pH 5.8. Growth of Myriococcum albomyces was poor on insoluble cellulose at all pH values tested, whilst growth of Stilbella was best at pH 4.0 and pH 5.0, with very little growth of this fungus on insoluble cellulose at any other pH value.

We have seen, therefore, that as far as extracellular cellulases of these thermophilic fungi are concerned, maximum cellulolysis of the 0.55% CMC solutions was produced at acidic pH values. When the same fungi were grown upon insoluble cellulose perfused with buffered nutrient salts, in the majority of cases, better growth and sporulation was produced at acidic pH, but solubilisation of the test strips was produced equally at acid and alkaline pH values. From this research can we state categorically that the thermophilic fungi which have been isolated from composting town waste were present in such material simply because they were also able to elaborate cellulase enzymes which were stable to alkaline pH values so that these fungi could compete with the other thermophilic, cellulolytic microflora? Such a question would be superfluous, in fact, at this point of time until further research is carried out into other aspects of the ecology and physiology of these fungi in town waste composting. What can be done, here, however, is to put this point of view forward for further investigation, as one possible reason for the paucity of thermophilic fungi in the process of town waste composting.

As previously mentioned, one of the many other aspects to be investigated here are the effects of one thermophilic, cellulolytic fungus upon the cellulolytic activity of another. Is it possible that although many of the thermophilic fungi in isolation are extremely cellulolytic, they have an antagonistic effect upon each other's cellulolytic activity when grown in combination?

CHAPTER 6

CHAPTER 6

Interaction between the thermophilic cellulolytic fungi

Introduction

The principle of microbial coexistence in nature was first propounded by Pasteur (1863), and later De Bary (1879) was the first to emphasise the significance of antagonistic relationships between micro-organisms when two microorganisms were grown upon the same substrate. The interaction between fungi has been studied by many workers (see general reviews by Waksman 1937, Garrett 1950, De Vay 1956 and Barnett 1964), and may be due to a wide variety of factors which, in combination, play a major role in the physiology and ecology of many fungi. Interaction between fungi occurs when one fungus parasitises another, produces volatile or diffusible metabolites which have a measurable effect upon the growth of another fungus, or when one fungus completely dominates another fungus on a specific substrate owing to its rapid growth rate and rapid utilisation of the substrate.

Barnett (1963) defined parasitism between fungi as "myco-parasitism". He went on to describe those fungi which obtain nutrients from other fungi without damage to the host as "biotrophic" mycoparasites, and those which obtain nutrients at the expense (or death) of the host as "necrotrophic" mycoparasites. The processes of mycoparasitism will not be mentioned here, but it is sufficient to say that many factors other than availability of nutrients may also play a part in this phenomenon for example some fungi are more susceptible to attack in total darkness rather than in

constant light (Butler 1957). Two other factors also play an extremely important part in this competitive behaviour, and these are (a) that certain fungi are able to produce substances which are toxic to other organisms, and which may reduce competition for the available substrate (Garrett 1950) and (b) that certain fungi are able to produce such rapid growth upon a substrate to the exclusion of other possible colonising fungi.

Toxic substances secreted by fungi may be volatile or non-volatile and exert their influence over quite considerable distances. Gaseous inhibitory products have been detected as by-products of many fungi (Hutchinson 1971). Hydrogen cyanide was discovered by von Lösecke (1871) from fructifications of Marasmius oreades and has been detected in other fungi by Bach (1948), Lebeau and Dickinson (1953) and Ward and Thorn (1965). Pratt (1924) found traces of alcohol, aldehydes, formaldehyde and ammonia produced by Fusarium sp; and similar products were found in Fusarium oxysporum by Robinson and Park (1966) and by Robinson and Garrett (1969) who, using gas chromatography, detected acetaldehyde, propionaldehyde, n-butyraldehyde, n-propanol, isobutanol, ethyl acetate, isobutyl acetate and acetone in the gaseous environment above cultures of this fungus. Ethylene gas has been found as an inhibitory by-product of many fungi for example Penicillium digitatum (Young et al. 1951); and Ilag and Curtis (1968) believe that ethylene is a common metabolic product of fungi. They verified that 22 species of fungi produced ethylene, and that 58 out of 228 species of fungi produced a gaseous compound with a retention time (gas chromatography) identical to authentic ethylene.

The majority of fungi however produce non volatile products which diffuse into the immediate environment and have an effect, synergistic

or inhibitory, upon neighbouring organisms. Some by-products render the environment virtually sterile and eventually kill the organism which produced them. Examples such as ethanol produced by yeasts and lactic acid by many species of Rhizopus and Mucor are well known in this respect. Other fungi produce acids such as citric, gluconic, itaconic, fumaric and oxalic (Lilly and Barnett 1951); all of which could exert an effect upon the growth of microorganisms in the immediate environment. Early work (Boyle 1924) showed that staled culture media of Fusarium sp. could completely inhibit germination of the spores of Botrytis sp. and that some of this inhibition could be removed by filtering the staled media through collodion membranes. Similar work was carried out by Pratt (1924b) who considered that in a staled medium there was a balance between toxic substances and available food. Many fungi produce antibiotics which inhibit the growth of bacteria and other fungi. Fleming (1929) discovered penicillin from P. chrysogenum, and other fungally produced antibiotics have been found - see reviews by Brian (1951) and Broadbent (1966). Of the common fungi Trichoderma viride produces a wide range of antibiotics such as gliotoxin, viridin (Brian 1944, Brian and McGowan 1945, Wright 1952, 1955), trichodermin and peptide antibiotics (Dennis and Webster 1971a) and "Antibiotic U-22324" (Meyer and Reusser 1967). Some workers however, failed to find gliotoxin and viridin in many strains of T. viride (Webster and Lomas 1964, Dennis and Webster 1971 a). Antibiotic "E" was produced by Phoma exigua (Logan and O'Neill 1970); whilst Bassett et al. (1967) detected fomannosin in culture filtrates of Fomes annosus. Psalliotin was isolated from another basidiomycete, Psalliota xanthoderma (Atkinson 1954); whilst other antifungal anitbiotics include griseofulvin from Penicillium nigricans (Wright 1955); aspergillic acid from Aspergillus flavus; citrinin from A. terreus and mycophenolic acid from P. brevi-compactum (Lilly and Barnett 1951). Other diffusible, toxic

substances which suppress fungal growth have been found by Dobbs and Hinson (1953) in soils; Park (1961, 1964) from Fusarium oxysporum; Stilwell (1966) from Cryptosporiopsis sp.; Ikediugwu and Webster (1970 a,b) from Coprinus heptemerus and by Dayal et al. (1971) from Trichoderma koningii. Physical factors are known to play a part in antibiotic production since some fungi require a high nutrient level before antibiotics are synthesised (Wright 1955), and some antibiotics are extremely labile under normal conditions (Park 1961) and can even be destroyed by light (Atkinson 1954).

Not all non-volatile products produced by fungi however, are inhibitory in their action. Certain fungi are known to produce auxins (Jump 1938) which certainly have a growth stimulating effect on the plant tissue which these fungi invade. Indole acetic acid is produced by Cladosporium herbarum (Valadon and Lodge 1970) and by Diplodia sp. (Gentile and Klein 1955); but very little is known about the possible effects of these auxins on other fungi. Some fungi are able to synthesise the vitamins riboflavin and vitamin B_{12} (see Lilly and Barnett 1951), and it might be argued that these compounds could have a synergistic effect upon the growth of neighbouring fungi, since riboflavin, at least, is an important constituent of several essential coenzymes.

The third important factor in the competition for available nutrients is that certain fungi are able to produce such rapid growth over a particular substrate that they virtually "swamp out" the growth of other potential colonisers. Ecological work by Malik (1970) and by many other investigators has shown that specific colonisation patterns exist when a piece of cellulose, for example, is exposed to the mixed microbial population of the soil.

Mixed microbial populations were studied by Waksman and Hutchings (1937 ') who found that mixed cultures of fungi brought about greater total degradation of alfalfa grass than did individual fungi, and that the presence of non cellulolytic fungi stimulated cellulose decomposition by the cellulolytic fungi. Wright (1955) showed that the yield of griseofulvin from Penicillium nigricans could be decreased by infection with other fungi and by the competition for nutrients between this fungus and other rapid growing fungi such as Mucor rammannianus; whilst Wastie (1961) believes that the success of a fungus in colonising a particular substrate in an agar plate from a mixed inoculum, can be predicted simply from the measurement of its linear growth rate in pure culture on the same substrate. He states that the struggle for colonisation is largely decided by the linear growth rates of all the competing fungi in an agar plate before significant antibiotic effects are produced.

If these hypotheses are true, could it explain the fact why certain thermophilic fungi such as Chaetomium thermophile, Humicola insolens, Humicola grisea and Torula thermophila are amongst the relatively few thermophilic fungi to be found in composting town waste? These fungi are all able to elaborate their cellulase systems at alkaline pH values and so it might also be interesting to observe their possible interaction effects with other memebrs of the thermophilic, cellulolytic fungi when grown together on a cellulose substrate. Very little work has been done on the interaction effects of the thermophilic fungi. It has been shown that Malbranchea pulchella can produce penicillin at a temperature of 52^{o}C (Rode, et al. 1947) but Lilly and Barnett (1951) quoting from previously published work state that penicillin is not an antifungal antibiotic, and so if penicillin is produced by this fungus growing on a cellulosic

substrate, it might not have any demonstrable effect upon the growth of other fungi. The thermotolerant A. fumigatus produces a variety of antibiotics, some of which are antifungal, and so it might be anticipated that fungi grown in combination with A. fumigatus on cellulose might be suppressed. Biran (1951) lists (mesophilic) A. fumigatus as producing spinulosin, fumigatin, gliotoxin and helvolic acid (fumigacin); and this fungus in its mesophilic state has been shown to completely inhibit the infection of Mucor recurvatus by Rhizoctonia solani (Butler 1957). The interaction between Humicola lanuginosa and Chaetomium thermophile was studied by Chang (1967), and she found that growth of H. lanuginosa was more vigorous when grown in combination with C. thermophile on cellulose, although H. lanuginosa is non cellulolytic. Since, however, H. lanuginosa was able to utilise the soluble sugars produced by C. thermophile Chang placed H. lanuginosa in the category of "secondary sugar fungi" - or those fungi which grown in close association with the hyphae of cellulolytic fungi and absorb some of the sugars as they are produced by the cellulase enzymes (Garrett 1963, Hudson 1972). More recently, Craveri et. al. (1972) isolated an antifungal antibiotic they named Thermozymocidin, from a thermophilic Mycelia Sterilia. They noted that this compound ($C_{21}H_{39}NO_6$) was highly effective against a large number of yeasts and against P. notatum, Aspergillus oryzae and Trichophyton mentagrophytes. Chang and Hudson (1967) also isolated a thermophilic Mycelia Sterilia from their wheat straw composts, and they noted that towards the end of composting this fungus was present on every piece of straw in all the composts. Although a thermophilic Mycelia Sterilia was not isolated in these studies it appears to be an ubiquitous thermophile and so it might be interesting, in future work, to see whether this fungus can withstand the parameters of town waste composting and whether under such conditions it is able to elaborate an antibiotic system.

Methods available for the study of fungal interaction include the testing of staled liquid culture media on the germination and growth of other fungi (Boyle 1924; Pratt 1924a,b; Park 1961, 1964); the testing of macerated mycelium against other fungi (Dayal et al. 1971); agar techniques designed to show inhibition of growth of one fungus by another (Dennis and Webster 1971, a) or hyphal interaction (Eckstein and Liese 1970, Ikediugwu and Webster 1970 a,b) or the effects of volatile components (Robinson and Park 1966, Dennis and Webster 1971,b); and studies designed to show the coiling and penetration of possible mycoparasitic fungi (Butler 1957, Aluko and Hering 1970, Dennis and Webster 1971,c). The interaction between fungi has also been studied on cellulosic substrates. Slifkin (1963) found that infectivity of Olpidiopsis incrassata depended entirely upon the hosts' nutrient requirements e.g. hosts grown on sugars with an α - glucoside linkage were susceptible to infection, whereas hosts grown on saccharides containing β -glucoside linkages were not susceptible. Wood was used as a cellulosic substrate by Stilwell (1966) who noted interaction effects between the hyphae of Cryptosporiopsis sp. and Fomes fomentarius when these two fungi were inoculated onto wood blocks. A classical study of the interaction of fungi on cellulose film was carried out by Tribe (1966). who studied the cellulolytic breakdown of the film and the interaction between pairs of fungi inoculated at opposite ends of small strips of cellulose film. Tribe found that competitive characteristics led to the dominance of one fungus over another and that cellulolysis was a secondary effect to this. He went on to discuss the merits of using cellulosic substrates for such studies, and also stated that the use of cellulose agars would be preferred to the use of sugar containing agars. Similar methods were used by Sharp (1969) and by Sharp and Eggins (1970) in their studies of the ecology of soft rot fungi. They used thin veneers of

beechwood, placed upon cellulose agar and inoculated at opposite ends with their test fungi. Interaction effects were noted by observing the absence or the appearance of hyphal intergrowth, and also by the effect upon the cellulolytic activity of the more cellulolytic of the two fungi when measured by comparing the breaking strengths of the veneers against controls. They found that if the hyphae of two species mixed then, without exception in the combinations investigated, the amount of decay in the area of mixing was equal to that normally produced by the more cellulolytic species of the two. Many examples of synergism were also found with one species increasing the wood degrading efficiency of another species. Similar work by Malik (1970) and Malik and Eggins (1970) showed that when two fungi were inoculated onto cellulose agar contained in Rautela-Cowling tubes there was no increase in the depths of clearing produced by any of their test fungi. They attributed this decrease in cellulolytic activity in part to the mycoparasitic nature of one of their test fungi. Gliocladium roseum. These authors also go on to describe a perfusion interaction technique in which the diffusible metabolites from one fungus can be carried around a U-shaped strip of chromatography cellulose by the perfusion current to affect the linear growth rate of another cellulolytic fungus. Using this system they studied the effects of the diffusible metabolites of G. roseum on six other cellulolytic fungi and found that the linear growth rates of five out of the six fungi were depressed, whilst that of Arthrobotrys sp. was enhanced.

To study the interaction effects between the thermophilic, cellulolytic fungi it was decided to use two methods each of which could be used to measure a different interaction parameter. The first method decided upon was that used by Malik (1970) in which interacting fungi were

inoculated onto cellulose agar contained in Rautela-Cowling tubes. This method should demonstrate possible enhancement or inhibition of cellulase production, and indicate a possible mycoparasitic relationship. Secondly the cellulolytic thermophiles were inoculated in pairs onto cellulose agar in plates. This method should allow the observation of hyphal intermingling, and of any possible morphological changes in one, or both members of each pair of interacting fungi.

The possibility of using a third technique, the interaction perfusion technique of Malik (1970) was also investigated since this system lends itself to the study of diffusible metabolites produced by one fungus on the growth rates of another fungus also growing upon a cellulosic substrate. However, difficulties such as rapid drying out of the cellulose strips at thermophilic temperatures, and the continual infection of the test strips with A. fumigatus brought disappointing results and therefore this technique was not pursued in the present investigation.

Materials and Methods

(1) Inhibition or Enhancement of Depth of Clearing

Pyrex boiling tubes (15 x 2.5 cm) were half filled with E & P cellulose agar, plugged with non absorbent cotton wool and autoclaved at 120°C for 10 minutes. The tubes, contained in wire baskets, were then taken directly from the autoclave and plunged into cold, running water in a sink. It was found that by using this method a rapid setting of the agar was achieved with an even distribution of the cellulose particles. In all cases Oxoid agar No. 3 was used to ensure that all results would be truly

comparable (Williams 1970). The tubes were then inoculated with 5m. m. discs of two actively growing thermophilic, cellulolytic fungi taken from cultures on E & P cellulose agar. The discs were positioned so that there was a 15 m. m. zone of agar between the inocula. The fungi were paired in all possible combinations and five replicates were set up for each pair, with the exception of H. grisea in combination, when seven replicates were made. Control tubes consisted of five replicate tubes for each fungus growing in isolation so that individual depths of clearing could be measured. The tubes were incubated at 48°C for 14 days and the depth of clearing was measured periodically. This was done by measuring from the top of the agar each time, with the tubes held against a strong light.

(2) Agar Plate Method

The twelve thermophiles were inoculated in all possible combinations onto E & P cellulose agar in plates. The two 12 m. m. discs of inocula were placed diametrically opposite each other and duplicate plates were made for each combination. The plates were incubated at 48°C for 10 days and then interaction between each pair of fungi was noted by recording (a) morphological changes, especially sporulation effects between the fungi and (b) whether the interacting hyphae showed antagonism (a clear front between the hyphae), marginal intergrowth (hyphae just merging but not completely intergrowing) or complete intergrowth.

Results

(1) Depth of Clearing of Cellulose Agar

The results for the depth of clearing of cellulose agar in Rautela-Cowling tubes by combinations of interacting thermophilic fungi

are shown in Table 11 and Figure 21 a - d. The % inhibition (or enhancement) of growth of the more cellulolytic fungus of the pair was calculated using the formulae:-

$$\% \text{ Inhibition} = \frac{X - Y}{X} \times 100$$

$$\% \text{ Enhancement} = \frac{Y - X}{X} \times 100$$

Where: Control depth of clearing after 14 days = X m.m.

Depth of clearing of interacting fungi after 14 days = Y m.m.

The results indicate that out of 66 possible combinations, cellulolytic activity of the more cellulolytic of the pair of fungi was enhanced by the partnership in only 13 cases. These were C. thermophile var. coprophile with Stilbella thermophila (+ 3.3%); Sporotrichum thermophile with Thielavia thermophila (+25%); Sporotrichum thermophile with M. pulchella (+31.5%); Sporotrichum thermophile with Stilbella thermophile (+ 29.4%); Sporotrichum thermophile with M. albomyces (+ 19.6%); Thielavia thermophila with A. fumigatus (+ 11.1%) ; Thielavia thermophila with Stilbella thermophila (+ 9.5%); H. grisea with H. insolens (+ 15.8%); H. insolens with M. pulchella (+ 46.6%); H. insolens with M. albomyces (+ 21.2%); H. grisea with M. albomyces (+ 5.3%); Torula thermophila with M. albomyces (+ 75.0%); and A. fumigatus with Stilbella thermophila (+ 3.3%).

It is interesting to note that the cellulolytic activity of Sporotrichum thermophile, the most cellulolytic thermophilic fungus, was greatly enhanced by the partnership with fungi such as M. pulchella, Stilbella thermophila and

A	A									
B	0*	B								
C	8.3*	20.0*	C							
D	10.0*	0*	+25.0*	D						
E	6.6*	27.5*	0*	29.5*	E					
F	72.0*	66.6*	73.8*	50.0*	68.8*	F				
G	49.0*	65.8*	76.0*	58.7*	75.4*	+15.8*	G			
H	20.8*	17.5*	9.8*	65.1*	49.4*	5.0*	15.0*	H		
I	52.5*	73.4*	31.5*	44.4*	78.8*	+46.6*	10.5*	25.0*	I	
J	30.0*	48.4*	10.9*	+11.1*	66.0*	16.6*	50.0*	6.7*	23.4*	J
K	2.5*	+ 3.3*	+29.4*	+ 9.5*	11.7*	17.4*	21.8*	34.8*	52.2*	+ 3.3*	K
L	40.0*	59.1*	19.6*	70.0*	76.5*	+21.2*	+ 5.3*	+ 75.0*	0*	16.7*	34.8*

A Chaetomium thermophile var. dissitum
B Chaetomium thermophile var. coprophile
C Sporotrichum thermophile
D Thielavia thermophile
E Cephalosporium sp.
F H. insolens
G H. grisea
H Torula thermophile
I Malbranchea pulchella
J A.fumigatus
K Stilbella thermophile
L Myriococcum albomyces

TABLE 11

% Inhibition (+ % enhancement) of depth of clearing of E & P cellulose agar in Rautela and Cowling's tubes by combinations of thermophilic cellulolytic fungi. Figures represent % inhibition (+ % enhancement) of the more cellulolytic fungus in each pair. Asterisk (*) shows more cellulolytic fungus of pair * left hand side - read off fungus in vertical column on left of table; * right hand side - read off fungus directly above.

FIGURES 21 (a) to (d)

Graphs show depth of clearing of E & P cellulose agar in Rautela and Cowling's tubes by combinations of thermophilic, cellulolytic fungi.

Key

Sp	Sporotrichum thermophile
Cd	Chaetomium thermophile var. dissitum
Cc	Chaetomium thermophile var. coprophile
Mb	Malbranchea pulchella
To	Torula thermophile
Hg	Humicola grisea
Hi	Humicola insolens
Ce	Cephalosporium sp.
St	Stilbella thermophila
Af	Aspergillus fumigatus
My	Myriococcum albomyces
Th	Thielavia thermophila

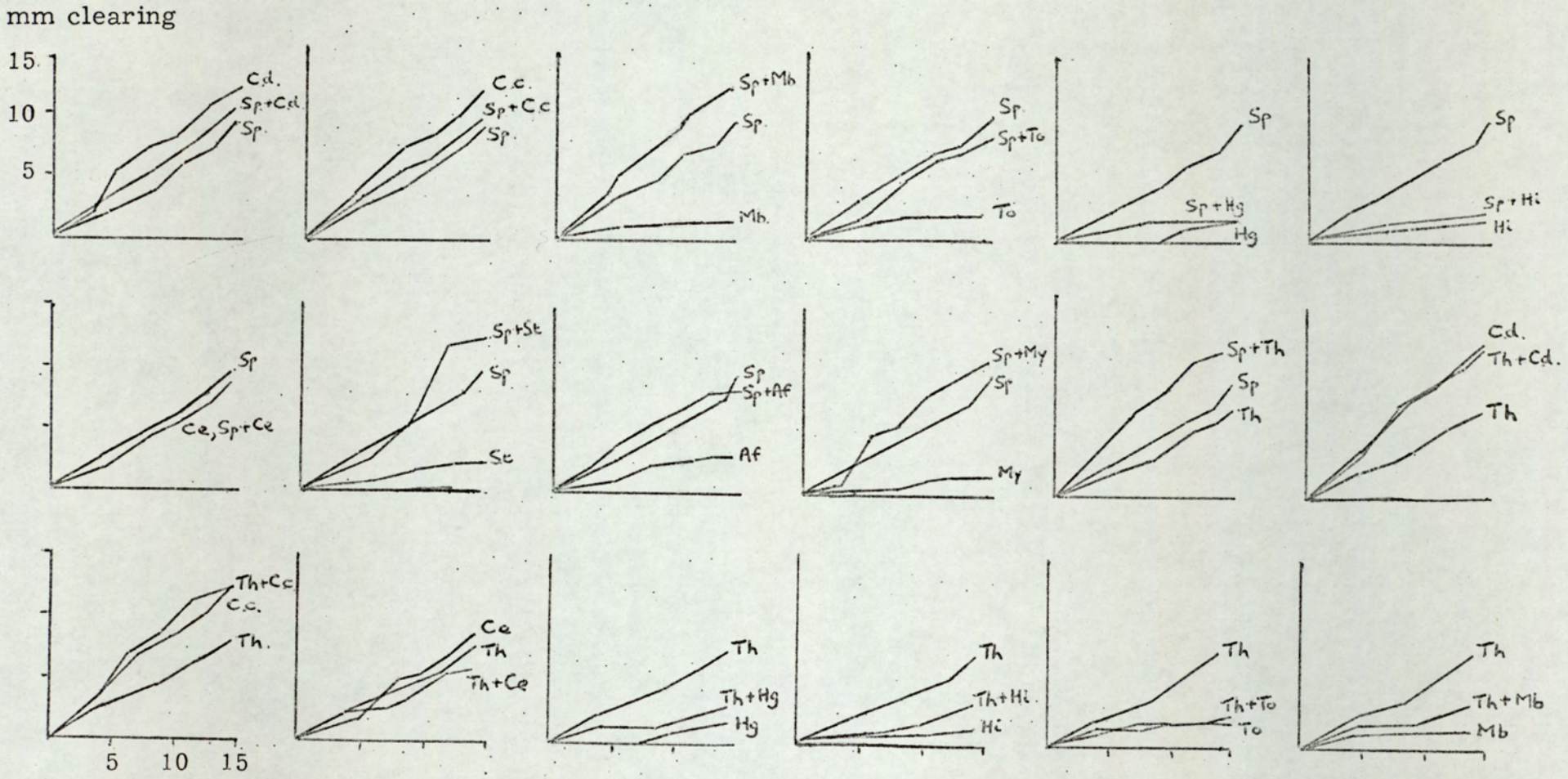

mm clearing
15
10
5
Cd.
Sp+Cd
Sp
Cc.
Sp+Cc
Sp
Sp+Mb
Sp
Mb.
Sp
Sp+To
To
Sp
Sp+Hg
Hg
Sp
Sp+Hi
Hi
Sp
Ce, Sp+Ce
Sp+St
Sp
St
Sp
Sp+Af
Af
Sp+My
Sp
My
Sp+Th
Sp
Th
Cd.
Th+Cd.
Th
Th+Cc
Cc.
Th.
Ce
Th
Th+Ce
Th
Th+Hg
Hg
Th
Th+Hi
Hi
Th
Th+To
To
Th
Th+Mb
Mb
5
10
15
Days of incubation

FIGURE 21 (a)

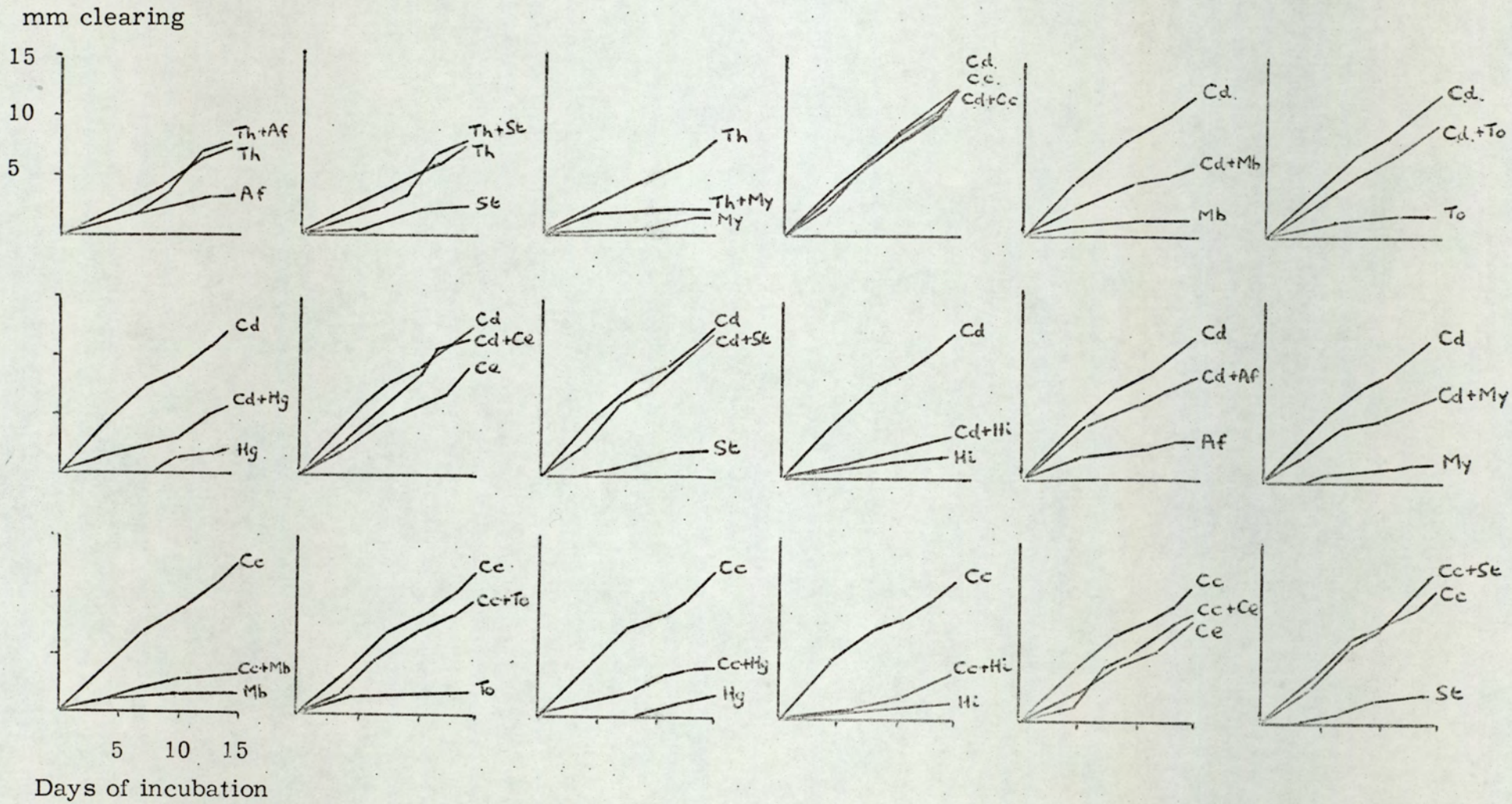

mm clearing
15
10
5
Th+Af
Th
Af
Th+St
Th
St
Th
Th+My
My
Cd.
Cc.
Cd+Cc
Cd.
Cd+Mb
Mb
Cd.
Cd.+To
To
Cd
Cd+Hg
Hg
Cd
Cd+Cc
Cc
Cd
Cd+St
St
Cd
Cd+Hi
Hi
Cd
Cd+Af
Af
Cd
Cd+My
My
Cc
Cc+Mb
Mb
Cc
Cc+To
To
Cc
Cc+Hg
Hg
Cc
Cc+Hi
Hi
Cc
Cc+Ce
Ce
Cc+St
Cc
St
5
10
15
Days of incubation

FIGURE 21 (b)

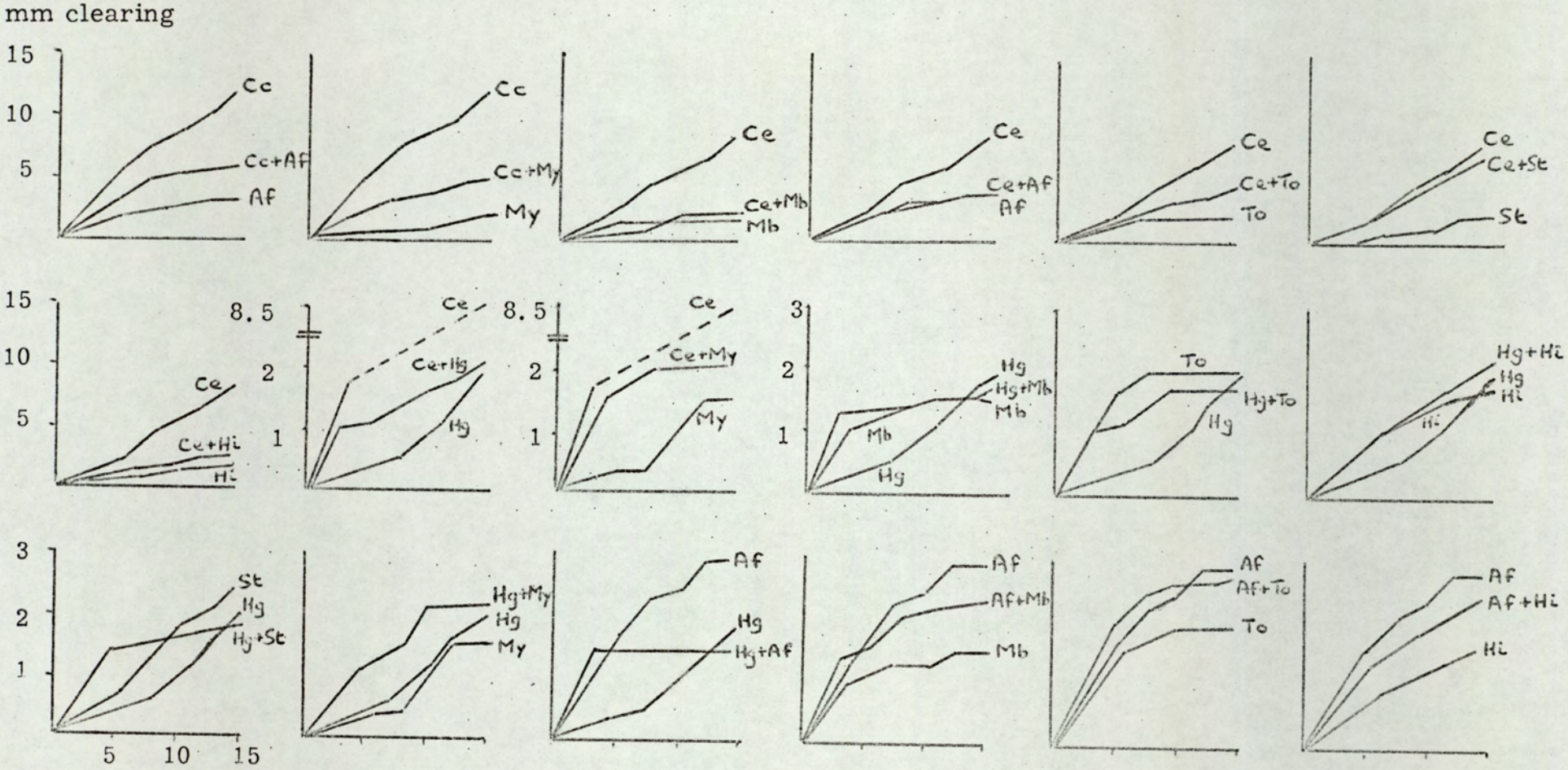

mm clearing
15
10
5
Cc
Cc+Af
Af
Cc
Cc+My
My
Ce
Ce+Mb
Mb
Ce
Ce+Af
Af
Ce
Ce+To
To
Ce
Ce+St
St
15
10
5
Ce
Ce+Hi
Hi
8.5
2
1
Ce
Ce+Hg
Hg
8.5
2
1
Ce
Ce+My
My
3
2
1
Hg
Hg+Mb
Mb
Mb
Hg
To
Hg+To
Hg
Hg+Hi
Hg
Hi
Hi
3
2
1
St
Hg
Hg+St
Hg+My
Hg
My
Af
Hg
Hg+Af
Af
Af+Mb
Mb
Af
Af+To
To
Af
Af+Hi
Hi
5
10
15
Days of incubation

FIGURE 21 (c)

mm clearing

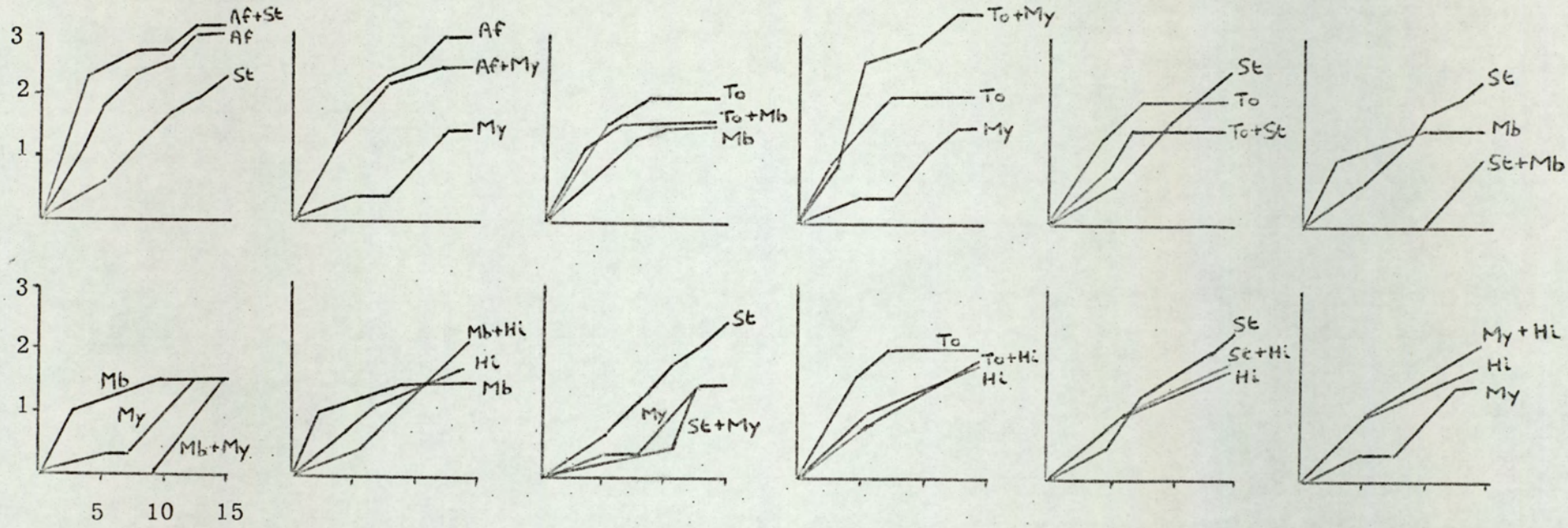


FIGURE 21 (d)

M. albomyces which produce very little clearing individually.. M. albomyces enhanced the cellulolytic activity of Sporotrichum thermophile, H. insolens, H. grisea and Torula thermophila, whilst Stilbella thermophila enhanced the activity of C. thermophile var. coprophile, Sporotrichum thermophile, Thielavia thermophila and A. fumigatus. It would be interesting to study the interaction effects of these two fungi in greater detail than was possible here to establish the nature of this "symbiotic" association.

There were four cases in which the partnership made no difference whatsoever to the depth of clearing produced by the more cellulolytic of the two fungi. This happened when the two varieties of Chaetomium thermophile; Sporotrichum thermophile and Cephalsoporium sp., C. thermophile var. coprophile and Thielavia thermophila and M. pulchella and M. albomyces were growth together. The depths of clearing of these pairs of fungi, with the exception of C. thermophile var. coprophile and Thielavia thermophila are very similar however and so one might not expect to find large interaction effects here.

For the remaining 49 combinations, the cellulolytic activity of the more cellulolytic of the pair of fungi was inhibited to a greater or lesser degree. Fungi which clearly stand out as being depressors of cellulolytic activity were H. insolens depressing the two varieties of Chaetomium thermophile (- 72% dissitum, - 66.6% coprophile), Sporotrichum thermophile (- 73.0%), Thielavia thermophila (- 50%) and Cephalosporium sp. (- 68%); H. grisea depressing the two varieties of Chaetomium thermophile (- 49% dissitum, - 65.8% coprophile), Sporotrichum thermophile (- 76%), Thielavia thermophila (- 58.7%), Cephalosporium sp. (- 75.4%), and A. fumigatus (- 50%); Torula thermophila depressing Thielavia thermophila (- 65.1%)

and Cephalosporium sp. (- 49.4%); Malbranchea pulchella depressing the two varieties of Chaetomium thermophile (- 52.5% dissitum, - 73.4% coprophile), Thielavia thermophila (- 44.4%), Cephalosporium sp. (-78.8%) and Stilbella thermophila (- 52.2%); Aspergillus fumigatus depressing C. thermophile var. coprophile (- 48%), and Cephalosporium sp. (- 66%); and finally Myriococcum albomyces depressing C. thermophile var. coprophile (- 59.1%), Thielavia thermophila (- 70%) and Cephalosporium sp. (- 76.5%).

It was observed that under the conditions imposed by the Rautela-Cowling tubes i.e. limited space for growth and partial anaerobiosis, the growth and sporulation of H. grisea, H. insolens, Stilbella thermophila and M. pulchella were greatly enhanced. This effect was so strong that these fungi, with the exception of Stilbella thermophila, tended to quickly overgrow and reduce the growth of most other fungi growing in combination with them. Malik and Eggins (1970) using such a system demonstrated the mycoparasitic nature of Gliocladium roseum on six other fungi and noted that the use of Rautela-Cowling tubes demonstrates the effects of non-diffusible metabolites. It would be interesting therefore to study fungi such as H. grisea, H. insolens and Malbranchea pulchella in greater detail then was possible here to establish whether these fungi are indeed mycoparasitic, or whether their faster growth under slightly anaerobic conditions is the contributory factor in the inhibition of the cellulolytic activity of the highly cellulolytic thermophiles such as Chaetomium thermophile, Sporotrichum thermophile, Thielavia thermophila and Cephalosporium sp. One must, of course, not forget that even in a system such as this, diffusible or aromatic metabolites could also play a part in these inhibition effects.

(2) Interaction Plates

The results of the interaction of the cellulolytic fungi are shown in Table 12, and indicate that for most combinations of fungi an interaction front was formed between the colony edges. However, there were some notable exceptions where marginal or complete intergrowth occurred. Taking each fungus in turn:- C. thermophile var. dissitum showed complete intergrowth with Thielavia thermophila, Cephalosporium sp., Torula thermophila, M. pulchella and Stilbella thermophila. Growth of Thielavia thermophila was slightly reduced but sporulation of both species was normal. Cephalosporium sp. produced much aerial mycelium above the very densly sporulating Chaetomium thermophile var. dissitum. When grown with Torula thermophila, both species produced a thick line of sporulation along their intergrowing margins; whilst normal growth and sporulation of Chaetomium thermophile var. dissitum was noted, but no sporulation of Stilbella thermophila was seen when these two fungi were grown in combination. Marginal intergrowth was produced by variety dissitum when grown with variety coprophile, Sporotrichum thermophile and M. albomyces, with a dense line of perithecia being produced by variety dissitum on meeting variety coprophile. Frontal formation was observed between variety dissitum and H. insolens, H. grisea and A. fumigatus. H. insolens did not produce spores whilst sporulation of variety dissitum and H. grisea appeared normal in all cases. Growth of A. fumigatus was reduced, and very heavy mycelial growth, with little perithecial production, was observed in variety dissitum along the mycelial margins.

Chaetomium thermophile var. coprophile gave complete intergrowth with Thielavia thermophila, Cephalosporium sp. and A. fumigatus.

1	1										
2	M	2									
3	M	F	3								
4	G	G	G	4							
5	G	G	F	M	5						
6	F	F	F	F	M	6					
7	F	F	M	G	F	F	7				
8	G	F	F	M	M	F	F	8			
9	G	F	M	F	F	F	F	F	9		
10	F	G	M	G	M	G	G	G	F	10	
11	G	F	F	F	F	F	G	F	F	G	11
12	M	F	M	M	M	F	F	F	F	G	F

1 Chaetomium thermophile var dissitum
2 Chaetomium thermophile var coprophile
3 Sporotrichum thermophile
4 Thielavia thermophile
5 Cephalosporium sp.
6 Humicola insolens
7 Humicola grisea
8 Torula thermophile
9 Malbranchea pulchella
10 Aspergillus fumigatus
11 Stilbella thermophile
12 Myriococcum albomyces

TABLE 12

Interaction effects between pairs of thermophilic cellulolytic fungi grown on E & P cellulose agar in petri plates.
F - Front between fungi formed. G - Complete intergrowth of both species. M - Frontal hyphae just merging but no definite intergrowth.

Very heavy perithecial production was observed when grown with Cephalosporium sp., but only submerged growth with no perithecial production was given when grown with A. fumigatus. Marginal growth was produced (as noted above) with variety dissitum and fronts were formed with all the other test fungi. With H. grisea the sporulation of variety coprophile was reduced, and growth of H. grisea was completely submerged with a black line of dark hyphae forming along the front. Growth of Torula thermophila was similarly submerged with only a small amount of aerial sporulation. When grown with Malbranchea pulchella, variety coprophile produced a dark line of perithecia along the front, whilst growth with Stilbella thermophila gave very heavy general perithecial production.

Sporotrichum thermophile gave complete intergrowth with Thielavia thermophila and marginal intergrowth with C. thermophile var. dissitum, H. grisea, Malbranchea pulchella, Myriococcum albomyces and A. fumigatus. The sporulation of H. grisea and Malbranchea pulchella was very heavy along the respective merging fronts, whilst growth of Myriococcum albomyces was reduced by the very rapid growth of Sporotrichu thermophile. Complete fronts were formed between Sporotrichum thermophil and C. thermophile var. coprophile, Cephalosporium sp., H. insolens, Torula thermophila and Stilbella thermophila. Growth of H. insolens was reduced by the very heavy growth and sporulation of Sporotrichum thermophil whilst Torula thermophila produced a thick, black line of sporulation along the front.

Thielavia thermophila gave complete intergrowth with the two varieties of Chaetomium thermophile, Sporotrichum thermophile, H. grisea and A. fumigatus. Sporulation of H. grisea was very heavy at the region of

intergrowth, whilst A. fumigatus completely overgrew and reduced the growth of Thielavia thermophila. Marginal intergrowth was observed with Cephalosporium sp., Torula thermophila and Myriococcum albomyces. Lateral growth of Cephalosporium sp. was reduced by very heavy growth of Thielavia thermophila, however mycelial production of Cephalosporium sp. was very heavy indeed. No sporulation was observed with Torula thermophila and growth of Myriococcum albomyces was reduced by the very heavy growth of Thielavia thermophila. Interaction fronts were formed between this fungus and H. insolens, M. pulchella and Stilbella thermophila with the growth of these latter two fungi being reduced by heavy growth and sporulation of Thielavia thermophila. In all cases, only asexual sporulation was observed in Thielavia thermophila on the E & P cellulose agar.

Cephalosporium sp. gave complete intergrowth with the two varieties of Chaetomium thermophile and marginal intergrowth with Thielavia thermophila, H. insolens, Torula thermophila, A. fumigatus and Myriococcum albomyces. H. insolens produced a band of sporulation at the meeting hyphal tips, whilst both Cephalosporium sp. and Torula thermophila formed thick bands of growth and sporulation along the fronts. The very heavy growth of A. fumigatus reduced the growth of Cephalosporium sp., whilst Myriococcum albomyces and Cephalosporium sp. mycelia merged mainly between their aerial hyphae. Complete fronts were produced between Cephalosporium sp. and Sporotrichum thermophile, H. grisea, Malbranchea pulchella and Stilbella thermophila. H. grisea produced heavy sporulation along the front and Stilbella thermophila produced very heavy mycelial growth. Slight aerial intermixing was noted with Malbranchea pulchella, however, a clear front was fromed at substrate level.

Humicola insolens was completely overgrown by only A. fumigatus, and showed a slight merging reaction with Cephalosporium sp. Complete fronts were formed between it and all other test fungi, with a large front (5 m.m.) being produced between this fungus and H. grisea. H. insolens produced very heavy sporulation along the front with M. pulchella Stilbella thermophila and Myriococcum albomyces, with normal growth being produced by these latter fungi.

Humicola grisea produced complete intergrowth with Thielavia thermophila, A. fumigatus and Stilbella thermophila. A. fumigatus completely overgrew and reduced the growth of H. grisea, whilst H. grisea itself overgrew Stilbella thermophila which only produced submerged growth. Some merging of hyphae was observed with Sporotrichum thermophile, but complete fronts were produced with all other test fungi. H. grisea did not sporulate when grown with Torula thermophila although growth of both and sporulation in Torula thermophila was normal. With Malbranchea pulchella very heavy sporulation of H. grisea was observed along the front.

Torula thermophila was overgrown only by A. fumigatus, and slightly by Chaetomium thermophile var. dissitum; with marginal intergrowth by Thielavia thermophila and Cephalosporium sp. Discrete fronts were formed between Torula thermophila and all other test fungi with heavy sporulation being produced by Torula thermophila along the front with M. pulchella and in general when grown with M. albomyces.

Malbranchea pulchella gave complete intergrowth with Chaetomium thermophile var. dissitum and marginal intergrowth with Sporotrichum

thermophile. Fronts were formed between it and all other test fungi, with A. fumigatus cutting down its growth.

Aspergillus fumigatus, the most "gregarious" of this group of fungi, completely overgrew and reduced the growth of all other test fungi except M. pulchella and C. thermophile var. dissitum. Slight intergrowth was produced between it and Sporotrichum thermophile and Cephalosporium sp., however in all cases sporulation of A. fumigatus was normal.

Stilbella thermophila was completely overgrown by C. thermophile var. dissitum, H. grisea and A. fumigatus; and definite fronts were formed between it and all other test fungi. In all cases, no sporulation was observed in Stilbella thermophila.

Myriococcum albomyces was overgrown only by A. fumigatus, but showed slight marginal intergrowth with Sporotrichum thermophile, Thielavia thermophila and Cephalsoporium sp. Fronts were produced between it and all other test fungi, and as previously noted (see Chapter 3) M. albomyces did not produce cleistothecia on this media or as an interaction effect.

Discussion

When the results from the two former interaction experiments are compared, several interesting facts being to emerge. If we take initially those cellulolytic thermophiles which have previously been isolated from composting town waste, viz: Chaetomium thermophile, H. insolens, H. grisea, Torula thermophila, M. pulchella and A. fumigatus,

we can see (Table 13) that the notable depressors of cellulolytic activity were H. insolens, H. grisea, M. pulchella and Torula thermophila; with a lesser effect from A. fumigatus. These fungi greatly depressed the depths of clearing of cellulose agar by the more cellulolytic varieties of Chaetomium thermophile. The two varieties of Chaetomium thermophile, however, were able to form either complete fronts or slight merging reactions with most of these depressors, and at the same time were able to reduce the growth and sporulation of Torula thermophila, H. insolens and H. grisea on the interaction plates. Variety differentiation within the one species Chaetomium thermophile was also observed when Malbranchea pulchella depressed the cellulolytic activity of both varieties but was able to intergrow with only variety dissitum; and when growth of A. fumigatus was reduced by variety dissitum, which in turn produced heavy mycelial growth, whilst perithecial production in variety coprophile was depressed. These observations, coupled with the fact that the two varieties of Chaetomium thermophile did not depress each other's cellulolytic activity, could account for the success of these two varieties in town waste composting. They are both able to elaborate their cellulase systems at alkaline pH values and they are both rapid growers.

The four thermophiles H. insolens, H. grisea, Torula thermophila and M. pulchella produced very little clearing of the cellulose agar themselves, but produced excellent growth and sporulation in the Rautela-Cowling tubes in combination and in isolation. It might be interesting to study the growth of these four fungi, in future work, under conditions of reduced oxygen tension to establish whether they are, in fact, able to grow better under such conditions. Conditions of partial anaerobiosis will certainly exist beneath the surface layers of composting town waste,

TABLE 13

Table 13 shows the combined results of the interaction experiments using Rautela-Cowling tubes and interaction plates

KEY

1	Chaetomium thermophile var. dissitum
2	Chaetomium thermophile var. coprophile
3	Sporotrichum thermophile
4	Thielavia thermophila
5	Cephalosporium sp.
6	H. insolens
7	H. grisea
8	Torula thermophila
9	Malbranchea thermophila
10	Aspergillus fumigatus
11	Stilbella thermophila
12	Myriococcum albomyces

	Enhanced D.C. by	Outstanding depressors of D.C.	Complete Intergrowth	Marginal Intergrowth	Front Formation
1		6, 7, 8, 9, 10, 12	4, 5, 8, 9, 11	2, 3, 12	6, 7, 10
2	11	6, 7, 8, 9, 12	4, 5, 10	1	3, 6, 7, 8, 9, 11, 12
3	4, 9, 11, 12	6, 7, 9, 11	4	1, 7, 9, 10, 12	2, 5, 6, 8, 11
4	10, 11	6, 7, 8, 9, 12	1, 2, 3, 7, 10	5, 8, 12	6, 9, 11
5		4, 6, 7, 8, 9, 10, 12	1, 2	4, 6, 8, 10, 12	3, 7, 9, 11
6	9, 12		10	5	1, 2, 3, 4, 7, 8, 9, 11, 12
7	6, 12		4, 10, 11	3	1, 2, 6, 7, 8, 9, 12
8	12	9	1, 10	4, 5	2, 3, 6, 7, 9, 11, 12
9			1	3	2, 4, 5, 6, 7, 8, 10, 11, 12
10	11	7, 9	2, 4, 6, 7, 8, 11, 12	3, 5	1, 9
11		8, 9	1, 7, 10		2, 3, 4, 5, 6, 8, 9, 12
12			10	1, 3, 4, 5	2, 6, 7, 8, 9, 11

TABLE 13

TABLE 13 shows the combined results of the interaction experiments using Rautela-Cowling tubes and interaction plates.

and so this could be one factor accounting for the success of these four fungi in composting. In combination with each other H. grisea was able to enhance the cellulolytic activity of H. insolens, and H. insolens was able to enhance the depth of clearing of Malbranchea pulchella, but all other combinations produced a reduction in cellulolytic activity of the more cellulolytic fungus of the pair. On the interaction plates H. insolens was overgrown only by A. fumigatus, and interaction fronts were formed between it and all other test fungi. Similar results were observed with H. grisea; however a very large interaction front was formed between it and H. insolens, even though a synergistic combination was produced between this pair of fungi in the Rautela-Cowling tubes. Torula thermophila was able to depress the sporulation of H. grisea, and sporulation of Torula thermophila was enhanced in the presence of Malbranchea pulchella. Aspergillus fumigatus, as might be expected, was able to intergrow with most fungi except Chaetomium thermophile var. dissitum and Malbranchea pulchella. Its cellulolytic activity was depressed by H. grisea, H. insolens and Malbranchea pulchella, but the very profuse sporulation of A. fumigatus was normal in all cases. Again it would be interesting to test the possible mycoparasitic nature of these fungi, and the staled media or the agar in the vicinity of their growth to see whether diffusible metabolites or antibiotics are being produced, and also whether A. fumigatus is able to elaborate its antibiotic systems at these high temperatures.

The results presented above indicate that the six species of fungi described are able, in general, to compete together on a cellulosic substrate. Most are able to produce cellulolysis at alkaline pH values, and all are able to sporulate freely and profusely thereby achieving a ready means of propagation and dissemination throughout a windrow of composting

town waste. The reduction in the cellulolytic activity of the two varieties of Chaetomium thermophile appeared to be balanced by the ability of these two varieties to reduce growth and sporulation of H. grisea, H. insolens and Torula thermophila, the notable depressors of cellulolytic activity. These facts, by themselves, go part of the way in explaining the success of these six species of thermophilic cellulolytic fungi in the process of town waste composting. Obviously many more ecological, biochemical and physiological studies must be carried out before a much clearer picture will emerge.

When we examine the effects of the above six fungi on the cellulolytic and growth effects of Sporotrichum thermophile, Cephalosporium sp. and Thielavia thermophila, it can be seen that the depths of clearing produced by all three fungi were greatly depressed by interaction with H. grisea, H. insolens and Torula thermophila. Sporotrichum thermophile was able to induce very heavy sporulation in H. grisea, M. pulchella and Torula thermophila; Thielavia thermophila produced heavy sporulation in H. grisea; and Cephalosporium sp. induced heavy sporulation in H. insolen Torula thermophila and H. grisea. The depths of clearing of Sporotrichum thermophile and Thielavia thermophila were enhanced only by interaction between themselves and between M. pulchella and A. fumigatus respectively. A. fumigatus, however, drastically cut down the growth and sporulation of Thielavia thermophila in the interaction plates, and also reduced the depths of clearing of Sporotrichum thermophile and Cephalosporium sp. It might be possible therefore that the effects of the interaction of these three fungi with H. insolens, H. grisea and Torula thermophila and the fact that Sporotrichum thermophile and Thielavia thermophila grow and sporulate better at low acidic pH values and Cephalosporium produces little aerial mycelium at any pH value on insoluble cellulose, might prevent the large

scale colonisation of composting town waste by these three highly cellulolytic thermophiles. The remaining two thermophiles, M. albomyces and Stilbella thermophila, are quite rare thermophiles and might not be expected to be isolated from such systems. Myriococcum albomyces was able to depress the cellulolytic activity of some of the highly cellulolytic thermophiles e.g. the two varieties of Chaetomium thermophile, Thielavia thermophila, Cephalosporium sp. and A. fumigatus, whilst Stilbella thermophila was able to enhance the activity of Sporotrichum thermophile and Thielavia thermophila. These two fungi however can be drastically affected by physical parameters which are present in the composting process. We have seen that Stilbella thermophila produces very little growth and sporulation at alkaline pH values on cellulose strips, and that its spores are quite susceptible to thermal death (Fergus and Ameluny 1971). Myriococcum albomyces, during this study, was never observed to sporulate on any cellulosic substrate, and this fact would also make the mycelium of Myriococcum albomyces susceptible to thermal death during the initial stages of heating in the composting process (also see Fergus and Ameluny 1971).

To sum up, it appears that to be successful in colonising the cellulosic substrates present within composting town waste, a thermophilic cellulolytic fungus has to be able to grow, sporulate and produce its cellulase enzymes at alkaline pH values, and has to be able to compete with interacting fungi under these conditions. We have seen that six species of thermophilic fungi go part of the way in fulfilling some or all of these conditions and that these six fungi are the thermophilic, cellulolytic fungi usually isolated from composting town waste.

CHAPTER 7

CHAPTER 7

The biodegradation of synthetic polymers and plasticisers by thermophilic fungi

Introduction

The first six chapters of this thesis have shown that at least twelve thermophilic fungi are able to attack and degrade cellulose, a naturally occuring polymer. Town waste also contains ever increasing amounts of synthetic polymers, or "plastics"; and although we have a certain amount of knowledge concerning the part played by the mesophilic fungi in attacking these synthetic polymers and their associated plasticisers, we know nothing about the effects of the thermophilic fungi on this group of "man-made" products. How susceptible are synthetic polymers and plasticisers to fungal attack, and can we use the same criteria as we used for measuring cellulose breakdown when considering biodegradation of these materials? These questions can be answered by considering what we already know about the biodegradability of these materials, and then trying to apply similar, or novel techniques with these materials under thermophilic conditions.

The fact that "plastic" materials were susceptible to biological breakdown became evident during the Second World War when large amounts of plastic-containing materials were moved into tropical regions of the world - areas more conducive to large scale biological attack. This initial discovery led to intensive research programmes - at first by government departments, and then later by plastics manufacturers themselves. Much of the earlier reported work (c. 1946) tended to be confusing, and growth

of a particular organism upon a plastic was taken as evidence of breakdown of that plastic. No distinction was made, in fact, between the susceptibility of the polymeric constituents, and the plasticisers and fillers making up the "plastic". The first major piece of research to be carried out on polymeric materials was that of Brown (1945) who tried to grow fungi on several unplasticised polymers. He came to the conclusion that the majority of the polymers in use at that time were resistant to fungal attack. Similar conclusions were reached by Abrams (1948) who tried to grow Aspergillus niger on pure polymers. These results were summarised by Wessel (1964) and are reproduced in Table 14.

The resistance of the majority of the synthetic polymers has since been expounded by a U.K. Ministry of Aviation Report (1962), Heap (1965), Allakhverdiev et al. (1967) and Booth and Robb (1968). The chemically reactive groupings in high molecular weight synthetic polymers are inaccessible to microbial enzymatic attack due to high intermolecular forces (Kulkarni 1965, Gagliardi and Kenney 1968), and also to the fact that the surfaces of most polymers represent a highly hydrophobic environment. Difficulties involved in assessing the susceptibility of a polymer include the extreme slowness of possible degradation reactions, and a lack of suitable methods for measuring these parameters. The only polymers to date which do show signs of susceptibility to microbiological attack are melamine-formaldehyde, cellulose-nitrate, polyvinyl acetate and polyurethanes (Brown 1945, Berk 1957, Ross 1963, Leeflang 1963, Evans and Levisohn 1968, and van der Toorn 1969).

Why then, do plastics materials break down under conditions

TABLE 14

Microbiological resistance of polymeric constituents of plastics

Polymer	Resistance
Polymethyl methacrylate	Good
Polyacrylonitrile	Good
Cellulose acetate-butyrate	Good
Cellulose acetate-propionate	Good
Ethyl cellulose	Good
Phenol formaldehyde	Good
Urea formaldehyde	Good
Nylon	Good
Polyethylene	Good
P. T. F. E.	Good
P. C. T. F. E.	Good
Polypropylene	Good
Polyisobutylene	Good
Polycarbonates	Good
Polystyrene	Good
P. V. C.	Good
Vinyl chloride/vinyl acetate copolymer	Good
Polyvinylidene chloride	Good
Polyvinyl butryl	Good
Expoxies	Good
Chlorinated polyethers	Good

conducive to microbiological attack? The answer seems to lie with the susceptibility of the other processing ingredients present within the "plastic" material. There appears to be no reported evidence of microbial breakdown of stabilisers e.g. antioxidants, incorporated into plastics. The little evidence available on accelerators in rubber (Rook 1955) showed that the accelerators, in normally applied amounts, would not prevent biodegradation of the rubber and hence were not biocidal in properties. Since many stabilisers in plastics are inorganic salts, usually of heavy metals such as lead and barium, it might be anticipated that such compounds would possess biocidal properties. Many of the earlier thermosetting plastics such as "Bakelite" used to be 'filled' with wood shavings, flour etc. and these fillers, if exposed to the environment would of course provide a source of nutrition for microorganisms. Present day plastics are filled with inorganic fillers or glass and these thermosetting plastics should be resistant. There are still, however, many plastic-coated cellulose based products on the market today, produced mainly by the textile industry. Polymers are used to make a material resistant to wrinkling, soiling, water and oil, and to microbial growth. Gagliardi and Kenney (1968) have shown, however, that unless the applied polymer forms linkages with at least some of the available -OH groups on the anhydroglucose molecules in cellulose, then the cellulosic material will still remain susceptible to microbiological breakdown.

It appears that the plasticisers influence the susceptibility of a plastic to microbiological attack. Removal of the plasticiser from plasticised plastics leads to deleterious changes in the physical properties of the plastic. Although the cellulosics were the first plasticised plastics it has been estimated that the majority of plasticisers now produced are

used by the vinyl industry (Klausmeier and Jones 1961). Chemically they are high boiling organic compounds, usually liquid, and predominantly esters of organic acids or phosphoric acid. Others are hydrocarbons, halogenated hydrocarbons, epoxides, ethers and various polymers. A large number of plasticisers will support microbial growth and are able to be broken down to a greater or lesser degree. A list of biodegradable and non-biodegradable plasticisers is presented in Table 15. This list was based on that of Heap (1965) and includes many more plasticisers which have been tested since that date. Where any doubt exists in the literature, the particular plasticisers has been placed in the biodegradable section.

Since thermoplastic resins not containing plasticisers are generally resistant to microbiological attack, it might be anticipated that internally plasticised polymers such as polyethylene should also be resistant. This fact was confirmed by Stahl and Pessen (1954) and their results are presented in Table 16.

Test Methods for Biodegradation

When considering suitable test methods to demonstrate possible biodegradability of polymers and plasticisers a number of test criteria can be used. These include: (1) Visual evaluation of the extent of fungal growth, production of biomass or changes in shape of the plastic test piece, (2) Weight loss of the test piece after attack, (3) Changes in mechanical properties of the plastic after attack, (4) Changes in electrical (insulation) properties, (5) Changes in the uptake of oxygen by organisms attacking plasticisers, (6) Measurement

TABLE 15

Biodegradable and Non-biodegradable Plasticisers

Biodegradable Plasticisers

Hydrogenated methyl abietate

Tri-n-butyl aconitate

Triethyl aconitate

Adipic acid ester P 103 N

Adipic acid ester P 204 N

Butylene glycol polyadipate

Di-isodecyl adipate

Di-isooctyl adipate

Di-(1,3 dimethyl dibutyl) adipate

Di-hexyl adipate

Di-iso-octyl azelate

Di-(ethylene glycol monobutyl ether) - azelate

Benzyl bensoate

Chlorinated hydrocarbons: Aroclor 1254: Aroclor 1263

Glyceryl triacetate

Diethylene glycol ethyl ether acetate

Diethylene glycol butyl ether acetate

Ethyl phthalyl ether glycolate

Methyl phthalyl ethyl glycolate

Methyl phthalyl methyl glycolate

Butyl phthalyl butyl glycoate

Butyl laurate

Ethylene glycol laurate

Ethylene glycol ethyl ether laurate

Diethylene glycol monolaurate

Diethylene glycol ethyl ether laurate

Glyceryl laurate

Sorbitol laurate

Biodegradable Plasticisers (cont.)

Dibutyl ammonium oleate

Ethylene glycol methyl ether oleate

Nitrile from oleic and linoleic acids (N. T. D. - 181.5 - B)

Sorbitol eleate

Dipentaerythritol hexapropionate

Pentaerythritol diacetate dibytyrate

Pentaerythritol diacetate dipropionate

Oleic Acid

Ricinoleic Acid

Fatty acid dimethyl amide

Triethyl tricarballylate

Tri-n-butyl carballylate

Tung oil

Castor oil

Cotton seed oil

Dehydrated castor oil

Refined Tall oil (Indusoil)

Triethanolamine dicaprylate

Expoxidised soya bean oil G62/Di-iso-octyl phthalate 15/35 mixture

Expoxyester ED3 (Lankro)/Di-iso-octyl phthalate 15/35 mixture

Pentaerythritol monoacetate tripropionate

Pentaerythritol triacetate monopropionate

Pentaerythritol tripropionate monomyristate

Pentaerythritol tetrabutyrate

Pentaerythritol tetrapropionate

Citric Acid tributyl ester

Biodegradable Plasticisers (cont.)

Triethylene glycol dipelargonate

Polymeric "Hexaplas" (I.C.I.) polypropylene adipate, end stopped with laurate

Acetyltributyl citrate (Pfizer Citroflex A4)

Epoxy plasticiser (Estabex 2307)

Alkyl ester of mixed dibasic acids

Triphenyl phosphate

Diphenyl mono-(p-tert-butyl phenyl) phosphate

Monophenyl di-(p-tert-butyl phenyl) phosphate

Diphenyl mono-o-exenyl phosphate

Tri-(2-nitro-2 methyl proply) phosphate

Tricresyl phosphate

Tri-2-ethyl hexyl phosphate

Hexyl phosphate

Diamyl phthalate

Dicapryl phthalate

Diphenyl phthalate

Ethyl-2-methyl-2 nitropropyl phthalate

Butyl isodecyl phthalate

n-octyl n decyl phthalate

iso-octyl iso-decyl phthalate

octyl phthalate

Methyl acetyl ricinoleate

Butyl acetyl ricinoleate

Ethylene glycol methyl ether acetyl ricinoleate

Biodegradable Plasticisers (cont.)

Glyceryl monoricinoleate

Glycol sebacate resin

Sebacic acid alkyd resins - Paraplex G 25

- Paraplex RG 2

- Paraplex RG 20

Paraplex X - 100

Ester type alkyd resin - Duraplex C 50 LV

Sebacic acid ester P 204 N

Di-2 ethyl hexyl sebacate

Di-iso-octyl sebacate

Polymeric Rheoplex (Geigy) polypropylene sebacate

Dimethyl sebacate

Dibutyl sebacate

Di-(1,3 - dimethyl butyl) sebacate

Di-(2 - ethyl hexyl) sebacate

Stearic Acid

n - butyl stearate

Cyclohexyl stearate

Butoxy ethyl stearate

Diethyl glycol ethyl ether stearate

Tetraethylene glycol monostearate

Tetraethylene glycol distearate

Non-biodegradable Plasticisers

Abietic acid

Dimethyl adipate

Di (2-ethylhexyl) azelate

Ethyl-o-benzoyl benzoate

Chlorinated diphenyls : Aroclor 1242

Aroclor 1248

Aroclor 1262

Aroclor 1270

Aroclor 5460

Clorowax

Cerechlor

N-alkyl caproanilides (25)

"phthalate-4-dicaprylate"

Tri-n-butyl citrate

Triethylene citrate

Diacetate of 2-nitro-2methyl - 1,3 - propanediol

Dipropionate of 2 - nitro - 2 methyl 1,3 - propanediol

Diethylene glycol dipropionate

Triethylene glycol

Hexoxymethyl ether of diethylene glycol

Triethylene glycol di-(2-ethylhexoate)

Triethylene glycol di-(2-ethylbutyrate)

β-chloroethyl γ-chloro- γ phenylpropyl ether

Polyethylene glycol 200

Non-biodegradable Plasticisers (cont.)

Polyethylene glycol 300

Polyethylene glycol 400

Polyethylene glycol 1500

Polyethylene glycol 6000

Polyethylene glycol di-(2-ethylhexoate)

Dipentaerythritol hexacetate

Dipentaerythritol hexabutyrate

Napthenic acid - naphthenecyclohexamide

Napthenic acid - monoethanolamide

Monochlorohydrin glycerol naphthenate

Triethyl phosphate

Tributyl phosphate

Tri exylenyl phosphate

Tri-(2 ethyl hexyl) phosphate

Phenyl bis (β chloroethyl) phosphate

Tris (chloroethyl) phosphate

Tributoxyethyl phosphate

Tricresyl phosphate

Diphenyl mono-(o-chlorophenyl) phosphate

Di-o-exnyl monophenyl phosphate

Tri-(p-tert butyl phenyl) phosphate

Tri-(o-chlorophenyl) phosphate

Tri-(o-xenyl) phosphate

Dimethyl phthalate

Diethyl phthalate

Di-n-propyl phthalate

Non-biodegradable Plasticisers (cont.)

Di-isopropyl phthalate

Di-isooctyl phthalate

Dibutyl phthalate

Di isobutyl phthalate

Dihexyl phthalate

Dioctyl phthalate

Di-(2 ethyl hexyl) phthalate

Di cyclohexyl phthalate

Di methoxy ethyl phthalate

Di ethoxy ethyl phthalate

Di butyoxy ethyl phthalate

Methyl-2-methyl-2-nitropropyl phthalate

Butyl-2-methyl-2-nitropropyl phthalate

Bis-(diethylene glycol ethyl ether) phthalate

Silicone oil (Fluid 500)

Diethyl succinate

Di-n-butyl tartrate

Ethyl-p-toluene sulphonate

o-cresyl-p-toluene sulphonate

o and p-toluene ethylsulphonamide

Sulphonated Oil (Naftolen R - 510)

Diphenyl

Diamylnaphthalene

Diamylphenoxyethanol

Benzophenone

Methyl amyldihexylcyclohexanone

Methylcylohexyl oxalate

Non-biodegradable Plasticisers (cont.)

Diphenyl sulphone

Triphenylguanidine

Supraflex IX

Flexol P.E.P.

Polymer Trade Name	Composition of Polymer	Test Results		
		Shake Flask	Soil Burial	Other Method
Kel-F	Monochlorotrifluoroethylene	R(G)	-	-
KF - VF2	(50% Monochlorotrifluoroethylene (50% vinylidene fluoride	R(G)	-	-
Nylon 66	Hexamethylenediamine, adipic acid	-	R(C)	R(C,N)
Polythene	Ethylene	R(G)	-	R(N)
Saran 115	(85% Vinyliden chloride (15% Vinyl chloride	R(P)	R(F)	-
Teflon	Tetrafluorethylene	R(Sh)	-	-
Tygon	Vinyl chloride/vinyl acetate	R(G)	-	-
Velon	Vinylidene chloride, small amount of vinyl chloride	-	-	R(N)
Vinyon	(90% vinyl chloride (10% vinyl acetate	R(F)	-	R(N)
0-123-184 (ERRL)	Vinyl stearate	R(G)	-	-
0-147-79-4,5 (ERRL)	(55% Vinyl stearate (45% Vinyl chloride	R(G)	-	-
0-147-53-3 (ERRL)	(30% Vinyl stearate (70% Vinyl chloride	R(G)	-	-

TABLE 16

Fungus susceptibility of internally plasticised resins. (From Stahl and Pessen 1954)

R = resistant, C = Cloth, F = Fibre, G = Granules, P = Powder, Sh = Shavings, N = Not specified.

of pH changes upon microbial breakdown of an ester-type plasticiser.

With these test criteria in mind we can now go on to describe the main test methods in use today for determination of the biodegradability of polymers and plasticisers.

(1) Agar Plate Methods

These are methods in which fungi or fungal spores are inoculated onto either a test strip of plastic on the surface of agar or onto agar containing homogenised plasticiser. From the large collection of literature on the subject it soon becomes obvious that the plastic most susceptible to biodegradation is plasticised P.V.C. which can contain up to 50% plasticiser. Plasticised P.V.C. has been used in this, and in the other techniques to be described, as a test plastic and indeed the petri plate method for testing plastics has become a standard test method (A.S.T.M. D1924 - 63, 1963) Berk (1950) placed P.V.C. strips on agar in bottles and exposed them to a Trichoderma sp. for 6 weeks at 29°C. He found that incorporation of dibutyl sebacate plasticiser increased the vulnerability to fungal attack and caused a 63% increase in tensile strength and a 67% loss in percentage elongation of the strips. In contrast, strips plasticised with dioctyl phthalate and butadiene-acrylonitrile showed very little fungal growth and no significant changes in tensile strength or percentage elongation.

Dayal et al. (1962) and later work by Nigam (1965) with strips of parachute nylon-66 on agar showed that Penicillium janthinellum was able to cause a pink staining of the nylon and an appreciable loss in tensile

strength after 42 days. This work was later reviewed, however, by Rogers and Kaplan (1971) who, using similar agar methods, contradicted the findings of the Indian authors and in fact confirmed the results of Gray (1945) who was unable to find one organism out of one hundred and one different organisms able to grow when Nylon-2 was used as the sole carbon source. The same test method was used by Burgess and Darby (1964, 1965) to evaluate the percentage loss in weight and the percentage shrinkage of P.V.C. strips, plasticised with different plasticisers, over a period of 14 days.

An interlaboratory experiment (Hazen 1967) was carried out in which P.V.C. test strips were placed on agar and sprayed with a mixed spore suspension of six fungi. Testing, by visual evaluation, weight loss and change in elasticity modulus was carried out and results from all participating laboratories were in good agreement, which would tend to suggest that this method might be a reasonable test method to use.

In another similar interlaboratory experiment (Dolezel 1967) the agar plate method was used to study the effect of various fungicides incorporated into plasticised P.V.C. These workers then assessed the suitability of evaluation criteria and found that elongation testing of the strips gave the best measure of fungal degradation whilst tensile strength and modulus measurements gave less sensitive results.

Thin slabs of polyurethane were placed on agar and inoculated with species of Penicillium, Phoma, Aspergillus, Fusarium, Cephalosporium, and Stemphylium by Evans and Levisohn (1968). These workers found that the Stemphylium degraded the polyurethane and made deep tunnels into the

material within two days. Similar observations were later made by Jones and Le Campion-Alsumard (1970) who examined the attack on submerged polyurethane by marine fungi.

Homogenised plasticiser in agar was used by Klausmeier and Jones (1961) to isolate organisms capable of degrading specific plasticisers. They included yeast extract as an additional source of microbial nutrient, and found that many so called "non-biodegradable" plasticisers could be broken down in the presence of these extra growth substances. This was obviously an important step in the testing of these plasticisers because if the "non-biodegradable" plasticisers had been used in plastics formulations, then the plasticisers could have become susceptible if the plastic article became coated with organic detritus capable of supplying additional microbial nutrients.

Plasticiser-agar was also used by Gamova-Kayukova and Pal'mova (1967) to test the resistance of 35 different plasticisers to a mixture of 12 fungal species; whilst Bomar (1968) used a similar method to test the susceptibility of four plasticisers to Aspergillus niger, in the presence and absence of fungicides.

Cavett and Woodrow (1968) used this technique to isolate organisms from a plasticiser-enriched river mud. They were able to isolate test organisms which could actually utilise plasticisers, rather than use test organisms picked at random.

(2) Soil Burial Methods

This type of test in which plastic test pieces are buried vertically

in soil has been used by various workers for long and short term testing work. It has the obvious advantage that the test strips are exposed to a wide variety of organisms under natural environmental conditions. The test can also be modified by enriching the soil with test plasticisers in order to select out organisms capable of degrading such plasticisers. For long term soil burial test work it is a wise precaution to periodically renew the soil around the plastic test strips (J. J. Elphick - personal communication). This method has been used by Klausmeier and Jones (1961), Dolezel (1967), Klausmeier (1968), Booth and Robb (1968), Cavett and Woodrow (1968) and Wendt et al. (1970) to provide decayed specimens for subsequent physical evaluation.

(3) Liquid Culture Methods

In these methods the plastic test strip is usually fully immersed in a nutrient salts solution which is then inoculated with test organisms (Hitz and Zinkernagel 1967, Booth and Robb 1968, Kestelman and Vilnina 1971). Plasticisers can be incorporated into nutrient salts, usually with added yeast extract (Williams et al. 1968); or leachates from compounded plastics can be tested in this way (Evans and Levisohn 1968, Rogers and Kaplan 1971).

(4) Respirometric Techniques

This test method was first published by Burgess and Darby (1964) as a rapid method for determining the susceptibility of plastic to microbial degradation, and was an adaptation of the methods used by Siu and Mandels (1950), Mandels and Siu (1950) and Siu (1951) for

determining the mildew susceptibility of various materials. Burgess and Darby used Barcroft differential manometers attached to large conical flasks containing nutrient salts, test pieces and organisms. In a later paper (1965) they used Pseudomonas aeruginosa as test organism and related oxygen uptake by this organism growing on several plasticised plastics to break down of the plastic when tested by the weight loss technique.

This method was later modified by Cavett and Woodrow (1968) to cut down the test time to two days by incorporating the plasticiser itself into nutrient salts contained in the flasks of a Warburg respirometer. They also standardised their inoculum of Pseudomonas sp using a rapid dry cell weight method. This overcame the "highly undesirable practice" of spraying on spore suspensions (Burgess and Darby 1964, 1965). These workers also went on to measure the endogenous respiration of the Pseudomonas cells in the presence of small plasticised P.V.C. discs by measuring $C^{14}O_2$ derived from uniformly C^{14} labelled cells. They found that the endogenous respiration correlated inversely with the degradability of the substrate and came to the conclusion that respirometric techniques (with this organism) cannot give absolute values for breakdown of a plastic or plasticiser due to the variability of the attendant endogenous respiration. However, they say that this method does have certain advantages:- (1) it is a rapid test method, (2) it is a real method of biological activity and (3) it offers the possibility of standardisation between laboratories. As such, it can be used as a rapid comparative method, in conjunction with other test methods.

(5) Enzymatic (esterase) Technique

A very large percentage of all plasticisers in use today are chemically esters, and it has been postulated that the microbial degradation of plasticised P.V.C. could be carried out by using extracellular esterases (Reese et al. 1955, Berk et al. 1957). The hydrolysis of dibutyl phthalate and dibutyl sebacate during microbial degradation had been confirmed in earlier work (Klausmeier and Jones 1961, Klausmeier 1966), and these authors went on to suggest that microbial enzymes could be used for such work to eliminate the time necessary for microbial growth and to decrease the test time.

Williams et al. (1968) made use of this fact and grew fungi in liquid shake culture containing an ester plasticiser to induce esterase production. They then tested the mycelia and the filtrate for esterase enzyme by incubation with a range of ester plasticisers, followed by titrating the liberated acids with 0.05N NaOH. They found that the esterase activity lay within the mycelium itself and not the filtrate. This method, in which results can be obtained within 24 hours , can be used to estimate the optimum temperature and pH level for plasticiser hydrolysis.

Evaluation criteria

The above methods are the main test methods in use today for the microbiological degradation of plastics and plasticisers. After exposure to the action of microorganisms one is then faced with the task of evaluating the extent of degradation. Test evaluation methods fall roughly into three main categories:- (1) Visual evaluation methods, (2) Physical testing methods, (3) Chemical testing methods.

(1) Visual evaluation

These evaluation methods are of little use in determining the extent of breakdown of a plastic and accounted for much of the confusion in the earlier literature. A fungus might produce excellent growth on a plastic test piece without actually removing the plasticisers or degrading the plastic to any significant extent. Visual evaluation is useful however in screening tests, when the extent of growth and production of biomass by a fungus growing on plasticiser-agar can be compared to that produced in controls.

(2) Physical test methods

These include the Clark flexibility test, in which the test strip of plastic is passed through rollers and then allowed to bend through 90^{o} under its own weight (Burgess and Darby 1964, Wendt et al. 1970). The length of plastic bending through this angle is then measured and related to the flexibility of the plastic. On fungal degradation P.V.C., for example, will become stiffer due to loss of plasticiser, and hence a longer length of plastic will be needed to bend through this angle. As might be anticipated, however, long and thin test pieces are required for this particular evaluation method.

Tensile strength methods are commonly used by polymer chemists to determine physical properties of plastics, and these methods have also been used by biologists to determine changes in physical properties of test strips after microbial degradation (Berk 1950, Dolezel 1967, Hitz et al. 1967). The plastic test piece is clamped between the jaws of cross heads

in a tensometer and the machine then records, usually graphically, the applied stress and the subsequent elongation of the test strip as the cross heads move apart at constant speed. From the resulting stress/strain curve (see Figure 23) several parameters can be read off and related to the degree of microbial breakdown of the strip.

The portion of the curve from the origin to the yield point covers the total useful range of stress and strin for most applications. Stress at the yield point corresponds to the stress at which "cold drawing" of the plastic commences. This happens when the entire extension of the plastic takes place at a localised region - the "neck" - and this gradually traverses the entire length of the specimen. During this process crystallite re-orientation takes place within the plastic and the drawn polymer is substantially stronger in the direction or orientation - this accounts for the rise in the graph from A to C. This further uniform stretching occurs until point C when the test strip breaks.

Tensile strength methods have been compared by Hitz et al (1967) who found that changes in tensile strength (at break) and change in elongation at break were less sensitive methods than either stress at 33% elongation or weight loss. The weight loss method was recently investigated by Wendt et al. (1970) who found that weight loss (of plasticised P.V.C. strips) was as sensitive or more sensitive a method than stress at 33% elongation, stress at break or Clark flexibility.

Changes in stiffness (flexibility) of test strips can also be tested by the "vibrating reed" method (Hazen and Waterman 1965), in which "the frequency corresponding with the fundamental of one of the

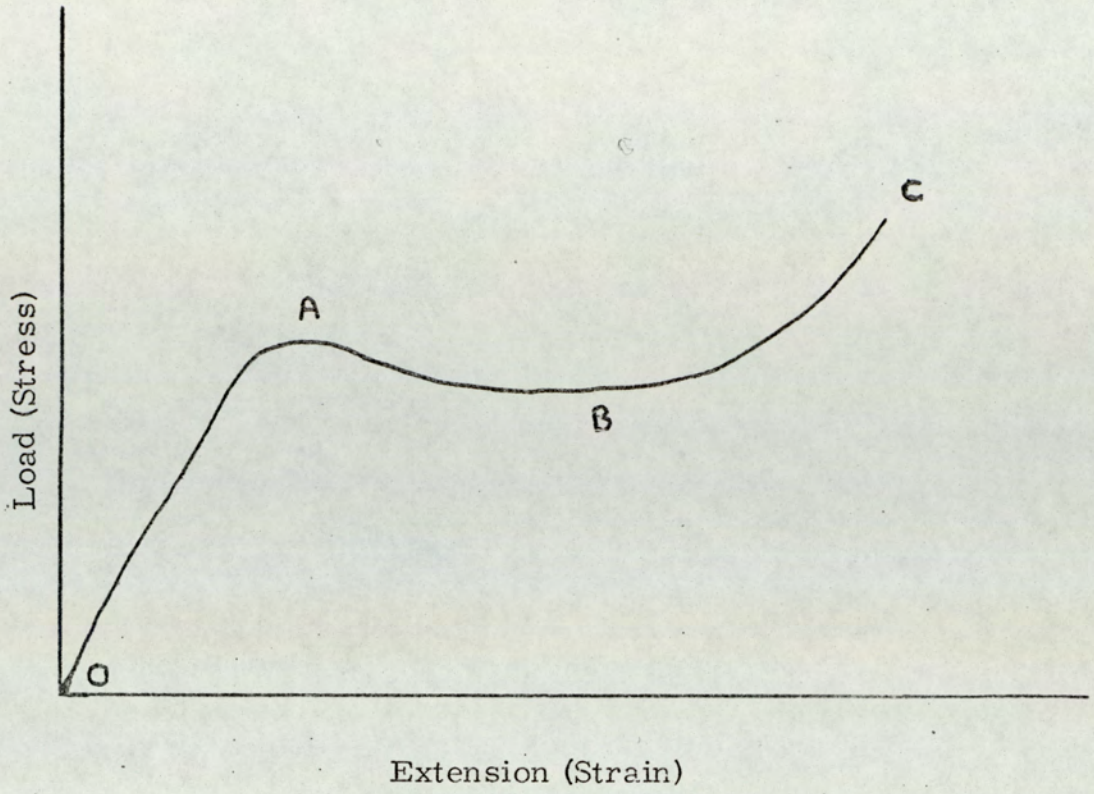


FIGURE 23

Typical Stress/Strain curve for polyethylene

A	corresponds to the Yield Point
A - B	the region of "cold drawing"
C	the fracture point
O	origin

overtones of the sample" is determined, and from this the elasticity modulus of the sample can be calculated. The method involves clamping the test strip into an electro magnetic driver attached to a variable frequency oscillator. The frequency of the oscillator is gradually increased until the strip begins to resonate. The elasticity modulus is then calculated by substituting values for resonance frequency, thickness length and density of strip into the appropriate equation. This method is not widely used by biologists however, probably due to its complexity and its lack of sensitivity when used with thicker samples (Hazeu 1967).

Other physical evaluation methods include torsional tests to measure resistance to a twisting force (Payen 1966); and methods which measure changes in the electrical insulation properties of a plastic - the surface and volume resistivity tests much favoured by workers in Eastern Europe (Allakhverdiev et al. 1967 a, b).

Finally we have nephelometric methods in which the amount of light passing through a suspension of microorganisms (usually bacteria) is related to the growth of the bacteria on certain plasticisers (Hitz and Zinkernagel 1967).

(3) Chemical test methods

These include, as previously mentioned, the titratable acidity produced during enzymatic hydrolysis of ester plasticisers, and the measurement of radioactive products from labelled plasticisers or polymers. Polymers for use in human transplant surgery have been labelled in this way (Kulkarni 1965).

Thin layer chromatography of plasticisers and their breakdown products can be used together with many other modern biochemical test techniques involving G. L. C., U/V and I/R spectrophotometry.

The choice of test methods

When faced with a complete variety of test methods and evaluation criteria one has the problem of trying to decide which method would be most appropriate to use under the conditions imposed by growth of the thermophilic fungi, since such test methods have not been used to date (to the author's knowledge) with the thermophilic microorganisms. From what has been said it appears that for polymeric material the agar plate method and the soil burial method might be good methods to use, whilst the weight loss together with the less sensitive tensile techniques, might prove good evaluation methods. It was, therefore, decided to try out these techniques, together with the perfusion technique - since this method might also lend itself to the production of potentially decayed specimens - using specimens of polyethylene, since this particular polymer represents a high percentage of all plastics ending up in town waste (Staudinger 1970).

When assessing plasticisers for potential degradation by thermophilic fungi it was decided to use several of the standard methods. The first method would involve the use of respirometry to find out whether a randomly chosen range of plasticisers could be utilised by a mixed thermophilic population in soil. Since this method is a real measure of biological activity (Cavett and Woodrow 1968), any increase in the rate of respiration of the soil population over normal endogenous values might

indicate the possible susceptibility of the particular plasticiser to breakdown by thermophilic fungi. The second method would be to then enrich the same soil with various plasticisers and attempt to isolate thermophilic fungi which could either tolerate or actively breakdown the enriching plasticiser. These two methods in combination should give some indication which plasticisers are potentially biodegradable, and which thermophilic fungi could be potential biodeteriogens. As a "back-up" method to these initial experiments it was then decided to test all of the thermophilic fungi in pure culture on plasticiser-agar to ensure that the individual fungi themselves were able to utilise the plasticisers for growth and not the breakdown products which might have been produced by other microorganisms in the soil enrichment tests.

Finally it was decided to evaluate the capabilities of each thermophilic fungus to produce esterase enzymes when grown on an ester plasticiser. This information could then be correlated to the previous data to obtain a reasonable picture of the action of thermophilic fungi on plasticisers.

Section 1. Polymeric Material

(1) Weight loss of polyethylene upon perfusion

Ten dumbell shaped polyethylene test pieces (3.125 x 0.5 x 0.0001") were cleaned of surface debris with 70% ethanol, washed in running water and blotted dry, and then placed in a desiccator for 48 hours. The strips were then weighed from the desiccator to avoid picking up atmospheric water and then placed within the glass fibre sleeving in a perfusion kit. Clent

soil was then placed on top of the sleeving and the strips were perfused with E & P nutrient salts for 37 days at 48°C. Two of the test strips served as controls i.e. no soil, and after incubation the strips were carefully removed from the kits, examined under the microscope and then washed in running water, rinsed in 70% ethanol, washed in running water, blotted dry and placed in a desiccator for 48 hours before weighing.

Results

Microscopic examination of the strips revealed no signs of fungal growth on any of the strips. The strips showed no signs of decay, cracking or shrinkage. The results for the weight loss of the strips are shown in Table 18, and from these results it appears that any slight weight loss observed is accounted for by corresponding results in the control strips. Indeed, the largest weight loss observed was with one of the control strips. It seems likely therefore that these small weight losses could have been due to physical conditions e.g. the leaching out of small quantities of material by the perfusing salts, rather than by microbial attack.

(2) Tensile testing experiments

Similar dumbell shaped specimens of polyethylene were cleaned of surface debris using 70% ethanol, washed in running water and blotted dry before being subjected to the following tests:-

(1) Twenty-nine strips were placed on the surface of

TABLE 1

Loss in weight of perfused polyethylene strips

Test Strip	Weight of Strip Before Test	Weight of Strip After Test	Weight Loss
1	0.0374 g	0.0371 g	0.0003 g
2	0.0189	0.0187	0.0002
3	0.0189	0.0183	0.0006
4	0.0195	0.0191	0.0004
5	0.0199	0.0193	0.0006
6	0.0200	0.0195	0.0006
7	0.0198	0.0190	0.0008
8	0.0179	0.0170	0.0009
Control 9	0.0187	0.0181	0.0006
Control 10	0.0192	0.0180	0.0012

E & P cellulose, glucose and YpSs agars and inoculated with crumbs of Clent soil, particles of composting town refuse and with pure cultures of eight randomly chosen thermophiles. The plates were incubated at 48°C for 37 days and controls consisted of 6 strips on agar at 48°C and 9 strips on agar at room temperature.

(2) Ten test strips were buried vertically in a mixture of Clent soil and composting town refuse contained within a plastic plant pot. Glass fibre sleeving with a central wire support ran from a nutrient reservoir, at the base of the pot, through the soil to the atmosphere. In this way a current of nutrient salts could be passed through the soil by evaporation of moisture from the length of sleeving exposed to the atmosphere. The "perfusion pot" was incubated at 48°C for 61 days (Figure 24).

(3) Perfusion kits were set up and E & P nutrient salts were perfused over 18 test strips for up to 49 days. The strips were inserted within the glass fibre sleeving and Clent soil and composting town refuse were used as sources of thermophiles.

(4) A new perfusion system was set up in which a miniature composting system could be perfused with E & P nutrient salts. The system consisted

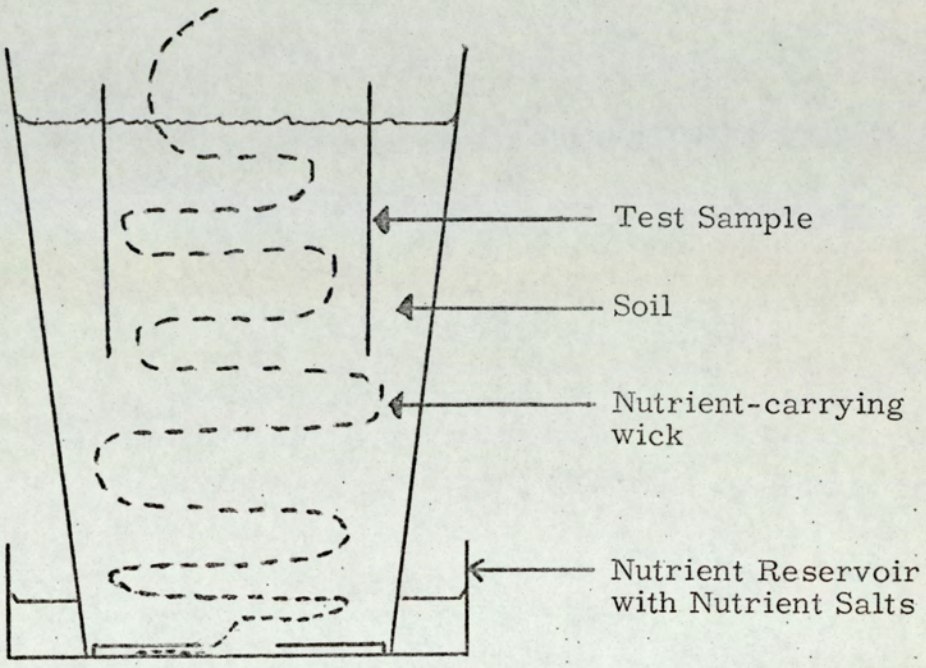


FIGURE 23

Construction of Soil Perfusion Pot

of a large pyrex boiling tube with a hole in the bottom and lined around the inside wall with glass fibre cloth. The cloth was attached to two evaporation wicks at the top of the tube and to two wicks supplying nutrients from a reservoir through the bottom of the tube. The centre of the tube was filled with composting town refuse and six test strips were placed between the wall of the tube and the glass fibre cloth. The advantage with this system over the normal perfusion systems was that direct visual examination of the strips could be made without disturbing the ecosystem within the tubes (Figure 24).

Test strips were also used in the above systems as controls (a) at room temperature, (b) at 48°C, (c) at 48°C in the perfusion tests with no source of organisms and (d) in agar with no source of organisms.

Results

(a) Visual evaluation

The test strips were examined microscopically, without treatment or staining, at x 50 and x 400. It was found that fungal growth had occurred in all the agar plates and that the test strips did not cause zones of inhibition around themselves. Of the eight thermophiles tested in pure culture Chaetomium thermophile, M. pusillus, Torula thermophila, H. insolens, H. lanuginosa and M. pulchella were found to

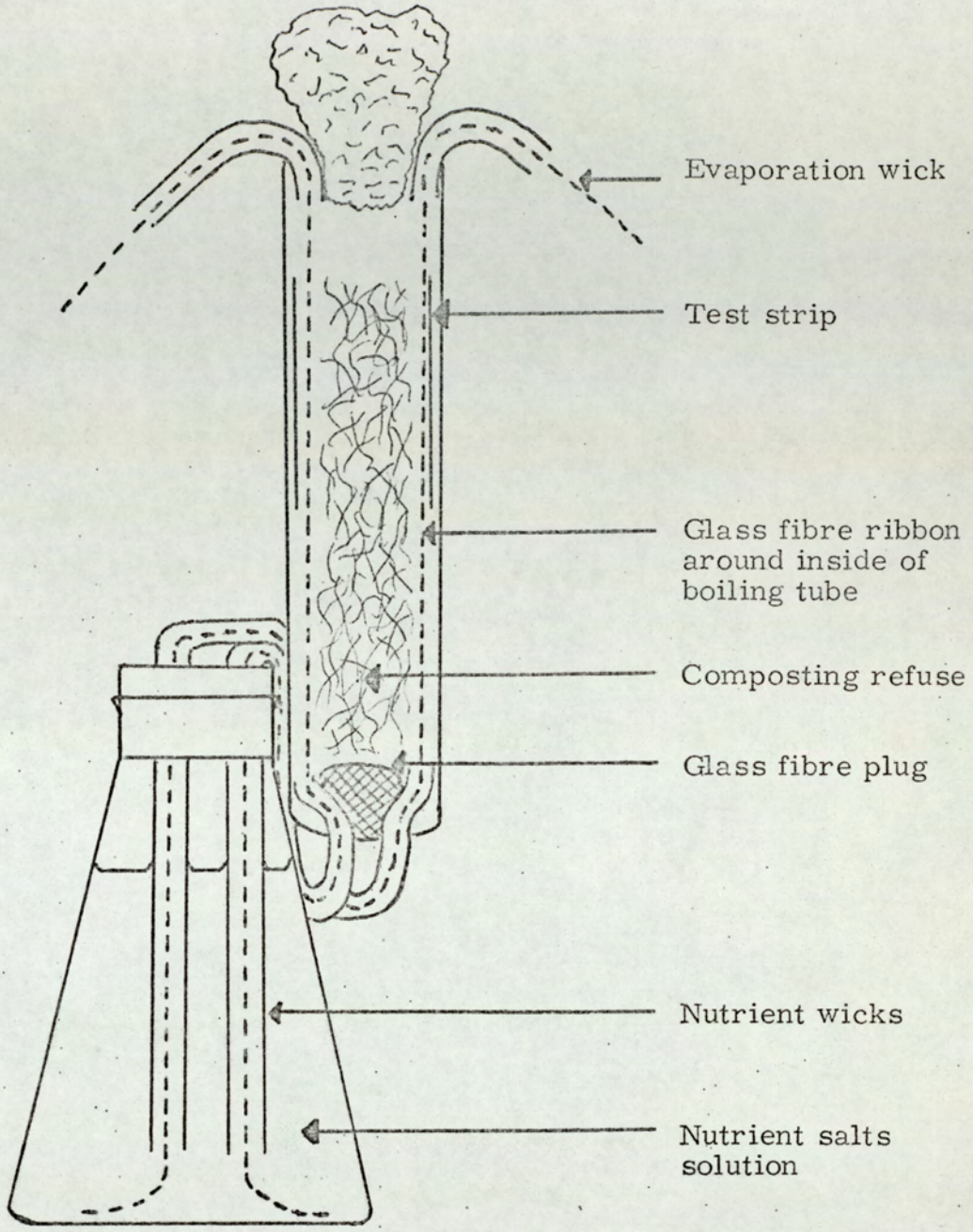


FIGURE 24

The Miniature Perfused Refuse System (MpRs)

Showing Composition of a Typical Unit

grow over and partially or wholly cover the test strips. Growth of several thermophilic actinomycetes was also observed in some of the soil inoculated plates. These were identified as Thermoactinomyces vulgaris, Streptomyces thermoviolaceus var. pingens and Micropolyspora virida.

It was found, however, that the fungal growth could easily be wiped off the test strips using a damp lens tissue, and that upon microscopic examination of the strips no further growth or penetration was observed.

In the perfusion kits, fungal growth was rarely observed on the test strips and contaminating soil could be easily wiped off the strips.

(b) Tensile testing

The tensile characteristics of the strips were determined on a "Tensometer Type E" machine at 20°C, using a crosshead speed of 5 inches per minute for separation of the jaws, and a 1:4 crosshead ratio. The machine automatically recorded the applied stress and subsequent elongation of the samples as the cross heads moved apart at constant speed. The strips were conditioned before testing for two hours at 20°C and all test strips had been previously washed in running water, dipped into 70% ethanol, washed in water and blotted dry. The results of the tensile testing are shown in Table 19 and a tracing of typical stress curves is shown in Figure 25.

The results show that of the 29 test strips exposed to thermophilic

TABLE 19

Results of tensile testing of polyethylene test strips exposed to potential decay by thermophilic microorganisms. All results are the mean of the number of tested samples shown in parenthesis.

	Stress at Yield Point (lb/0.1sq")	Stress at 33% Elongation (lb/0.1sq")	Stress at Break (lb/0.1sq")	Elongation at Break (inches)
61 day Perfusion Pot	2.53 (10)	2.43 (8)	4.13 (10)	6.42 (8)
MpRs	2.61 (3)	4.79 (3)	5.62 (6)	2.99 (3)
41 day Perfusion			4.58 (11)	
36 day Perfusion			4.27 (7)	
48°C Perfusion Controls	2.69 (11)	4.18 (8)	6.54 (11)	4.55 (9)
25°C Perfusion Controls	2.62 (7)	3.46 (4)	5.56 (10)	4.94 (4)
Strips on Nutrient Agar 37 days			5.55 (29)	
Control Strips, 48° C. Nutrient Agar			5.25 (6)	
Control Strips 25°C Nutrient Agar			6.49 (9)	

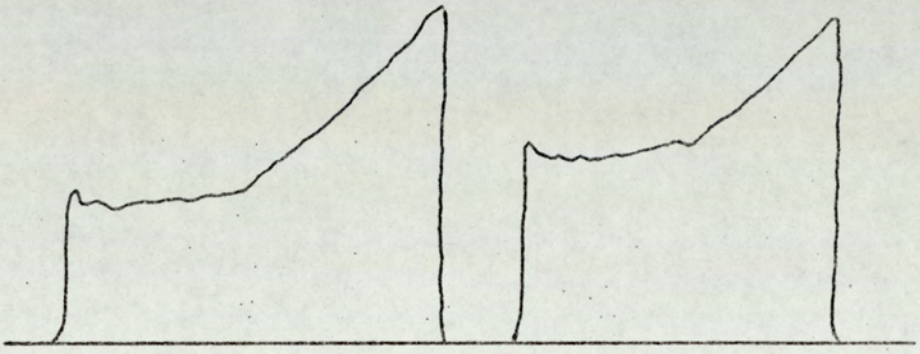


FIGURE 25

Typical Stress-Strain curves produced by the tensometer used. These represent the particular results obtained from two strips after soil burial in the perfusion pot system for 61 days at 48°C.

microbial action on nutrient agar media the mean stress at break was 5.55 lbs per 0.1 sq. inches with a range of results from 3.88 to 7.0 lbs. Control strips on agar averaged 5.25 lbs at 48°C and 6.49 lbs at 25°C with ranges of 4.0 to 6.75 lbs and 4.5 to 8.0 lbs respectively. It appears that the 48°C incubation temperature had a slight effect by lowering the breaking stress of the strips - compare results for the 48°C and the 25°C controls. When the results of the exposed strips are compared to the results of the 48°C controls it can be seen that exposure to potential microbial attack caused very little change in the breaking strengths after 37 days.

The results for the strips perfused with nutrient salts and exposed to potential soil deteriogens for 36 and 41 days show, again, very close similarities between their breaking strengths (4.27 lbs and 4.58 lbs respectively). These strips had ranges of breaking strength between 3.25 and 6.0 lbs (36 days) and between 2.75 and 5.75 lbs (41 days), and the fall in the breaking strength of these strips could be explained by the fact that the perfusing salts could have leached out various stabilisers from the polyethylene during the incubation period.

When one examines the average breaking strengths of the perfused control strips it can be seen that the results are higher than those of the exposed strips, both at 25°C (range 3.44 to 7.82 lbs) and at 48°C (range 3.58 to 8.34 lbs). The only explanation that can be given here for these results it that some chemical factor was leached out of the soil by the perfusion current and affected the properties of the polyethylene.

The results for the strips buried in soil in the perfusion pot

show that the same low values for breaking strength were obtained (range 3.25 to 4.83). The strips themselves showed no signs of microbiological attack after 61 days and again one must conclude that some factor or factors from the soil were responsible for these test results.

The strips in the MpRs showed slightly higher mean breaking strengths (range 1.87 to 8.67 lbs) and these results are similar to those obtained for the exposed strips on agar. They are also within the limits of values obtained for all the control strips.

When the results for the breaking strengths of the strips are compared it can be seen that there is a large range of values within any one group of tested strips. The lowest breaking strength recorded was 1.87 lbs and the highest 8.67 lbs. These results would tend to indicate that a range of tensile strengths is to be expected under thermophilic conditions and that mean results may be more significant than individual results. It must be noted here that absolute values for breaking stress are not simple properties of the plastic, but also depend on the previous thermal history of the specimen, which can retain moulding and extrusion strains (Barker 1957). These particular results would also tend to confirm the observations of Hitz et al. (1967) that changes in tensile strength were less sensitive methods than weight loss or change in stress at 33% elongation.

It was only possible (for technical reasons) to measure the stress at yield point, stress at 33% elongation and elongation at break for the 61 day perfusion pot, the MpRs and the perfused 25 and 48°C controls. The stress at yield point for the two sets of exposed, perfused strips came very close to the values obtained for the perfused controls. Stress at

33% elongation results for the strips in the perfusion pot were less than the results obtained for either set of perfusion controls. However, it must be remembered here that such results were not compared to results obtained from strips buried in a sterile soil, since it is impossible to sterilise a soil completely without altering at least some of its chemical components (Dr. J. Hickman - personal communication). The values of this parameter for the MpRs are within the range (or slightly higher) than the control values.

When the results for elongation at break are examined, it can be seen that similar values for the two sets of perfusion controls were obtained but that slightly higher results were obtained for the strips in the perfusion pot. Exposure to a thermophilic soil environment in this case led to a slight softening of the strips. Results for the strips in the MpRs were low, however the mean of only 3 strips was calculated and so this value might not be a representative value.

From a consideration of all the results presented here, it appears that changes in tensile properties of polyethylene are to be expected when subjected to thermophilic environments, and that these changes can probably be accounted for by physical rather than microbiological attack. Since such a large range of values was obtained for any one parameter it appears that tensile testing (of polyethylene) after exposure to thermophilic conditions may give misleading results, since this polymer has been widely reported to be resistant to (mesophilic) microbiological attack. It would appear, therefore, that polyethylene is also resistant to microbial attack at thermophilic temperatures.

(2) Studies on P. V. C. and P. V. A. polymers

As an additional experiment it was decided to study two more polymers, one which has been reported to be resistant (P. V. C.) and one which has been reported to be susceptible to microbial attack (P. V. A.). Samples of these two polymers, in lattice form, were kindly supplied by Mr. C. A. Brighton of B. P. Chemicals (U. K.) Ltd. , London. One P. V. C. lattice was a straight chain polyvinyl chloride with a particle size of 0. 25 μ, in an emulsion of an alkyl benzene sulphonate. The other P. V. C. lattice was a copolymer of vinyl chloride containing about 20% acrylic ester. The P. V. A lattice was a copolymer of vinyl acetate and ethylene.

The lattices were spread over the surface of cleaned microscope slides and gently heated to fuse the polymer particles into a film, stuck to the surface of each slide. Fifteen slides containing films of each polymer were placed in perfusion kits and covered with a layer of glass cloth. Clent soil was used as a source of thermophiles and the kits were incubated at 48°C for 28 days.

In the second experiment slides containing the films of each polymer were placed in perfusion kits and inoculated with ten randomly chosen thermophilic fungi. The kits were incubated at 48°C for 20 days.

The third experiment consisted of burying slides containing the films of each polymer in Clent soil in perfusion pots. The pots were incubated at 48°C for 20 days.

In the fourth experiment the P. V. A. lattice itself was incorporated into agar at a concentration of 1% in Rautela-Cowling tubes. The tubes were inoculated with 5 m. m. discs of the same ten thermophilic fungi and incubated at 48°C for 30 days.

Results

The slides and tubes were examined microscopically after their respective periods of incubation. The perfused P. V. C. and P. V. A. films all showed no signs of fungal growth after washing the surface in running water. Physical degradation of these films was also lacking. In the perfusion pots the P. V. C. films were completely undamaged and showed no signs of microbial growth, whilst the films of P. V. A. had become totally degraded under these conditions and had become transformed into a highly viscous solution on the surface of the slides. When the Rautela-Cowling tubes were examined however, no evidence of clearing of the P. V. A. particles was observed. The thermophilic fungi would not grow on this media - however it was not known what effect the emulsifier had on the growth of these fungi in this particular experiment.

These results would tend to confirm what is already known about these two polymers i. e. that the unplasticised P. V. C. is resistant, and that a combination of physical and microbial attack in the soil can cause degradation of P. V. A. at thermophilic temperatures. It was impossible at this stage to determine whether thermophilic microorganisms within the soil, or the chemical environment of the soil itself was responsible for breakdown of the P. V. A.

(3) Thermal degradation of polyethylene

From the preceeding work it has been demonstrated that the polymers currently used in packaging i. e. polyethylene and P. V. C. are resistant to biodegradation. It appears therefore that in order to break down plastics waste in refuse completely some initial attack on the high molecular weight polymer itself is needed, followed by microbial degradation of the lower molecular weight products of this attack. One such method of initial attack could be by heating polyethylene to partially oxidise it.

Polyethylene in the absence of oxygen is stable up to temperatures of about 290°C (Oakes and Richards 1949). At higher temperatures there is a decrease in molecular weight and at about 370 to 400°C gaseous products are formed, namely n-alkanes, n-dienes, n-alkenes and cyclic hydrocarbons. At higher temperatures unsaturation increases. However, the energy of activation in the absence of oxygen is greater than that for normal oxidation of polyethylene (Myers 1952), and oxidation can be achieved by heating the molten polymer at 140 to 250°C on mixing mills for 2 - 10 hours in the presence of atmospheric oxygen. This treatment yields carboxylic acids of molecular weights similar to that of the initial polymer (Whittaker and Forsyth 1946).

If methods of partial oxidation could be employed to produce fatty acids then it might be anticipated that this could be one method for dealing with the ever mounting levels of synthetic polymeric material within town waste. The oxidation of polyethylene would break down the high molecular weight polymer and produce oxygen containing products which could then be

put back into the composting system for final degradation by micro-organisms. Such a system would in fact recycle the unavailable carbon present within these high molecular weight polymers.

(a) Initial experiments

Dumbell shaped strips of polyethylene were cleaned of surface debris with 70% ethanol, washed in water and blotted dry. The strips were then placed on the surface of cleaned microscope slides using sterile forceps to avoid contamination of the polyethylene by sweat present on the fingers. The slides were then placed in a small muffle furnace and heated to 200^{o}C in the presence of atmospheric oxygen, initially for one hour and then from one up to five hours.

On removal of the slides it was noted that the polyethylene had stuck firmly to the surface of the slides and that the surface of the films was a light to dark brown in colour, depending upon the time of oxidation. The strips were gelatinous when touched with the end of a sterile spatula and contained numerous small pits over their surfaces. The slides were then placed in a desiccator for 24 hours before weighing. They were then placed in perfusion kits, covered with a layer of glass fibre cloth and Clent soil was used as a source of thermophiles. The kits were incubated at 48^{o}C for 25 days and the slides were then examined microscopically before being placed in a desiccator for 24 hours before weighing.

Results

Upon microscopic examination of the films after incubation, it

was found that fungal and actinomycete growth had occurred on all the films. Growth was restricted to the light brown regions of the film and little growth was observed on the dark brown or on the clear regions. Aspergillus fumigatus was dominant on every strip, and Mucor pusillus, Malbranchea pulchella and a Streptomyces sp. were also observed. When an attempt was made to dislodge the fungal growth it was observed that hyphal penetration had occurred to some extent.

Weight losses of the slides were quite small (Table 29) and the number of hours of oxidation of the films did not seem to make any difference to the weight losses incurred. This was the first time, however, that fungal growth and penetration had been observed on modified polyethylene, and this fact in itself is quite significant (See Plates 2, 3 and 4). Whittaker and Forsyth (1946) mention that this kind of oxidation produces carboxylic acids of molecular weight 5,000 to 20,000, plus small quantities of very low molecular weight acids and in particular butyric acid. It seems likely therefore that the fungi were utilising these very low molecular weight acids for growth. If the polyethylene could be more completely oxidised therefore, it might be possible to produce products which can be completely utilised by the thermophilic fungi, and in this way we might be able to achieve our recycling goal.

One way of further oxidising polyethylene is to heat it in the presence of an oxidising agent such as nitric acid, oxygen and ozone, hydrogen peroxide, benzoyl peroxide, diethyl peroxide, ammonium persulphate, sodium hypochlorite, potassium chlorate, chromic acid or potassium permanganate (Whittaker and Forsyth 1946). The method preferred here was the one using nitric acid, since carboxylic acids of

TABLE 29

Showing weight losses (in mg.) of slides containing films of oxidised polyethylene after 25 days incubation in perfusion kits at 48°C.

OXIDATION TIME						1 HOUR OXIDATION			
1 HOUR	2.5	1.8	0.9	0.8	0	0	0.2	0.5	0.6
2 HOURS	0.2	0.3	0	1.5	0.8	0.2	1.2	26.7	0.3
3 HOURS	0.4	1.7	0.2	1.0	0.9	1.6	0.8	1.2	0.4
4 HOURS	0.4	0.6	0.3	0.4	0.1	0.5	0.1	0.1	0.3
5 HOURS	0.4	0.8	0.8	0.9	0.5	0.1	0.1	25.0	0.3

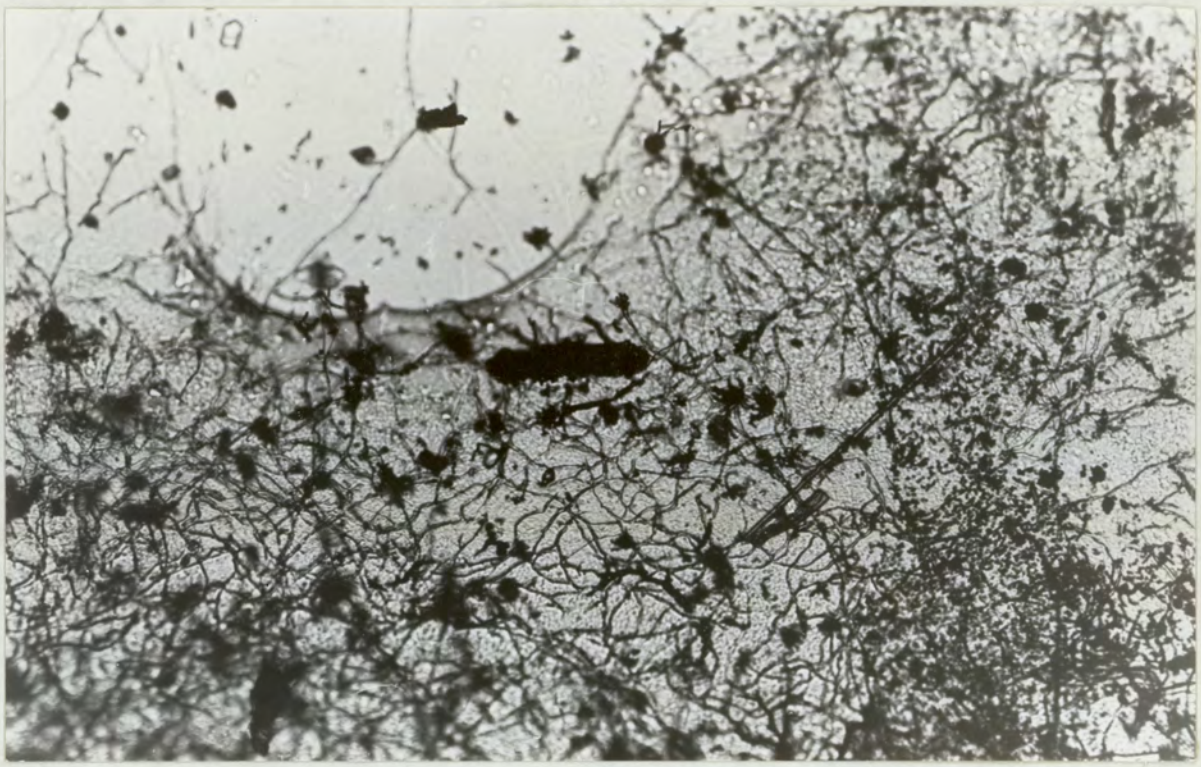


PLATE 2

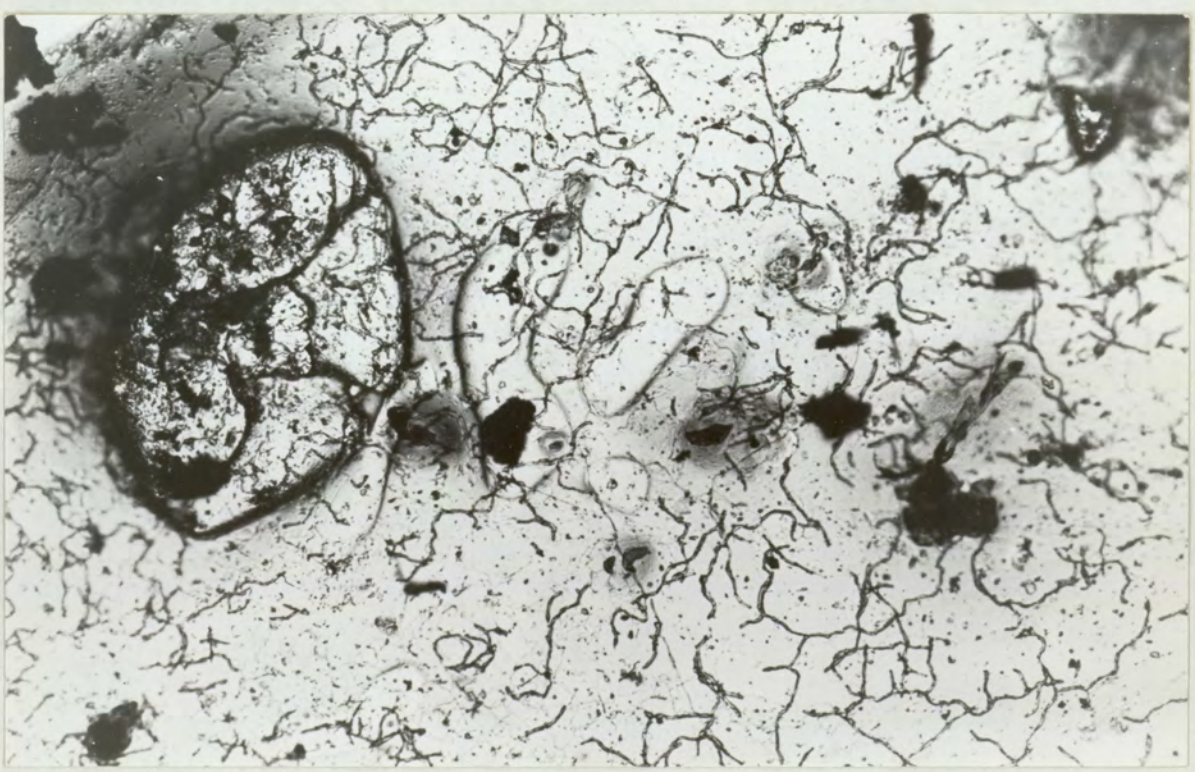


PLATE 3

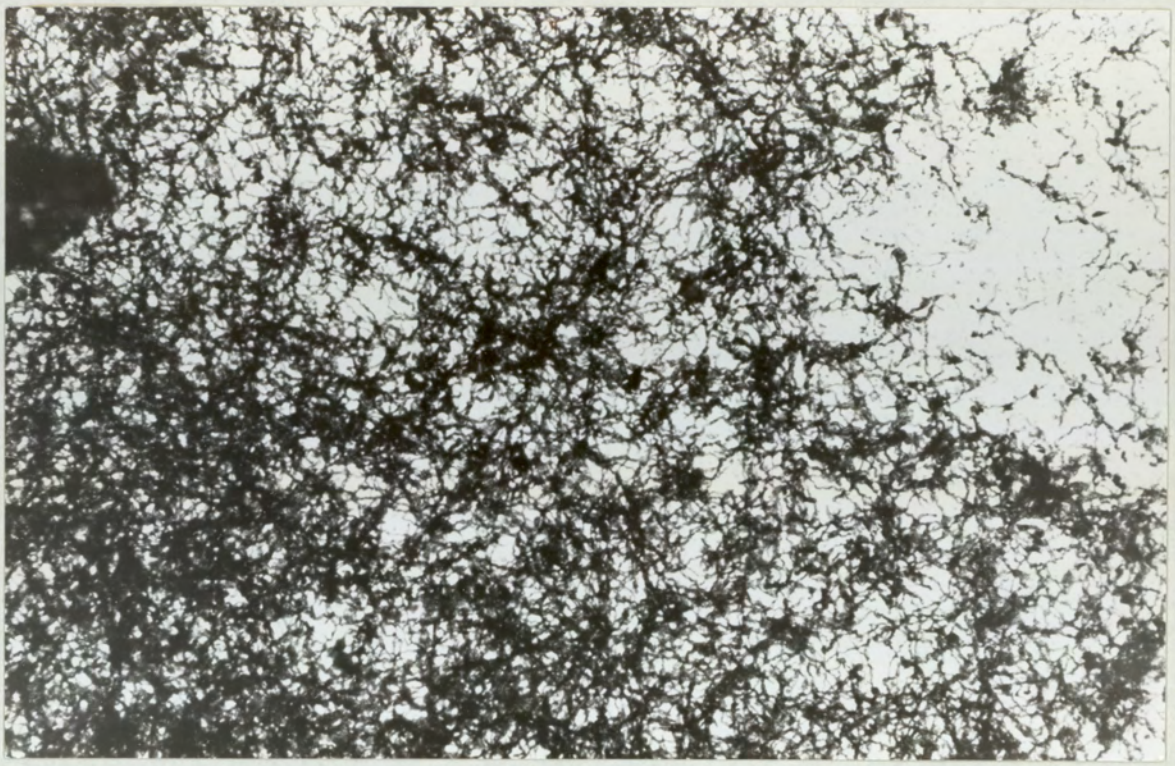


PLATE 4

PLATES 2, 3 and 4

Photomicrographs of air oxidised polyethylene strips showing growth of thermophilic fungi. Fungi isolated from the strips were A. fumigatus, M. pulchella and M. pusillus.

mean molecular weight 250 are produced when this oxidising agent is used.

(b) Nitric acid oxidation of polyethylene

Twenty grams of ICI grade "Alkathene" polyethylene with a molecular weight range of 10,000 to 50,000 was heated to 130°C in a round bottom flask with 250 ml of 60% nitric acid for 24 hours. The flask was fitted with a double reflux condensor and a tube to lead away any fumes. The polyethylene dissolved completely in the nitric acid within the first three hours, and after 24 hours the solution was allowed to cool and the carboxylic acids were precipitated by diluting the solution with large amounts of distilled water. The emulsion was then carefully shaken in separating funnels with diethyl ether to separate off the acids and the ether layers were washed with distilled water to remove traces of nitric acid. The diethyl ether was then removed by rotary evaporation and the resulting waxy acids were incorporated into agar media and liquid culture.

(1) Agar cultures

Duplicate plates of E & P nutrient salts agar containing 1% (v/v) of the fatty acids were inoculated with individual thermophilic fungi. Controls consisted of nutrient salts agar alone, and all plates were incubated at 48°C for 14 days.

Results

The results are indicated in Table 20 and show that some of

TABLE 20

Growth of thermophilic fungi on agar supplemented with 1% fatty acids.

	FATTY ACIDS	CONTROLS
A. fumigatus	3+	+
Cephalosporium sp.	-	-
C. thermophile var. coprophile	-	3+ P
C. thermophile var. dissitum	2+ P	3+ P
H. grisea	2+ S	3+ S
H. insolens	2+ S	-
H. lanuginosa	2+ S	-
H. stellata	-	+ S
M. pulchella	2+ S	2+ S
M. miehei	3+ S	3+
M. pusillus	3+ S	3+
M. albomyces	-	-
P. duponti	-	-
Sporotrichum thermophile	+	-
Stilbella thermophila	-	-
T. emersonii	-	-
T. aurantiacus	2+	-
Thielavia thermophila	-	-
Torula thermophila	-	3+

Key:-

- \- No growth
- \+ light growth around inoculum
- 2+ light growth over half plate
- 3+ light growth over all plate
- P perithecial production
- S asexual sporulation

the fungi produced better growth on the fatty acids than on the control plates. In general, however, growth of the fungi was quite light and the results were uninspiring.

(2) Liquid cultures

Growth studies were carried out in duplicate 250 ml Erlenmeyer flasks containing 50 ml E & P mineral salts solution plus 0.5% of the fatty acids as sole carbon source. The pH of the medium was adjusted to pH 6.4 (Lewis and Johnson 1967) and the flasks were autoclaved at 110°C for 20 minutes. Inocula consisted of 2 m.m. discs of mycelium from ten thermophilic fungi. The flasks were incubated at 48°C before filtering off mycelial growth and drying to constant weight at 80°C. Controls consisted of inoculated E & P mineral salts alone.

Results

Results of the dry weights of fungal mycelium produced in response to the fatty acids are shown in Table 21.

Growth of the thermophiles in the controls was negligible. Very vigorous growth of M. pusillus, Chaetomium thermophile var. coprophile, Cephalosporium and Sporotrichum thermophile was noted, and these fungi produced "pellets" of growth from the original 2 m.m. inoculum up to 3.8 cm. in diameter.

From this initial set of experiments it appeared that most of the thermophiles tested for growth on the fatty acids were capable of

TABLE 21

Showing dry weight of fungal mycelium (minus dry weight in controls) produced in response to 0.5% fatty acids in E & P mineral salts solution

FUNGI	BIOMASS (mg)	
Cephalosporium sp	7.0	9.7
C. thermophile var. coprophile	7.6	6.3
Humicola grisea	3.0	3.9
Humicola insolens	2.2	2.2
Humicola lanuginosa	2.6	2.5
Malbranchea pulchella	4.8	6.2
Mucor pusillus	7.2	5.9
Penicillium duponti	4.4	4.4
Sporotrichum thermophile	9.5	10.0
Torula thermophila	3.8	5.8

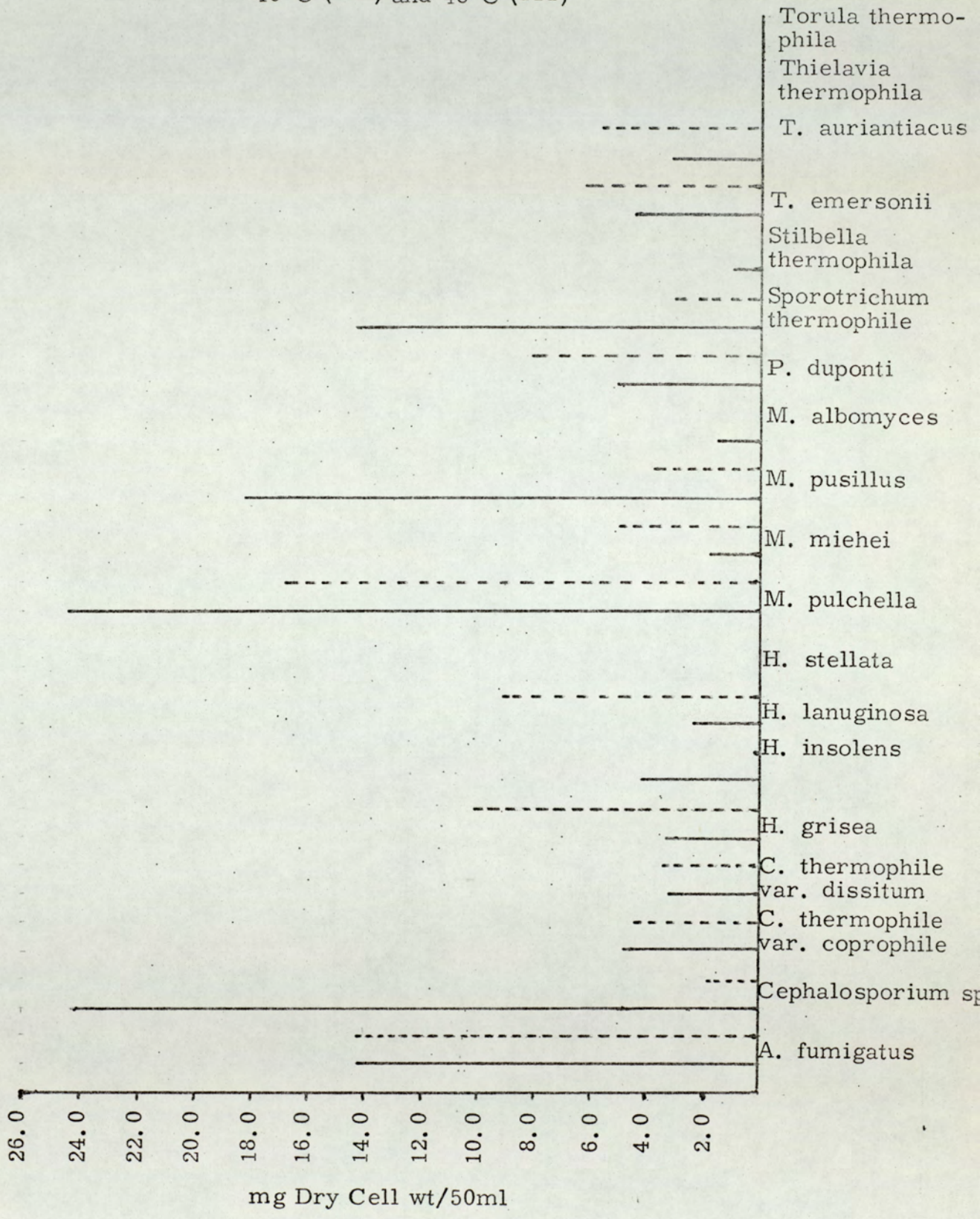
producing growth over and above that in controls. In the second set of experiments a new batch of oxidation products was prepared, in this case using 10g Alkathene and 200 ml 60% nitric acid since it was thought that by using less polyethylene the oxidation reaction might be more complete and produce carboxylic acids of even lower molecular weight. After 24 hours the fatty acids were separated off as above, and then converted into their potassium salts by the addition of 250 ml of 0.2N KOH solution. The soluble potassium salts, it was found, were far easier to handle than the waxy, sticky acids. The salts were incorporated at a 1% level (v/v) in 50 ml of E & P mineral salts solution in Erlenmeyer flasks. The pH of the medium was adjusted to pH 6.4 and all flasks were autoclaved at 115°C for 20 minutes. Inocula again consisted of 2m.m. discs of all the thermophilic fungi (with the exception of H. stellata) and the flasks were incubated at 40°C and 48°C for 19 days. Two replicates were made at each temperature, and after incubation the mycelia were filtered off, washed with boiling distilled water and dried to constant weight at 80°C. Controls consisted of mineral salts solution alone.

Results

The mean mycelial dry weights (minus weights produced in controls) are shown in Figure 26. Very little growth was produced by any fungus in the E & P mineral salts control flasks, whilst several fungi produced substantial amounts of growth on the potassium salts of the fatty acids. M. pulchella, M. pusillus, Cephalosporium sp. and Sporotrichum thermophile all produced good growth at 40°C, with a maximum dry weight of 35.2 mg. being recorded for one flask of M. pulchella. Several fungi produced better growth at 48°C; for

FIGURE 26

showing dry weights in mg/50ml of fungi grown on oxidation products of polyethylene at 40°C (——) and 48°C (---)



example H. grisea, H. lanuginosa, M. miehei, P. duponti, T. emersonii and T. aurantiacus; whilst no growth was observed in any flask of H. stellata, Thielavia thermophila and Torula thermophila at either temperature. H. insolens and Stilbella thermophila produced mycelial biomass at 40°C, but no growth was observed in any flask of these fungi at 48°C. It was noted that growth of A. fumigatus was quite good at both temperatures and that normal sporulation was clearly visible in all flasks. Since this fungus is widely found in composting town refuse it was decided to test its growth on these fatty acids at different pH levels.

In this study the pH of the E & P mineral salts was adjusted from pH 3.0 to pH 9.0, and controls consisted of similarly adjusted salts minus the 1% fatty acids solution. Inocula consisted of 2m.m. discs of the actively growing fungus on agar, and of one 2 m.m. diameter loopful of spore suspension in distilled water. The flasks were incubated for 19 days at 40°C and growth was recorded at each pH level as dry weight of filtered mycelium produced by the mycelial inocula and by the spore inocula.

Results

The results are indicated in Figure 27 and show that the growth of A. fumigatus was better at neutral to slightly alkaline pH values - in fact the sort of pH values which would be found within a windrow of composting town refuse. Spore germination was not inhibited by the carboxylic acids at any pH value tested, and mycelial dry weight produced by the spore inocula was roughly equivalent to that produced by mycelial inocula, except at pH 9 when spore germination was depressed. No growth

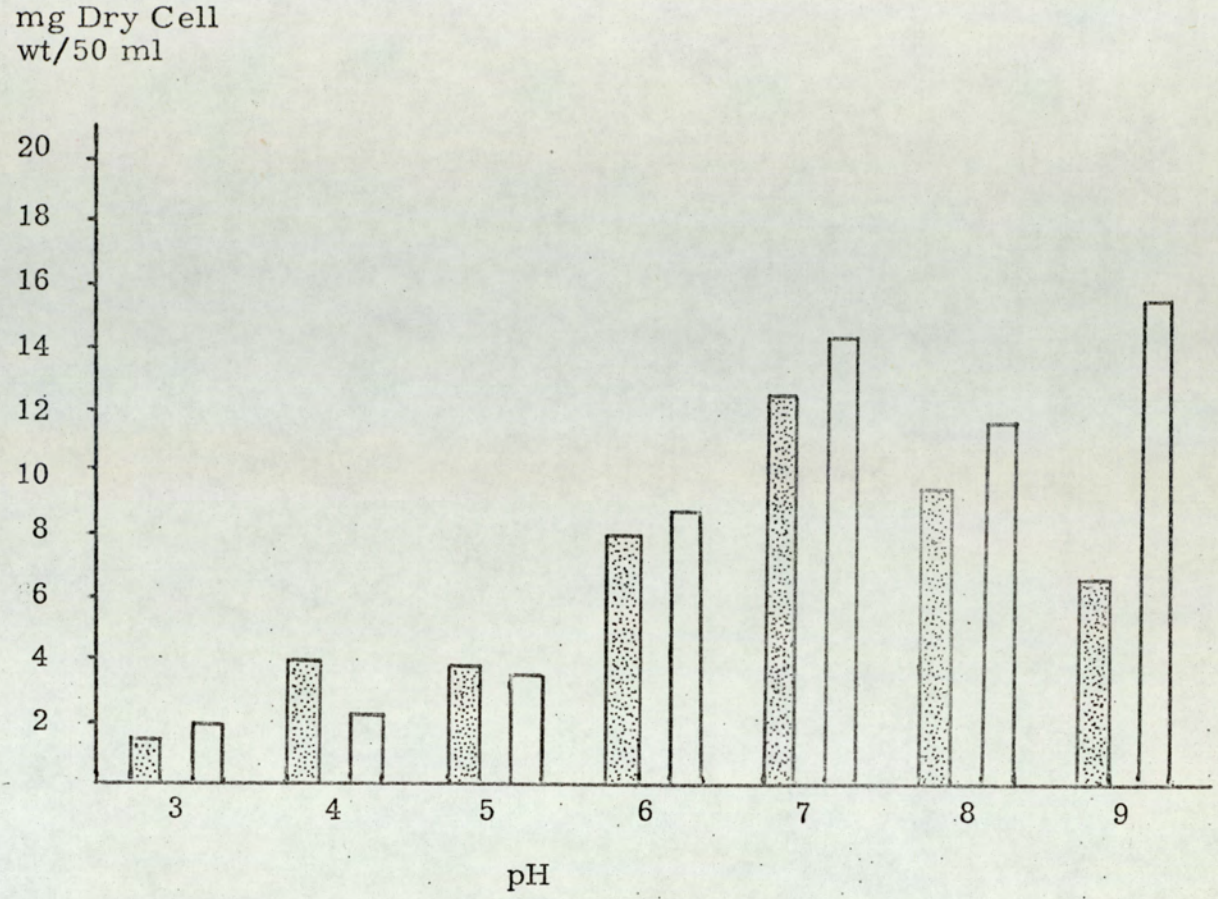


FIGURE 27

A. fumigatus. Production of mycelial biomass at various pH levels. Incubation temperature 40°C.

Spore inocula

Mycelial inocula

was recorded in any of the control flasks of nutrient salts alone.

Summary of experiments on Polymers

The results of the experiments presented here would tend to confirm the fact that synthetic polymers such as polyethylene and P.V.C. are resistant to thermophilic as well as mesophilic microbial degradation. It seemed likely, therefore, that a new approach to the problems posed by the recalcitrance of these materials was needed, and this was partially achieved by the use of an initial physico/chemical attack on the high molecular weight molecule of polyethylene, with subsequent microbiological attack by thermophilic fungi on the low molecular weight, oxygen containing products.

If experiments such as these could be carried further than was possible here, it might be possible to completely degrade all existing plastics' waste ending up in town refuse, so that, once again, the carbon locked up within plastics could be re-introduced into the biosphere.

Footnote

The oxidation products of polyethylene have been assayed by Dr. B. S. Brown and Mr. J. Hulse at the University of Manchester, Dept. of Medical Biochemistry. The acids were converted to their methyl esters and assayed in a Perkin-Elmer F11 gas liquid chromatogram using diethylene glycol succinate on Chromosorb W/HMDS as column material, at an isothermal temperature of 150°C. The acids discovered in the oxidised polyethylene when compared to authentic standards were oxalic, malonic, succinic, glutaric, adipic, pimelic, suberic and azeleic acids.

Section Two

The biodegradation of plasticisers by thermophilic fungi

We have already seen that polymers such as polyethylene and P.V.C. are resistant to thermophilic fungi but we do not know whether plasticisers and, in particular, plasticisers used in plasticised P.V.C. are resistant to thermophilic attack. In this section we will examine the possible biodegradation of these compounds by thermophilic fungi, following the plan of work outlined in the introduction to this chapter.

Materials and Methods

(1) Manometric method

The manometric technique of Cavett and Woodrow (1968) was used to measure the oxygen uptake of a soil thermophilic population enriched with various plasticisers, in order to determine whether such plasticisers were susceptible to thermophilic microbial degradation. The soil used in these experiments was Clent soil, which had been previously incubated at 48^{o}C for 5 days after adjusting the moisture content of the soil to 25 - 30% with E & P nutrient salts solution, to encourage the development of the thermophilic population. The apparatus used was the "Circular Warburg Apparatus, Model V166" supplied by Shandon Ltd., and single sidearm flasks were used throughout in duplicate. The centre well of each flask contained a standard size filter paper wick and 0.5 ml of 20% KOH solution to absorb CO_2. The total volume of liquid in each flask was 3.5 ml (see later), and thermobarometers were set up simultaneously

for each plasticiser tested. These consisted of flasks containing 2.75 ml distilled water plus 0.25 ml plasticiser, with 0.5 ml KOH in the centre well. It was felt that the use of a "sterile soil solution" in the thermobarometers would not represent a true control for the reasons already outlined concerning "sterile soil".

Six flasks containing 2.5 ml of 30% (w/v) soil solution and 0.5 ml distilled water were also run to measure the endogenous respiration of the soil thermophilic population; in this case using 3 ml of distilled water in the thermobarometers. The water bath of the Warburg was set at 48°C and the flasks were shaken at 75 strokes per minute with the taps of the flasks open to equilibriate the contents. After 30 minutes the taps were closed and readings taken periodically, resetting the manometers when necessary. Manometer constants were calculated according to the formula below and mean results for the duplicate flasks were calculated according to the method of Morris (1968).

$$K = \frac{\frac{273}{T} + Vf \times \alpha}{Po}$$

Where K = Manometer constant

Vg = Volume of available gas space in µl.

T = Experimental temperature in degrees Absolute

Vf = Volume of fluid in manometer flask in µl

α = Absorption coefficient of oxygen in water at experimental temperature.

Po = Atmospheric pressure in m.m. of manometer fluid

(In this case T = 48°C = 321°A, α = 0.0219, Po = 10,000 m.m.)

Two initial experiments were carried out to determine (a) the amount of plasticiser needed in each flask and (b) the time span of the experiment.

In the first experiment using various concentrations of plasticise it was found that 0.25 ml of plasticiser per flask indeed represented an "excess of substrate giving zero order kinetics" (Cavett and Woodrow 1968), and this concentration (8.35%) was in fact close to the concentration of plasticiser used by these authors (10%). In the second experiment it was found that there appeared to be a lag time of about 6 hours before the plasticisers started to be respired - or in fact just passively tolerated, since the curves obtained appeared to follow the curve of the endogenous soil controls (Figure 28) over the first 9 hours or so.

On the basis of these two initial sets of experiments it was decided that all future work would be run with a plasticiser concentration of 0.25 ml (making up the 3.0 ml reacting volume of each flask with 0.5 ml distilled water), and using a time scale of 72 hours to ensure that plasticiser respiration would be detected after endogenous respiration had levelled off. In all cases, the plasticiser was used as supplied and not previously sterilised.

Results

The results for the oxygen uptake of a microbial thermophilic soil population enriched with twelve plasticisers are shown in Figure 29. From these results it can be seen that of the twelve plasticisers tested, three depressed the microbial respiration below its normal endogenous

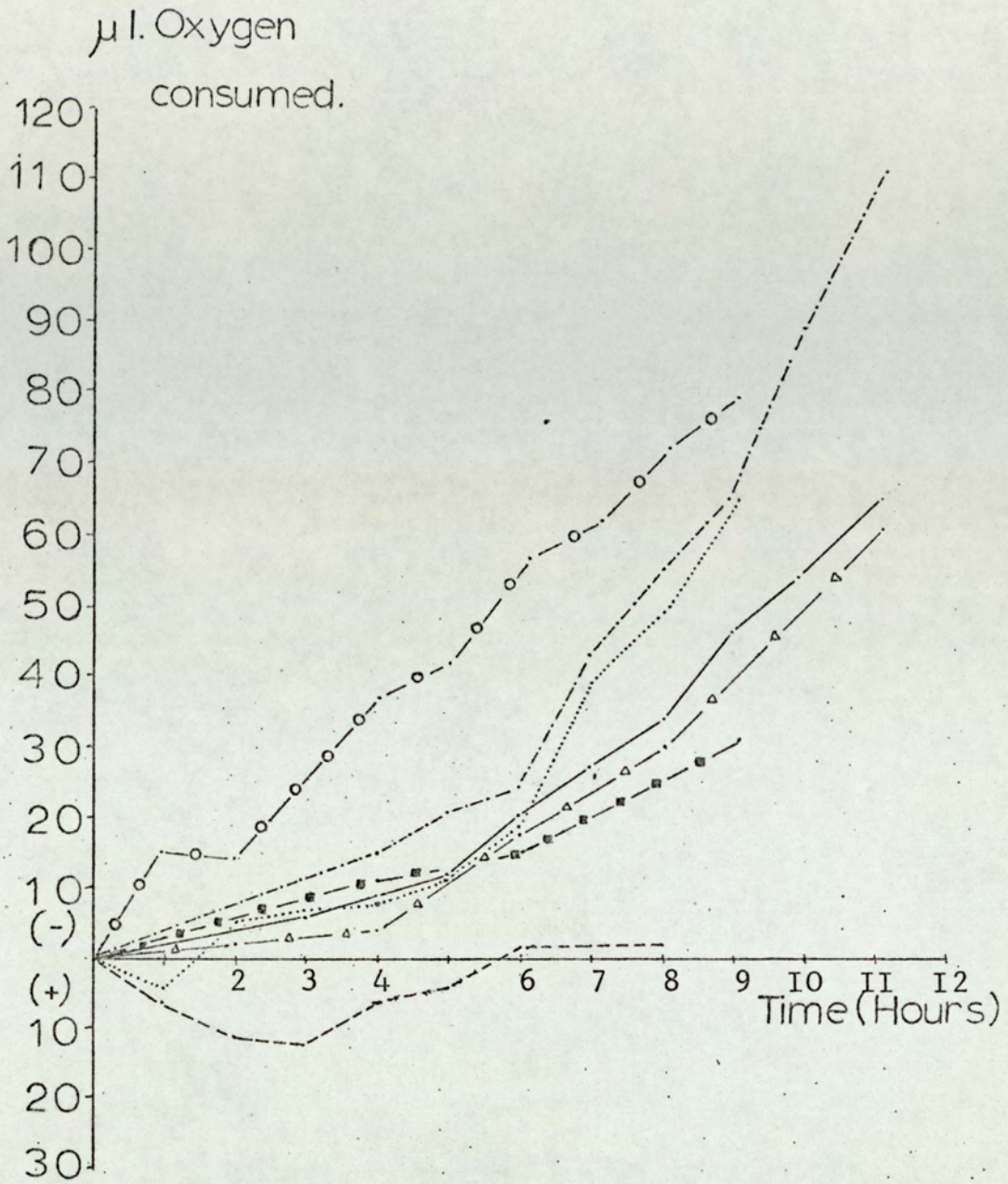


Figure 28

Legend :

Endogenous soil respiration.

Di-octyl sebacate.

Benzyl benzoate.

Di-octyl phthalate.

Tri-ethyl ortho phosphate.

Di n-butyl tartrate.

Glycerol triacetate.

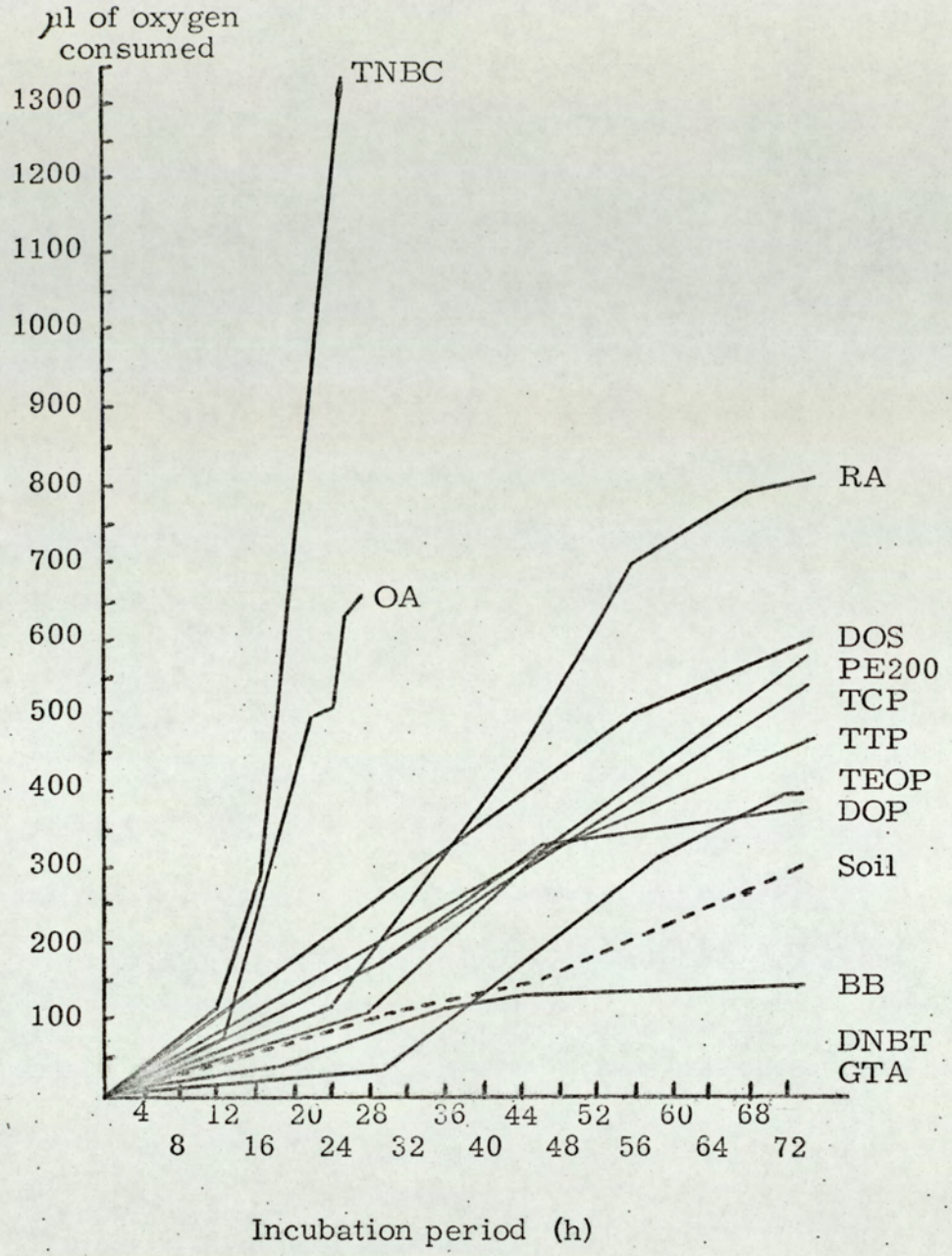


FIGURE 30

The uptake of oxygen at 48°C by the thermophilic microbial population of a pastureland soil enriched with various plasticisers

levels, and two of these, di n-butyl tartrate (DNBT) and glyceryl-triacetate (GTA), inhibited soil respiration altogether. Benzyl benzoate (BB) inhibited soil respiration by 50% (see Table 2a). The remaining nine plasticisers /or plasticiser breakdown products in the case of oleic acid (OA) and ricinoleic acid (RA)/ enhanced the respiration of the soil population. Tri n-butyl citrate (TNBC) which has been reported to support no fungal growth (Brown 1945) produced a 1390% increase in the rate of soil respiration within 24 hours, whilst di octyl sebacate (DOS), a plasticiser known to be degradable and much used in testing work, increased respiration by 100% after 72 hours. Polyethylene glycol 200 (PE 200), tri cresyl phosphate (TCP), tritolyl phosphate (TTP), tri ethyl orthophosphate (TEOP) and di octyl phthalate (DOP) have all ~~be~~ been reported to be resistant to (mesophilic) fungal growth, but all enhanced the respiration of the soil thermophilic population. Oleic and ricinoleic acids were also actively metabolised and it might be postulated that plasticisers containing these acids would be actively degraded by thermophilic microorganisms.

Discussion

From these results it can be seen that two "degradable" plasticisers (BB, GTA) and one "resistant" (Table 1b) plasticiser (DNBT) were able to depress the levels of respiration of soil microorganisms at thermophilic temperatures; whilst the other nine, made up of five "resistant" (TNBC, PE200, TCP, TEOP, DOP), three "degradable" (DOS, OA, RA) and one "unknown" (TTP) plasticiser were all able to enhance levels of respiration above endogenous levels. To find out whether the thermophilic fungi were actively involved in the breakdown of these plasticisers, the compounds

TABLE 23

Comparative cumulative oxygen uptakes by soil microorganisms in a pastureland soil enriched with various plasticisers

Plasticiser	Cumulative oxygen uptake at 72 hours (μ litres)	% Inhibition (-) or Enhancement (+) of soil respiration due to plasticiser
Tri n-butyl citrate	1338.0 *	+ 1390 *
Di octyl sebacate	586.4	+ 100
Polyethylene glycol 200	561.7	+ 91.5
Tri cresyl phosphate	539.9	+ 84.5
Tri tolyl phosphate	464.7	+ 59.0
Tri ethyl orthophosphate	396.5	+ 35.0
Di octyl phthalate	377.9	+ 28.5
Soil Endogenous Respiration	292.5	Control
Benzyl benzoate	134.2	- 54.3
Di n-butyl tartrate	0	(Completely
Glycerol triacetate	0	(inhibit respiration
Products of Plasticiser Breakdown		
Oleic acid	648.8 *	+ 620 *
Ricinoleic acid	805.0	+ 170

* After 24 hours

were then introduced into soil in an enrichment procedure.

(2) Soil enrichment tests

Samples of Clent soil (3g) were placed in petri dishes and moistened to a 25 to 30% level with distilled water. The soil samples were then enriched with the same plasticisers by spraying 1.5 ml of each plasticiser over the surface of the soil. The plates were incubated for 12 days at 48°C with plates containing Clent soil alone, as controls. After incubation, samples of soil were plated out onto E & P cellulose and glucose agars using the method of Warcup (1952) and the plates were incubated for 7 days when developing thermophiles were recorded.

Results

Results are presented in Table 23 and it can be seen that this technique selected out organisms which were able to tolerate the presence of the plasticiser or were able to utilise it as a carbon source. Several fungi, and in particular Thermoascus and T. emersonii were not isolated from control plates but appeared on several plates containing individual plasticisers. Other plasticisers such as RA, OA, DNBT, BB, TEOP and PE200 rendered the enriched soil practically sterile, and these results seem to contradict the results obtained for these plasticisers (with the exception of DNBT) in the respirometric experiment. However, the amount of plasticiser used might have had the effect of depressing the soil organisms, since it had been previously observed that amounts of plasticiser greater than 0.25 ml in the Warburg experiment, also depressed the level of soil respiration. If this was the case, then the other fungi isolated in this particular

TABLE 23

Isolation of thermophiles from plasticiser-enriched soil. *Dominant species.

	E & P Glucose Agar	E & P Cellulose Agar
Benzyl Benzoate	NO GROWTH	NO GROWTH
Tri cresyl phosphate	A. fumigatus H. lanuginosa * Actinomycetes M. pulchella	A fumigatus H. lanuginosa * Actinomycetes
Di n-Butyl Tartrate	NO GROWTH	NO GROWTH
Ricinoleic Acid	NO GROWTH	NO GROWTH
Di octyl Sebacate	T. aurantiacus * T. emersonii M. pusillus Actinomycetes	T. aurantiacus * T. emersonii * M. pusillus Actinomycetes
Glycerol Triacetate	NO GROWTH	NO GROWTH
Tri n-Butyl Citrate	T. aurantiacus *	Actinomycetes T. duponti
Tri tolyl phosphate	H. lanuginosa * T. aurantiacus Actinomycetes C. thermophile A. fumigatus	H. lanuginosa * T. aurantiacus Actinomycetes M. pulchella
Tri ethyl ortho phosphate	NO GROWTH	NO GROWTH
Di-octyl phthalate	C. thermophile * A. fumigatus Actinomycetes M. pulchella	T. aurantiacus * Actinomycetes
Oleic Acid	NO GROWTH	NO GROWTH
Polyethylene glycol 200	NO GROWTH	NO GROWTH
CONTROLS (Clent soil incubated 12 days at 48°C before plating out)	A. fumigatus * H. lanuginosa * Actinomycetes Cephalosporium sp	C. thermophile * H. lanuginosa * Actinomycetes Torula thermophile Sporotrichum thermophile * H. grisea

experiment must have been able to, at least, tolerate the presence of large amounts of the other plasticisers.

It appears then, that several of the thermophilic fungi are able to either grow upon, or tolerate some of the plasticisers used in these experiments. These, and the other thermophilic fungi, were then grown in isolation on plasticisers in agar media.

(3) Plasticiser-Agars

Plasticiser agars were made by aseptically emulsifying sterile plasticiser (after autoclaving at 110°C for 10 minutes) at a 1% level in E & P mineral salts agar, and in E & P nutrient salts agar containing 0.5g/l L-asparagine and 1g/l yeast extract. The particular plasticiser was added to the molten agar when the agar had cooled to 45°C, and the bottle containing the agar was then vigorously shaken to emulsify the plasticiser before pouring. The same twelve plasticisers were used with the inclusion of di n-butyl sebacate (DNBS), and duplicate plates were inoculated with 5m.m. discs of each fungus before incubation at 48°C for 14 days. Controls consisted of the fungi growing on nutrient and mineral salts agar alone i.e. no plasticiser. After incubation the plates were examined for sporulation characteristics of each fungus and for amounts of growth and possible clearing produced.

Results

Results are indicated in Table 24 and are signified according to the scheme shown below:-

TABLE 24

Showing growth of individual thermophilic fungi on agars enriched with 1% plasticiser. Upper figures in each column refer to growth on enriched mineral salts agar, and lower figures in each column refer to growth on enriched agar containing the additional growth factors Yeast Extract and L.-Asparagine.

KEY

PLASTICISER		TEST ORGANISM	
1.	Oleic acid	a	Aspergillus fumigatus
2	Di n-butyl tartrate	b	Cephalosporium sp
3	Benzyl benzoate	c	Chaetomium thermophile var. coprophile
4	Polyethylene glycol 200	d	Chaetomium thermophile var. dissitum
5	Tri n-butyl citrate	e	Humicola grisea var. thermoidea
6	Ricinoleic acid	f	Humicola insolens
7	Glyceryl triacetate	g	Humicola lanuginosa
8	Tri ethyl orthophosphate	h	Humicola stellata
9	Tri cresyl phosphate	i	Malbranchea pulchella var. sulphurea
10	Di octyl sebacate	j	Mucor miehei
11	Tri tolyl phosphate	k	Mucor pusillus
12	Di octyl phthalate	l	Myriococcum albomyces
13	Di n-butyl sebacate	m	Talaromyces duponti
14	Controls	n	Sporotrichum thermophile
	**********	o	Stilbella thermophila
+	light growth around inoculum	p	Talaromyces emersonii
2+	light growth over half plate	q	Thermoascus aurantiacus
3+	light growth over all plate	r	Thielavia thermophila
4+	heavy growth around inoculum	s	Torula thermophila
5+	heavy growth over half plate		
6+	heavy growth over all plate		
-	no growth		
CZ	clearing zone produced		
S	asexual sporulation		
C	cleistothecial production		
P	perithecial production		
NT	not tested		

TABLE 24

Pl / T	1	2	3	4	5	6	7	8	9	10	11	12	13	14
a	4+ S +	- -	2+ S 4+	+ +	+ C +	5+ S 4+ S	4+ 5+S	+ C -	+ +	2+ S 2+ CZS	- -	+ 2+ S	2+ S 6+ S	- +
b	+ -	- -	+ S -	+ S -	- -	+ +	- -	- -	- -	2+ 2+ S	- -	- -	5+ S 6+ S	- -
c	- 4+ P	- -	- -	- -	- -	- 4+ P	- 6+ P	- -	- 2+ P	2+ 2+ P	- 2+ P	- 3+ P	2+ P 6+ P	- 3+ P
d	- -	- -	- -	+ 2+ P	- -	+ 4+ P	- 6+ P	- -	- 3+ P	2+ 2+ P	- -	- -	+ P 3+ P	- 3+ P
e	- +	- -	- -	- 3+ S	- -	- 4+ S	- 6+ S	- -	- 3+ S	- 2+CZS	- 3+ S	- 2+ S	- 2+ S	- 3+ S
f	- -	- -	- -	- -	- -	+ -	- +	- -	- -	- 2+	- -	- -	+ +	- -
g	2+ +	- -	+ S 4+	- -	- -	5+ -	4+ 2+	+ -	- 3+	2+ +CZ	- -	- -	+ 4+ S	2+ S -
h	- +	- -	- + CZ	+ S + CZ	+ + SCZ	+ +	- +	+ +CZ	+ S NT	+ S NT	+ S NT	+ S NT	NT + CZ	4+ S + S
i	3+ 5+	- -	2+ 5+	+ +	+ CZ 2+	+ 5+	- 5+	- -	+ +	2+ CZ 2+ CZ	+ +	- -	+ S 5+ S	+ 2+ S
j	2+ +	- -	- + S	+ -	- -	2+ 5+ S	- 5+	- -	- +	+ 3+ CZ	- -	- +	3+ S 6+ S	+ 3+
k	2+ 5+	- -	- -	- + S	- -	3+ 5+ S	- 6+ S	- -	- +	4+ S 3+ CZ	- -	+ S -	3+ S 6+ S	3+ 3+
l	- -	- -	- 5+	- -	+ CZ -	+ -	- -	- -	- -	+ CZ -	- -	- -	- 5+	- -
m	- -	- -	- +	+ -	- -	4+ C 5+ S	4+ C 2+	- -	- -	2+ 3+	- -	- -	+ S 5+ S	- -
n	4+ 4+ S	- -	2+ +	+ +	+ CZ -	4+ 4+	4+ 6+	- -	- +	+ CZ 2+ CZ	- +	- +	- 5+	- -
o	+ -	+ -	- -	+ S -	- -	+ -	- -	- -	- -	+ CZ -	- -	- -	- 4+	- -
p	3+ +	- -	4+ +	+ S -	+ CZ -	6+ S 5+ S	6+ SC 6+	2+ -	- -	3+ S +	- -	- -	6+ SC 6+ SC	2+ S -
q	- -	- -	3+ C 6+ C	- -	- -	5+ C 6+ C	6+ C 6+ C	- -	- 3+ C	3+ C + CZ	- -	- -	6+ C 6+ C	4+ S -
r	- -	- -	- -	- -	- -	+ +	- 5+ S	- -	- -	+ CZ -	- -	- -	- +	- -
s	- +	- -	- -	- 3+ S	+ -	- + S	- 5+ S	- -	- -	+ CZS -	- -	+ S 2+	+ 2+	- 3+

Pl - Plasticiser T. - Test organism

The upper results in each column box refer to growth of each fungus on mineral salts-plasticiser agar, and the lower results refer to growth on nutrient salts-plasticiser agar.

When these results are studied, it appears that, in general, better growth and sporulation of individual fungi was obtained when the plasticiser enriched agar was also supplemented with L-asparagine and yeast extract. It would appear therefore that the observations of Klausmeier and Jones (1961) on the effects of additional microbial nutrients also hold true for the thermophilic fungi; and that some plasticisers which would support no fungal growth at all when incorporated into mineral salts agar, did support growth when supplemented with these additional essential nutrients. These results have been summarised in Table 25, and it can be seen that all the plasticisers tested were able to support the growth of at least some of the thermophilic fungi. The most "fungicidal" plasticiser was DNBT which supported the growth of one fungus, Stilbella thermophila, alone. Those supporting the growth of nearly all the thermophiles in mineral salts agar were DOS, DNBS and RA: with the same three plasticisers plus GTA supporting most growth in nutrient salts agar. Plasticiser-agar which benefitted from addition of yeast extract and L-asparagine was that containing TCP, TTP, DOP, BB, GTA, DNBS and OA.

A large number of the test fungi were able to grow and sporulate normally, and in some cases, to actually clear the globules of plasticiser. A. fumigatus was able to grow on seven plasticisers, and to clear DOS. It produced cleistothecia on TNBC and TEOP and this is a very rare sight indeed for this fungus. In this case the mycelium was white in colour and normal conidia were not produced. Cephalosporium sp produced feeble

TABLE 26

Showing numbers of thermophilic fungi (out of 19) able to grow on plasticiser-enriched agar in the absence and presence of additional essential nutrients.

Plasticiser	Mineral Salts Agar	Nutrient Salts Agar
TNBC	6	3
DOS	17	14
PE 200	10	8
TCP	3	10
TTP	2	4
TEOP	4	1
DOP	4	6
BB	6	10
DNBT	1	0
GTA	6	16
DNBS	13	19
OA	9	11
RA	16	15

growth on six plasticisers, with the exception of DNBS. No growth was produced at all in the control plates. Growth patterns of the two varieties of Chaetomium followed each other reasonably closely except in four cases, when one variety grew and sporulated on a particular plasticiser and the other did not. Growth in the nutrient salts controls was good, however, for both varieties, and it appears therefore that the fungi were utilising the yeast extract and L-asparagine rather than the plasticisers in the plates containing PE200, TCP, TTP, DOS and DOP. Variety coprophile could therefore utilise four plasticisers (OA, RA, GTA and DNBS), whilst variety dissitum was able to grow on two plasticisers (RA, GTA). H. grisea was unable to utilise any plasticiser when the plasticiser represented the sole carbon source. Increases above control values were seen on RA and GTA in the presence of extra nutrients and a clear zone was observed on DOS. In this case growth was less than that observed in control plates, however, dissolution of the DOS globules indicated that H. grisea was actively utilising this plasticiser. Very scanty growth was produced by H. insolens on RA, GTA, DOS and DNBS; and no growth at all was observed in the control plates.

Good growth was produced by H. lanuginosa on RA and GTA in mineral salts agar, with heavy submerged growth on RA. On nutrient salts media no growth of this thermophile was observed in the control plates, but growth was given on OA, BB, GTA, TCIP, DOS and DNBS, with clearing zones being produced on the plates of DOS.

The only thermophile which had proven extremely difficult to maintain during the period of this research was H. stellata, andyet this fungus grew on and cleared six plasticisers (BB, PE200, TNBC, RA, TEOP, DNBS), producing clearing zones 4 to 5 mm from the edge of the colony. Growth of

H. stellata was good in the control plates, and here again we have the case of a fungus whose growth was cut down by a particular substrate to below control values, when the fungus was actively metabolising the compound.

M. pulchella produced clearing of TNBC and DOS and, in general, better growth was produced in the presence of additional yeast extract and L-asparagine. The fungus grew well on five plasticisers (OA, BB, RA, GTA DNBS), but sporulation was only observed on DNBS. A yellow staining of the plasticiser globules was observed on mineral salts agar plus DOP and TCP, whilst a yellow pigment was given on nutrient salts plus TNBC, TCP and DOP. In the presence of nutrient salts plus OA, Malbranchea produced a pink staining of the agar.

The two species of Mucor gave almost identical results on all plasticisers. Growth exceeding that in controls was given on OA (M. pusillus RA, GTA, DOS (M. pusillus) and DNBS, with clearing of DOS being given by both species.

M. albomyces gave no growth at all in the control plates but grew on five plasticisers with clearing zones produced on TNBC and DOS. Better growth, in general, was produced on nutrient salts/plasticiser agar.

Cleistothecia were produced by T. duponti on RA and GTA, and good growth was produced in the presence of these two plasticisers and DNBS.

Clearing zones were produced by Sporotrichum thermophile on TNBC and DOS, and good growth was observed on OA, BB, RA, GTA, DOS

and DNBS. In all cases where growth occurred on nutrient salts/ plasticiser medium, a green pigment, which diffused down into the agar, was produced. Very little sporulation was noted, however, on any of the plasticisers.

Stilbella thermophila only produced reasonable growth on nutrient salts plus DNBS, whilst feeble growth was observed on mineral salts plus OA, DNBT, PE200, RA and DOS. On the plates of DOS, large (30 m.m. diameter) clearing zones were produced and, in this case, the esterase enzymes must have diffused quite a considerable distance from the growing mycelium.

Good growth was produced by T. emersonii on OA, BB, RA, GTA, DOS and DNBS, with clearing zones being produced on TNBC. Cleistothecia and conidia were produced on GTA and DNBS, whilst only conidia, and yellow staining of the globules, was observed on RA.

T. aurantiacus grew well on BB, RA, GTA and DNBS and clearing was observed on DOS.

In contrast, Thielavia thermophila was able to grow on very few plasticisers, but definite clearing zones were observed on plates of DOS when dissolution of several large plasticiser globules was clearly visible. Some growth was produced on RA and DNBS, and heavy growth was observed on GTA in the presence of nutrient salts.

Finally, Torula thermophila produced definite clearing zones only on DOS, although feeble growth was given on TNBC, DOP and DNBS in mineral salts agar. Good growth was produced in the control plates of

nutrient salts and better growth was produced only on GTA. In the presence of DOP the spores of Torula were produced on a totally submerged mycelium.

If these results are presented in the form of a table (Table 26) showing the number of plasticisers able to support growth of individual fungi over and above control levels, or to induce the production of clearing, then it can be seen that the fungi which were able to grow on the greatest number of plasticisers were A. fumigatus and Sporotrichum thermophile. Aspergillus grew better on mineral salts/plasticiser agar whilst Sporotrichum produced better growth on nutrient salts/plasticiser agar. Six fungi viz: Ce phalosporium sp., H. lanuginosa, H. stellata, M. pulchella, T. emersonii and T. aurantiacus were able to grow on approximately half the total number of plasticisers tested, with T. emersonii alone producing equal growth in the absence and presence of additional nutrients.

Discussion

The results presented here show that growth of thermophilic fungi can be supported by plasticisers incorporated into an agar enrichment technique. Of the thirteen plasticisers tested, six have been previously reported to be unable to support (mesophilic) fungal growth (TNBC, PE200 TCP, TEOP, DOP and DNBT) but in fact did support the growth of some of the thermophiles, whilst six others known to be able to support mesophilic fungal growth also supported growth of the thermophiles (DOS, BB, GTA, DNBS, OA, RA). No information was available on the remaining plasticiser TTP, however, from these results it would appear that it can support the growth of at least four of the thermophilic fungi.

TABLE 26

Showing numbers of plasticisers able to support growth of individual thermophilic fungi.

FUNGI	MINERAL SALTS + PLASTICISER	NUTRIENT SALTS + PLASTICISER
A. fumigatus	11	6
Cephalosporium sp.	6	3
C. thermophile var. coprophile	2	4
C. thermophile var. dissitum	4	2
H. grisea	0	2
H. insolens	2	3
H. lanuginosa	2	6
H. stellata	0	6
M. pulchella	4	6
M. miehei	3	4
M. pusillus	1	4
M. albomyces	3	2
T. duponti	5	5
Sporotrichum thermophile	6	10
Stilbella thermophila	5	1
T. emersonii	7	6
T. aurantiacus	3	6
Thielavia thermophila	2	3
Torula thermophila	4	1

Since clearing was produced by some fungi growing on the plasticiser/agars, it would appear that they might be able to elaborate enzymes capable of hydrolysing several of the ester-type plasticisers. Williams et al (1968) measured acid production from the enzymatic hydrolysis of ester plasticisers using mycelial homogenates of fungi. Accordingly, their method was used to evaluate the nineteen thermophilic fungi for esterase production.

(4) Esterase production by thermophilic fungi

Materials and Methods

The culture medium used for esterase induction was E & P mineral salts containing 0.05% L-asparagine and 1% yeast extract. 49 ml of medium was used per flask and the flasks were sterilised by autoclaving at 120°C for 10 minutes. Upon cooling, the nutrient salts were supplemented with 2% (i.e. 1ml) of di n-butyl sebacate (DNBS) sterilised by millipore filtration. Each flask was then inoculated with 0.5 cm discs of mycelium taken from actively growing cultures of the thermophilic fungi on E & P cellulose or YpSs agars. The flasks were incubated at 48°C on a shaking incubator (150 r.p.m.) for 7 days before harvesting the mycelium of each fungus by filtration through Whatman No. 1 filter papers. Williams et al. (1968) found that optimal esterase activity for A. glaucus esterase was given at pH 7.0 to pH 8.0 and so, after washing with sterile distilled water, the mycelia were suspended in 60 ml of phosphate buffer at pH 7.0 and homogenised in a sterile high speed homogeniser at 0°C. The homogenised mycelium was subsequently stored at 4°C until the time of testing.

Esterase assays were carried out by titrating the acid liberated from DNBS by the homogenised mycelium after 21 hours incubation at 48°C. The assay medium was prepared by emulsifying 0.25 ml of DNBS and 0.125 g of gum acacia in 15.75 ml of sterile distilled water. The emulsion (16 ml) was then incubated with 8 ml of the homogenised mycelium of each thermophilic fungus in buffer at pH 7.0. Controls consisted of emulsified plasticiser plus 8 ml of phosphate buffer at pH 7.0. After incubation the liberated acid was titrated with 0.05N NaOH and the titratable acidity reported as the number of millilitres (corrected for control values) of 0.05N NaOH necessary to readjust the pH of the incubated assay to pH 8.0 (Williams et al. 1968).

Results

After 7 days of initial incubation in DNBS induction medium, growth of most of the thermophiles was very good indeed, with (visually estimated) production of several hundred milligrams of mycelium for most of the fungi. No growth at all was observed in the flasks containing Sporotrichum thermophile, Stilbella thermophila and H. Stellata and repeating the experiment gave similar results. These particular results are surprising since all three fungi were able to produce growth and clearing on ester plasticisers in agar. When the mycelial homogenates were tested for esterase production it was found that 16 out of the 19 fungi were able to produce a titratable acidity over and above control values (Table 28). In some cases e.g. Cephalosporium sp., H. insolens, M. miehei M. albomyces, T. aurantiacus, and Thielavia thermophila this effect was quite small, but in others e.g. Torula thermophila, H. grisea, A. fumigatus, M. pusillus and T. emersonii the observed titratable acidity was large

TABLE 28

Showing corrected titratable acidity (ml of 0.05N NaOH) necessary to bring the pH of the assay medium to pH 8.0

N. G. - No growth of fungus in DNBS induction medium.

Fungus	Corrected Titratable Acidity
A. fumigatus	6.85 ml
Cephalosporium sp.	1.05
C. thermophile var. coprophile	3.45
C. thermophile var. dissitum	1.95
H. grisea	7.65
H. insolens	0.40
H. lanuginosa	1.65
H. stellata	N. G.
M. pulchella	2.45
M. miehei	0.65
M. pusillus	7.40
M. albomyces	1.10
T. duponti	2.25
Sporotrichum thermophile	N. G.
Stilbella thermophila	N. G.
T. emersonii	4.90
T. aurantiacus	1.00
Thielavia thermophila	0.60
Torula thermophila	6.75

and these results compare quite favourably with the data of Williams et al. (1968).

When these results are correlated with the previous sets of results it can be seen that A. fumigatus and T. emersonii were able to grow upon a high proportion of the tested plasticisers and were both able to produce considerable hydrolysis of the ester plasticiser di n-butyl sebacate. On the other hand, Torula thermophile, H. grisea and M. pusillus, although producing good hydrolysis of DNBS, were able to produce growth on very few of the plasticisers when emulsified in agar. All three fungi did produce clearing zones on DOS, showing that they do in fact actively produce esterase enzymes, but mycelial production on this plasticiser was very light. Of the remaining fungi, Sporotrichum thermophile was able to produce clearing zones on TNBC and DOS and good growth on at least six plasticisers. This fungus, however, was unable to produce any growth in the shake flask cultures with DNBS, although very good growth was produced on this plasticiser when emulsified in agar. This result, and similar results by H. stellata and Stilbella thermophila, tend to indicate that growth and esterase production might have been better in liquid culture on an ester plasticiser other than DNBS. These latter two fungi produced clearing zones on several plasticisers, again demonstrating the existence and production of esterase enzymes. The two varieties of Chaetomium thermophile with Cephalosporium sp., Talaromyces duponti and H. insolens were unable to produce clearing zones on any of the emulsified plasticisers, but all produced growth and sporulation on some plasticisers, and all were able to bring about hydrolysis of DNBS, showing that they are all able to produce esterase enzymes.

It appears from these results that all 19 thermophilic fungi are able to elaborate esterase enzymes at 48°C and grow upon and utilise a range of ester plasticisers and two plasticiser breakdown products, oleic and ricinoleic acids.

Discussion

In this chapter we have seen that several synthetic polymers, which were known to be resistant to mesophilic biodegradation, were also resistant to thermophilic fungal degradation. Visual evaluation of the polyethylene strips showed that several thermophilic fungi could indeed produce growth and sporulation on this polymer, but physical test methods eliminated the possibility of biodegradation. Studies on the P.V.C. and P.V.A. polymers again showed that P.V.C. was resistant to thermophilic attack, but that the physical properties of P.V.A. were greatly modified, especially in the thermophilic soil burial tests. These results correlate well with information currently available on the mesophilic biodegradation of these three polymers.

Since thermophilic fungi were unable to attack the polyethylene molecule, it appeared that a new approach was needed in order to bring about the degradation of this particular synthetic polymer which is largely responsible for the increases in plastic materials within town waste. This was partially achieved by pretreating the existing polymer with oxidising agents, especially nitric acid, to form low molecular weight dicarboxylic acids which could then be used as a source of carbon by thermophilic fungi. One advantage of this method of treatment is that all of the carbon locked up within the polymer molecule can be ultimately utilised, or in fact

recycled. As a commercial process, however, it would probably be expensive since separation and sorting of plastics from town waste would be involved. As we have seen however, valuable dicarboxylic acids can be produced which could be separated off, purified and sold, or simply sprayed back into the composting system to be further degraded. In this way total utilisation of the synthetic polymer would be achieved, in contrast to photodegradable plastics where a high molecular weight powder is formed from a polymer which happens to be exposed to the U/V fraction of sunlight. It appears that the only real application of these photodegradable polymers will be when the plastic wrapping or container becomes litter and not refuse.

In the second section of this chapter several standard techniques were applied at thermophilic temperatures to establish whether the thermophilic fungi were able to elaborate esterase enzymes in order to grow upon and utilise several synthetic plasticisers. It was found that all of the thermophiles were able to either produce clearing zones in plasticised agar or produce hydrolysis of an ester plasticiser in an enzymatic technique, thereby demonstrating the existence of esterase enzymes in all 19 of the thermophilic fungi under test. Six of the plasticisers under test: DNBT, TNBC, PE200, TCP, TEOP and DOP, are all mentioned in the literature as being non-biodegradable plasticisers under mesophilic conditions. These six plasticisers however, and one "unknown" plasticiser, TTP, all proved to support the growth of various species of thermophilic fungi at 48^{o}C. DNBT proved to be the most resistant plasticiser, allowing only the growth of Stilbella thermophila. In general the cellulolytic thermophiles, with the exception of A. fumigatus, Sporotrichum thermophile and Malbranchea pulchella, did not produce as much growth on the plasticisers as the non cellulolytic fungi. Of this latter group Talaromyces emersonii

proved to be most adaptive and produced excellent growth and sporulation on six of the plasticisers. Similar results were achieved using Thermoascus aurantiacus. Plasticisers which supported the growth of the majority of the thermophilic fungi were DOS and DNBS, both esters of sebacic acid, and the breakdown product RA which supported excellent growth of at least 16 thermophiles.

It would appear therefore that plasticisers known to be resistant to mesophilic microbial attack might become susceptible to thermophilic microbial attack in plastics exposed to thermophilic temperatures. This group of organisms must not be overlooked by plastics' manufacturers therefore in the testing of their plasticised plastics for biodeterioration. Respirometric and soil burial experiments have shown that several plasticisers are able to enhance the respiration and support the growth of thermophilic microorganisms, but it is probably unlikely that any large scale destruction of plasticised plastics would be brought about in the relatively short period of time the plastics are present within composting town waste. When one talks to people concerned with town waste composting about plastics the same answer is given time and time again, that plastics go through the whole composting process completely unchanged. This is not an unreasonable observation since the majority of plastics, and especially plasticised plastics, contain certain quantities of biocide to prevent biodeterioration. The content of plastics in town waste compost is certainly a disadvantage in the marketability of a saleable product but, after all, it is a disadvantage which can easily be rectified. It should be far easier to extract plastics waste from town waste after the process of composting is completed, and so composting could still produce a soil conditioner relatively free of these synthetic materials. The humus present

within such a compost could be supplemented with inorganic nitrogen, phosphorus and potassium to produce a highly effective soil conditioner/ fertiliser. Concern is growing in this country, and elsewhere in the world, that the addition of inorganic fertilisers to the soil is gradually causing the erosion of the soil structure, since the soil's organic content is being gradually depleted. It would seem a sensible proposition therefore to implement some of the proposals outlined above, so that in the long term we may benefit from such measures.

We know very little of the part played by the thermophilic fungi in the production of humus substances. The experiment carried out in Chapter Two produced a compost with a C/N ratio of about 19 and an "earthy" smell; in fact quite a reasonable compost. Are the thermophilic fungi actively involved in producing humus substances and thereby playing another active role in the formation of a soil conditioning product? This problem will be investigated in the next chapter.

CHAPTER 8

CHAPTER 8

The production of humus substances by thermophilic fungi

Introduction

The end product of the composting of organic matter is humus. Wallerius (1761) first defined humus in terms of decomposed organic matter, although the term "humus" dates back to Roman times when it was used to describe the soil as a whole (Waksman 1936). Although the precise chemical nature of humus has not yet been described, it has certain specific properties which were summarised by Waksman (1936) as:-

(1) Humus possesses a dark brown to black colour.

(2) Humus is practically insoluble in water, although it dissolves to a large extent in alkali. Certain constituents of humus may also be soluble in acid and may be precipitated at their isoelectric points.

(3) The carbon content of humus is approximately 55% of the total.

(4) Humus contains 3 to 6% nitrogen.

(5) The proportions of carbon to nitrogen in humus are in the order of ten to one, although this ratio will vary with the nature and stage of decomposition of the humus.

(6) Humus is in a dynamic condition, since it is constantly being formed from animal and plant residues and is continuously being decomposed by microorganisms.

(7) Humus serves as a source of energy for micro-organisms and during its decomposition carbon is given off as a steady stream of CO_2.

(8) Humus is characterised by a high capacity of base exchange, of combining with inorganic soil constituents, of absorbing water and by many other properties which make it a highly valuable constituent of substrates which support plant and animal life.

These physical, chemical and biological properties of humus help to modify the colour, texture, aeration and moisture retaining capacities of the soil, whilst also providing a source of nutrients for microbial and plant growth.

Humus was first isolated from peat by extraction with alkaline solutions by Achard (1786); and Vauquelin (1797) extracted humus substances from the wood of fungally infected elm using a similar method. Both Achard (1786) and Sprengel (1826) acidified their alkaline extracts of humus and noted the precipitation of a dark amorphous substance, "humic acid". Sprengel established the acidic nature of "humic acid", which, when added to alkaline soils combines with bases, rendering this type of soil neutral and increasing its fertility (Kononova 1966). Much confusion exists in the

literature concerning the term "humic acid", however a generally accepted definition is "that portion of humus which is soluble in aqueous alkali and precipitated by acidification of the alkaline extract" (Felbeck 1971) (Haworth 1971). This simple definition of "humic acid" will be accepted for the purposes of the experimental work to be carried out in this chapter, although its limitations are fully appreciated. Similar extraction methods have been used by a variety of workers (see Kononova 1966), and although objections have been raised by Tinsley and Salam (1961) and Dubach and Mehta (1963) that alkali extraction methods involve degradation of humus substances, later work by Schnitzer and Skinner (1968) showed that no significant changes to the organic matter occurred during extraction. An extraction method based on that used by Stevenson (1956) will be used in the experimental work to be presented here. The precise chemical nature of "humic acids" has not been defined, although many workers have shown that characteristic breakdown products from this fraction can be obtained (Murphy and Moore 1960; Steelink et al. 1960; Farmer and Morrison 1960; Visser 1962; Dubach and Mehta 1963; Kononova 1966; Haworth 1968, 1971; Swift et al. 1970; Felbech 1971). The nitrogenous fraction of "humic acid" was shown to be derived from proteins (Doyarenko 1901, Suzuki 1906 - 1908, Jodidi 1910 - 1913, Kelly 1914), although Burges (1960) believes that the presence of nitrogen is due to a secondary combination of "humic acid" with amino acids or protein. Sestini (1902) established the aromatic nature of the "humic acid" fraction; whilst Trusov (1916) thought that the aromatic compounds could be converted into humus substances by means of microbial enzymes. Whatever the true chemical nature of "humic acids" might be, (for arguments concerning this problem see Kononova 1966), these "compounds" are now widely accepted as being heteropolycondensates of phenolic substances, with or without the inclusion of amino acids (Burges

et al 1963), and may constitute 50 to 80% of the humus (Hurst and Burges 1967). The origin of the phenolic substances was postulated by Burges et al (1963) to be from three sources; phenolics leached from plant debris, compounds formed from lignin biotransformations and from substances synthesised by microorganisms utilising carbohydrates. The origin of "humic acids" by this latter pathway has been supported by Kononova and Aleksandrova (1958), Kang and Felbeck (1965), Haider and Martin (1967) and Martin and Haider (1968). Products actively formed by microorganisms with chemical properties similar to soil "humic acids" have been described by Kononova and Aleksandrova (1958), Kang and Felbeck (1965) and Martin et al. (1967); and Kononova and Aleksandrova (1958) showed that Aspergillus niger and Penicillium spp. could produce phenol oxidase enzymes and actively synthesise aromatic compounds from glucose as the sole carbon source. They showed that these compounds, together with autolysis products from the fungi did not differ basically from soil humic substances.

It would appear therefore that microorganisms are actively involved in the role of humus formation, and that in addition to synthesising several of the basic structural units of humus, they themselves are also able to contribute to the production of this fraction by their own autolytic products.

Humus substances are produced in soils and in composts by the activities of microorganisms which are involved in the decomposition of organic matter. These substances act as a "storehouse" of nutrients for microbial and plant growth as well as helping to improve the quality of the soil. Specific, beneficial effects of humus substances on the growth of soil microorganisms, including nitrogen fixing microorganisms, have

been noted by Grunda (1970) and by Bhardwaj and Gaur (1969); whilst the inactivation of substances toxic to plant growth by humus fractions, was described by Plchotova and Lastuvka (1971).

It was noted in earlier chapters that there was little demand in this country for town waste compost since it had little value as a fertiliser. This disadvantage, however, could easily be overcome since inorganics could still be added to town waste compost to produce a fertiliser. There is no doubt that the addition of town waste compost, and of humus substances prepared from composts, to certain soils can have a beneficial effect upon the structure of that soil, upon crop growth and yields, and upon mineral uptake by crops grown in such soil. The Commonwealth Bureau of Soils list at least twenty-three references to this effect in their annotated bibliography on "Town Refuse Fertilisers" (1968), and at least twenty-nine references to this effect in their annotated bibliography on "Humate Fertilisers" (1969). The increase in plastics, and possibly the increase in trace and harmful elements within town waste makes town waste compost an unattractive sales commodity to the farmer; however it seems not unreasonable to postulate that in the near future town waste compost could be produced with acceptable levels of synthetic and harmful elements, and, in fact, be supplemented with additional inorganic substances. Such a fertiliser, containing soil conditioning humus substances, might provide one answer to the problem of soil erosion, now being experienced by many farmers in this country.

The last chapter in this thesis will be concerned with the possible role played by the thermophilic fungi in the production of humus substances. We have seen that certain of these fungi could be actively

involved in the biodegradation of cellulose within composting town waste, it is still unknown whether these fungi are able to actively produce humus substances.

Materials and Methods

The substrate chosen for this study was beechwood, since it was felt that this substrate could provide a source of carbon, in the form of cellulose, and also other essential metabolites for humus production. The "humic acid" fraction of the humus was prepared since this fraction was easy to manipulate, and it represented 50 to 80% of the "total humus" (Hurst and Burges, 1967).

The "humic acid" extraction method used here was based on that of Stevenson (1956)* Fifty millilitres of 0.1N sodium hydroxide was added to each test sample contained in a 100 ml conical flask. The flasks were tightly stoppered, with non rubber stoppers, to prevent possible oxidation of the compounds, and shaken for 18 hours at room temperature. The solid matter was then removed by centrifugation and the dark brown alkaline extract acidified with 10% HCl until a pH of about 2.0 was achieved. At this pH a fine precipitate of "humic acid" settled out, and this process could be speeded up by heating the flask in a water bath at 50°C for several minutes. The precipitate was then removed by filtration through a small Whatman Grade 1 filter paper that had been previously dried over calcium chloride in a desiccator for two days and then weighed. The filtered "humic acid" was then washed with distilled water and dried by this same method, and the filter paper reweighed. The difference in weights was taken as the amount of "humic acid" extracted.

*N.B. This reference is erroneous (see Bibliography)

An initial experiment was carried out to determine whether the extraction method used was comparable to methods outlined by Kononova (1966), in which humus substances were extracted from soils by standing the soil in aqueous NaOH overnight. The results from this experiment are presented in Table 28, and show that more "humic acid" was extracted from the Clent soil using the shaking method than by standing the soil in NaOH. Moreover, when approximately 1mg of each "humic acid" sample was pressed into a pellet with KBr and scanned in a Unican SP200 infrared spectrophotometer, almost identical traces for the two samples were given, demonstrating that the humus products had not been substantially altered by the shaking method employed (Figure 30).

The thermophilic cellulolytic fungi were grown on beechwood veneers measuring 2.25 x 0.5 inches and having a thickness of 0.6 m.m., in the perfusion system described by Sharp and Eggins (1969). The samples were continuously supplied with E & P nutrient salts, and duplicate perfusion pots for each fungus were incubated at 48°C for up to 30 days. In a second experiment, seventeen thermophilic fungi, including many non cellulolytic fungi, were each grown on four strips of beechwood measuring 50 x 6 x 0.6 m.m. and placed on the surface of YpSs agar (Cooney and Emerson 1964). The plates were incubated at 48°C for up to 30 days, and samples of four strips per fungus were taken periodically, together with two strips per fungus from the perfusion experiment, for determination of "humic acid" content. Control strips consisted of uninoculated beechwood veneers on agar, or perfused with nutrient salts at 48°C.

TABLE 28.

A comparison of the "standing" and "shaken" methods of "humic acid" extraction.

	Shaken Soil	Standing Soil
Wet weight soil samples	2.0130 g 2.0208 g	2.0085 g 2.0177 g
% moisture	18.2%	18.2%
∴ dry weights of soil samples	1.6470 g 1.6528 g	1.6435 g 1.6520 g
Dry weight "humic acid"	66.4 mg 42.8 mg	22.1 mg 22.2 mg
% "humic acid" extracted	4.04 % 2.59 %	1.35 % 1.34 %

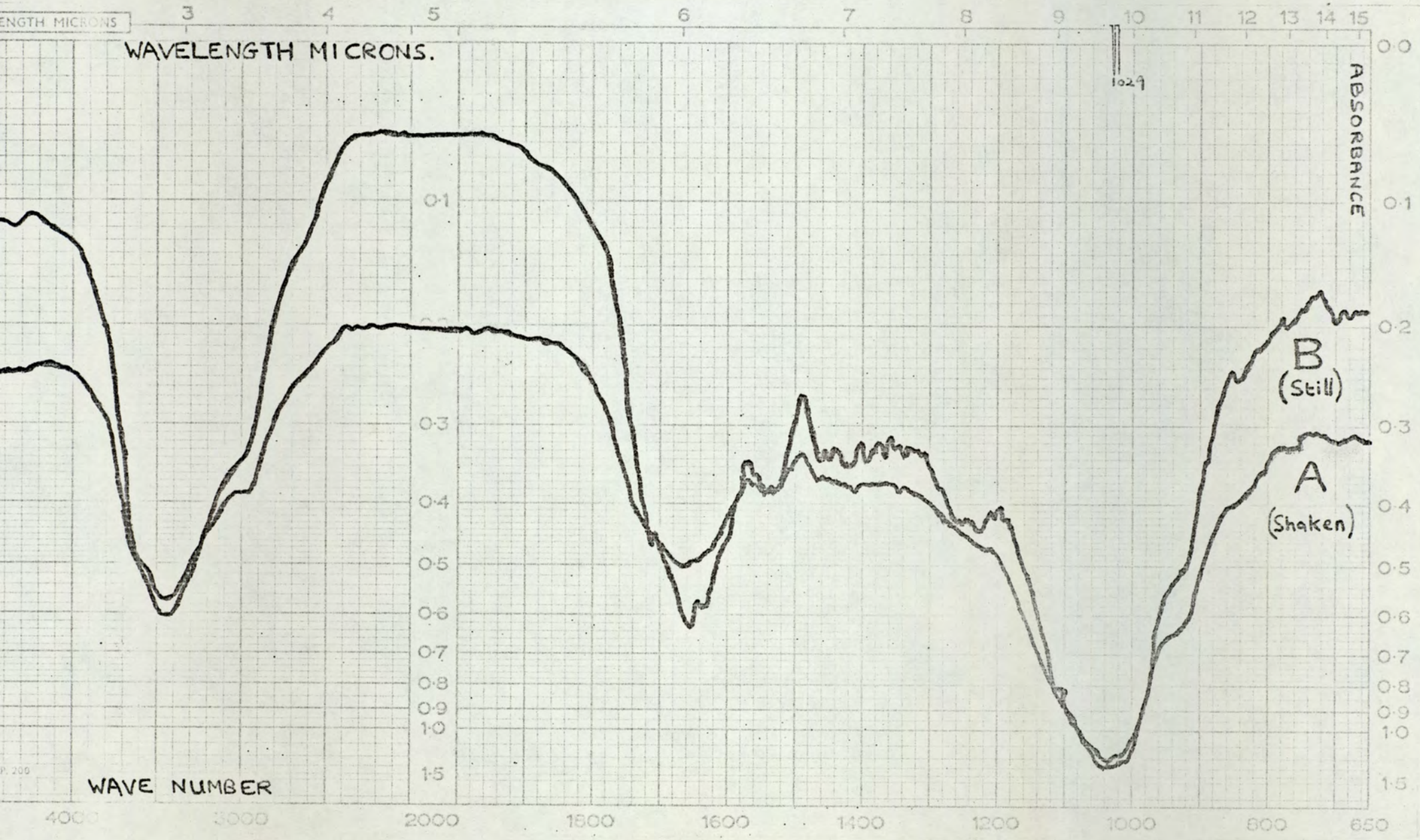

WAVELENGTH MICRONS.
3
4
5
6
7
8
9
10
11
12
13
14
15
1029
ABSORBANCE
0·0
0·1
0·2
0·3
0·4
0·5
0·6
0·7
0·8
0·9
1·0
1·5
B
(Still)
A
(Shaken)
WAVE NUMBER
4000
3000
2000
1800
1600
1400
1200
1000
800
650

FIGURE 3D

Results

The results for the dry weights of "humic acids" produced by the twelve cellulolytic thermophiles in the perfusion experiment are shown in Table 29, and those for the beechwood veneers on agar are shown in Table 30 .

Growth of all cellulolytic fungi upon the perfused strips was good. Cephalosporium sp. and Sporotrichum thermophile both produced blue staining of the strips, and after six to twelve days the strips disintegrated upon handling. Similar degradation was noted by both varieties of Chaetomium thermophile, whilst M. pulchella produced very heavy surface growth over the strips but caused little degradation (measured in terms of rigidity of the strips when subjected to bending between two pairs of forceps). Good surface growth, sporulation and degradation of the strips was produced by H. grisea and H. insolens, but Torula thermophila produced growth and sporulation within the conducting vessels of the wood. Degradation produced by this latter fungus on the strips was good, and the dark mycelium and spores gave a black appearance to the beechwood. Myriococcum albomyces produced good aerial mycelial growth on the perfused strips, and also produced cleistothecia - the first time that sporulation within this fungus had been observed!

Growth of the seventeen thermophilic fungi on the strips supported on YpSs agar followed the pattern of growth of these fungi on YpSs agar alone. The non cellulolytic M. pusillus, M. miehei, H. lanuginosa, T. emersonii, T. duponti and T. aurantiacus tended to quickly overgrow the whole surface of the agar, thereby obscuring observation of possible growth effects on the

TABLE 29

Fungi \ Days	6	12	18	24	30
Chaetomium thermophile var dissitum	4.3, 4.3	1.3, NT	5.1, 5.6	6.2, 9.7	5.8, 7.1
Chaetomium thermophile var coprophile	5.6, 3.6	3.5, 3.3	8.7, 6.6	6.0, 7.5	10.8, 6.8
Sporotrichum thermophile	4.3, 4.6	5.0, 4.8	13.0, 8.2	7.4, 9.1	10.0, 7.1
Thielavia thermophila	6.5, 3.0	4.3, 5.0	3.5, 5.0	6.2, 4.1	4.6, 5.8
Cephalosporium sp.	4.6, 3.1	7.6, 3.7	4.9, 6.7	6.0, 8.4	3.6, 8.7
Torula thermophila	5.8, 2.9	7.7, 6.0	3.8, 5.2	4.2, 6.7	6.8, 5.3
H. grisea	4.9, 3.9	6.9, 5.8	11.2, 7.2	7.8, 6.4	7.9, 7.8
H. insolens	5.2, 3.2	5.5, 3.5	1.0, 4.9	3.5, 6.2	7.2, 9.0
M. pulchella	4.0, 3.6	1.3, 6.6	2.5, 4.0	3.9, 2.7	4.1, 2.7
M. albomyces	4.0, 2.3	3.9, 1.5	4.5, 3.5	7.0, 4.8	5.6, 2.1
A. fumigatus	3.9, 3.9	1.8, NT	1.3, 3.7	4.8, 7.0	6.5, 6.3
Stilbella thermophila	3.8, 3.7	2.9, NT	2.6, 2.0	3.1, 4.3	3.9, 3.2
Control	1.1, 2.3	3.4, 1.8	1.3, 2.9	2.6, 3.7	1.0, NT

Showing the dry weights (mg) of "humic acids" extracted from perfused beechwood veneers inoculated with thermophilic cellulolytic fungi and incubated for up to 30 days at 48°C.

TABLE 30

Showing the dry weights (mg) of "humic acids" extracted from beechwood veneers supported on YpSs agar, inoculated with thermophilic fungi, and incubated for up to 30 days at 48°C.

	3	6	9	12	15	18	21	24	27	30
A. fumigatus	4.2	6.0	6.2	16.0	16.0	NT	7.6	7.5	13.4	NT
Cephalosporium	7.4	0.6	6.5	12.8	4.4	5.7	15.7	12.7	12.7	16.2
Chaetomium thermophile var. coprophile	2.9	12.1	5.5	14.9	5.1	2.8	8.2	12.4	4.1	7.9
Chaetomium thermophile var. dissitum	9.4	4.4	5.0	12.2	4.7	5.9	9.3	11.0	4.1	8.8
H. grisea	11.0	4.9	2.9	19.2	12.6	2.4	13.2	14.7	10.6	9.7
H. insolens	0.9	8.3	8.1	19.5	9.2	3.8	8.7	13.0	5.4	12.0
H. lanuginosa	5.5	3.7	3.7	9.7	9.2	NT	8.1	8.4	NT	9.1
M. pulchella	11.5	0.6	9.1	12.8	6.6	3.0	4.7	10.8	6.5	8.5
M. pusillus	9.0	1.7	3.0	5.4	5.1	2.5	4.1	3.7	2.7	7.5
M. miehei	4.6	1.3	0.6	12.2	5.0	NT	4.8	2.2	2.2	4.8
M. albomyces	0.5	7.2	8.9	9.5	4.8	4.1	7.9	NT	4.4	5.6
Sporotrichum thermophile	7.6	0.7	10.5	13.6	13.3	7.4	12.9	18.9	6.5	9.3
Stilbella thermophila	6.9	6.1	8.8	13.8	12.0	10.5	13.3	13.5	5.3	18.0
T. duponti	10.6	0.8	4.5	9.0	4.3	3.9	3.9	6.3	7.9	7.9
T. emersonii	10.2	3.3	5.5	11.3	4.4	NT	9.1	0.3	3.7	6.8
T. aurantiacus	10.0	3.9	6.0	8.5	0.8	1.1	5.6	3.8	0.5	2.2
Torula thermophila	7.9	7.9	7.4	14.3	4.5	1.2	8.5	14.8	4.4	6.3
Control	5.4	0.0	3.8	4.1	7.1	6.2	3.5	10.0	5.0	7.4

strips. The cellulolytic fungi, with the exception of A. fumigatus which overgrew the whole agar and the strips, preferred to grow upon the strips and, in general, produced better aerial growth and sporulation than they did in the perfusion kits. In this case, however, additional nutrients were available for fungal growth in the YpSs agar.

When samples from the perfusion pots were extracted with alkali and acidified, characteristic precipitation patterns were noted for each thermophilic cellulolytic fungus, fifteen minutes after acidifying. The "humic acid" extracted from the strips bearing Chaetomium thermophile var. dissitum was light brown in colour and remained in suspension, whilst that from the strips bearing variety coprophile was of a similar colour but precipitated more quickly. The "humic acids" from the strips bearing Sporotrichum thermophile and H. grisea was very dark brown in colour and floated upon the surface, and Malbranchea pulchella produced a floating mass of medium brown "humic acid" and also some which remained in suspension. The strips bearing Cephalosporium sp., A. fumigatus and Stilbella thermophila produced a light brown "humic acid" which remained in suspension, whilst that from strips bearing H. insolens, Thielavia thermophila, M. albomyces and Torula thermophila was medium brown in colour and mostly precipitated, with very little remaining in suspension.

The mean dry weights of "humic acids" extracted from the perfused strips, minus the control values, are presented in Figure 32. The results indicate that under conditions of fungal cellulolysis of the strips, humic substances can be isolated, and that, in general, the amounts of these substances increase with increasing degradation of the

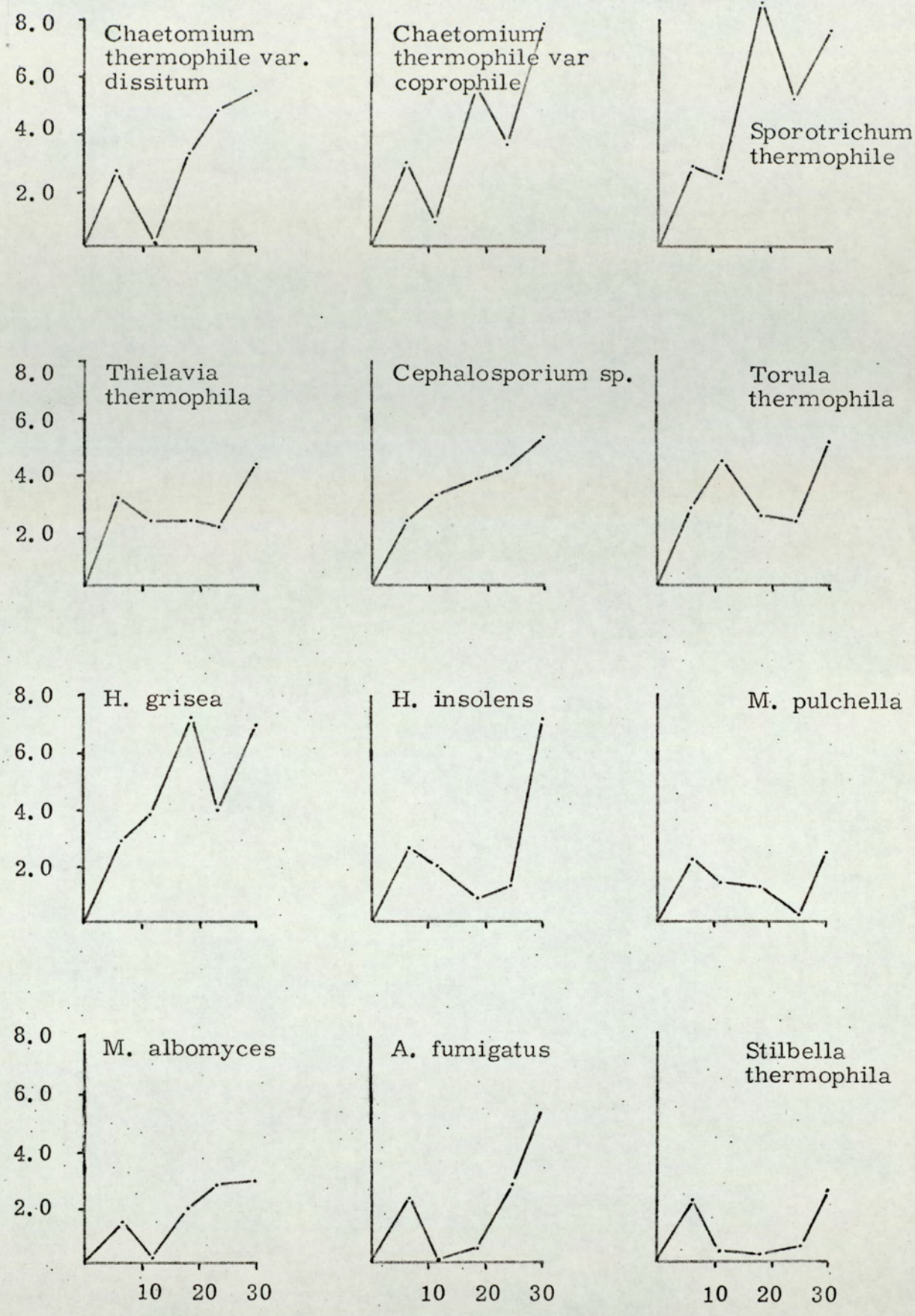


FIGURE 31

Figure showing dry weights of "humic acid" (mg), minus control values, isolated from perfused beechwood veneers in the perfusion system of Sharp and Eggins (1969) and inoculated with thermophilic cellulolytic fungi.

strips. The strips bearing the more cellulolytic fungi e. g. the two varieties of Chaetomium thermophile, Sporotrichum thermophile, Cephalosporium sp., Torula thermophila, Thielavia thermophila, H. grisea, H. insolens and A. fumigatus were found to yield increasing amounts of "humic acid" with time, whilst smaller increases were noted for those strips bearing M. pulchella, M. albomyces and Stilbella thermophila.

Whilst the results for the perfused beechwood veneers showed small overall levels of "humic acids" in the control strips, due in part to the probable leaching away of these compounds or their components by the perfusion current, the control levels of "humic acids" in the strips placed on agar were considerably higher. This fact led to the production of negligible amounts of "humic acids" from many strips on certain days, especially days 15 and 18. The resulting graphs, Figure 33 a to c, gave the impression that humic substances were being actively utilised at certain times by all of the test fungi, and being actively synthesised at other times. Similar results were observed for many of the cellulolytic thermophiles in the perfusion kits (Figure 31). Smaller amounts of "humic acids" were produced by the strips bearing the non cellulolytic thermophiles, but the fact that levels above those in controls were produced might be taken as an indication of the role of fungal autolysis in the production of these compounds. Over the period of incubation of the non cellulolytic fungi it was noticeable that fungal growth over the strips declined with increasing culture time. The high amounts of "humic acids" extracted from the control strips could possibly be explained by the fact that several of the structural components of these compounds were probably present in the YpSs agar surrounding the strips, and that diffusion into

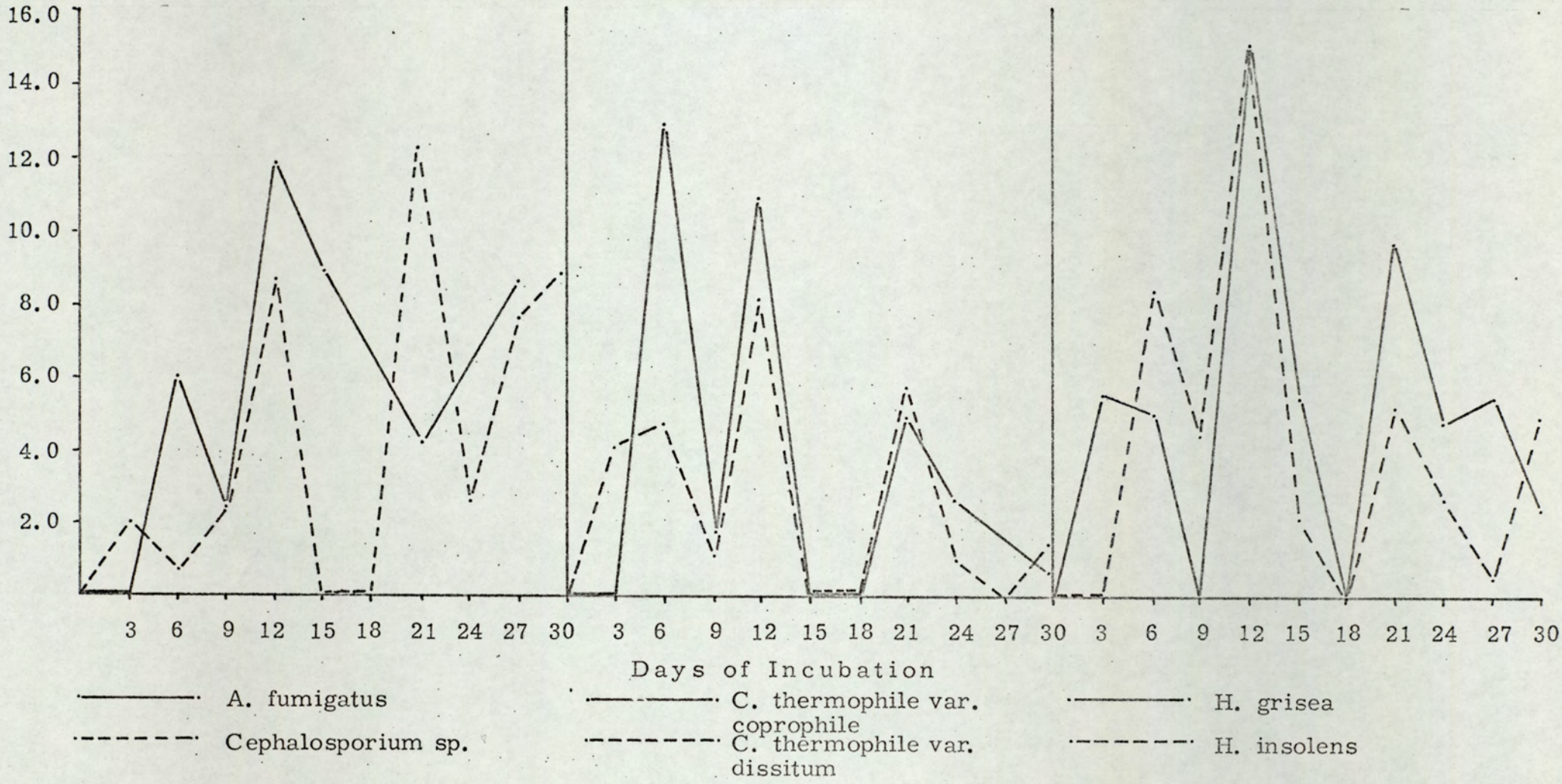


FIGURE 38a to c

Showing dry weights (mg) of "humic acids", minus control values,
isolated from beechwood veneers supported on YpSs agar and inoculated with thermophilic fungi

FIGURE 38a

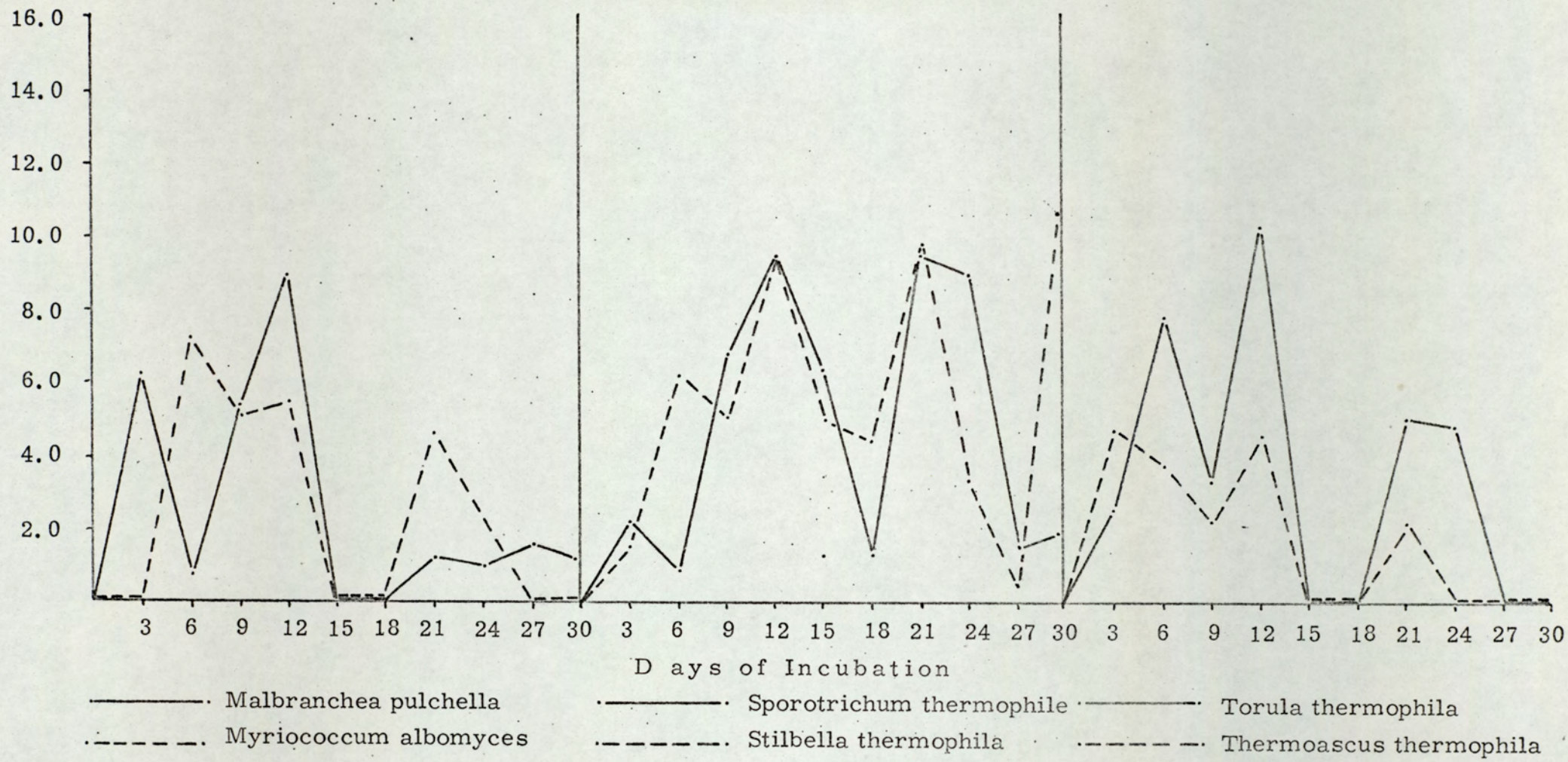

16.0
14.0
12.0
10.0
8.0
6.0
4.0
2.0
3 6 9 12 15 18 21 24 27 30 3 6 9 12 15 18 21 24 27 30 3 6 9 12 15 18 21 24 27 30
Days of Incubation
Malbranchea pulchella
Sporotrichum thermophile
Torula thermophila
Myriococcum albomyces
Stilbella thermophila
Thermoascus thermophila

FIGURE33b

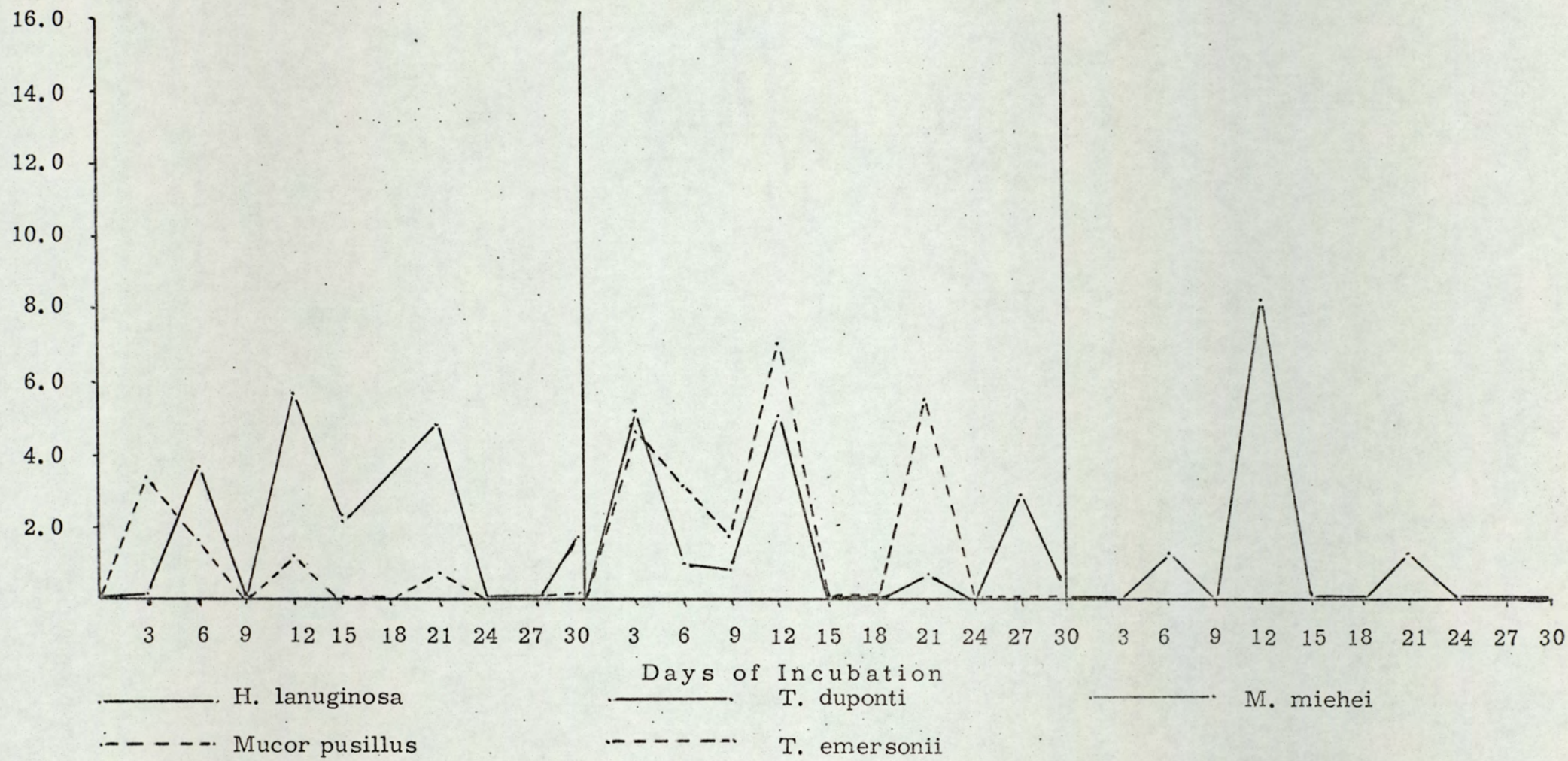

16.0
14.0
12.0
10.0
8.0
6.0
4.0
2.0
3 6 9 12 15 18 21 24 27 30 3 6 9 12 15 18 21 24 27 30 3 6 9 12 15 18 21 24 27 30
Days of Incubation
H. lanuginosa
T. duponti
M. miehei
Mucor pusillus
T. emersonii

FIGURE 33c

the strips of these components, and chemical reactions within the strips led to these high levels of "humic acids".

Although the "humic acids" from the strips bearing the thermophilic fungi were not further analysed, an analysis was carried out into the spectral characteristics of the "humic acid" isolated from the perfused beechwood control strips and that isolated from samples of the town waste compost produced in earlier experiments (Chapter 2). In the former case the shaking isolation method was used to extract the "humic acids" and in the latter case samples of compost were extracted using the "standing" and the shaking methods.

The results indicated that more "humic acids" could be extracted from the compost using the shaking method (mean value of 4.45%) than using the "standing" method (mean value of 1.50%), but that, in this case, a better defined I/R spectrum was given (Figure 33) with the "humic acids" prepared by the "standing"method. In comparison, the perfused beechwood veneers extracted by the shaking method yielded a "humic acid" showing well defined peaks (Figure 34) in the I/R region. Many of the peaks in both samples coincide exactly and it would appear that the "humic acids" formed in the beechwood controls and in the compost had very similar structures. When these two spectra are compared to the spectra produced by the soil "humic acid", and especially that produced by the "standing" method (Figure 30) again many of the peaks coincide exactly, even though the newer formed "humic acids" in the wood and the compost are better defined. These qualitative comparisons, on what was very crude humic material, give some indication that humus substances formed within differing materials may be very similar in composition; and similar

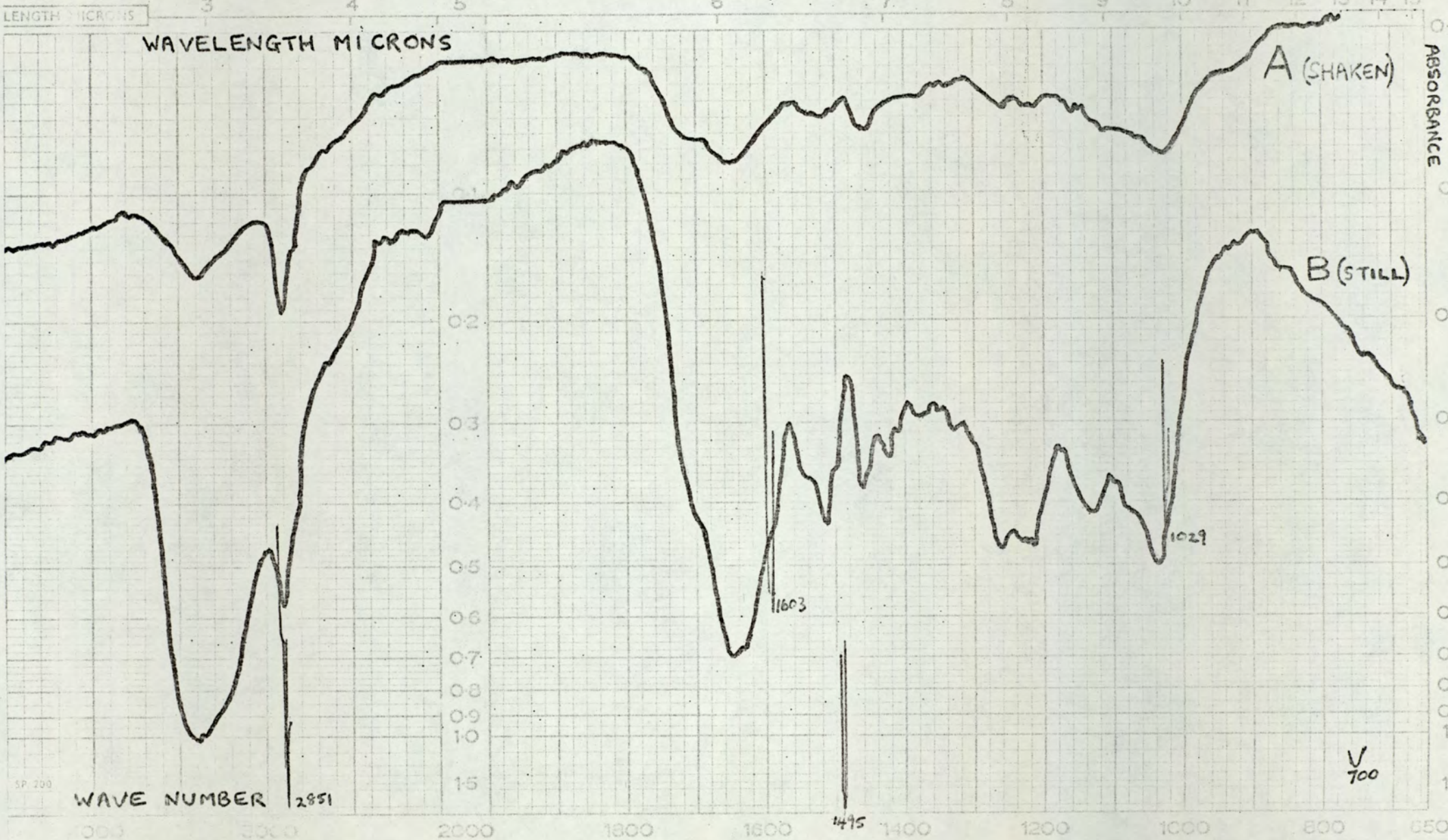

WAVELENGTH MICRONS
A (SHAKEN)
B (STILL)
ABSORBANCE
WAVE NUMBER
2851
1603
1495
1029
V 700

FIGURE 37

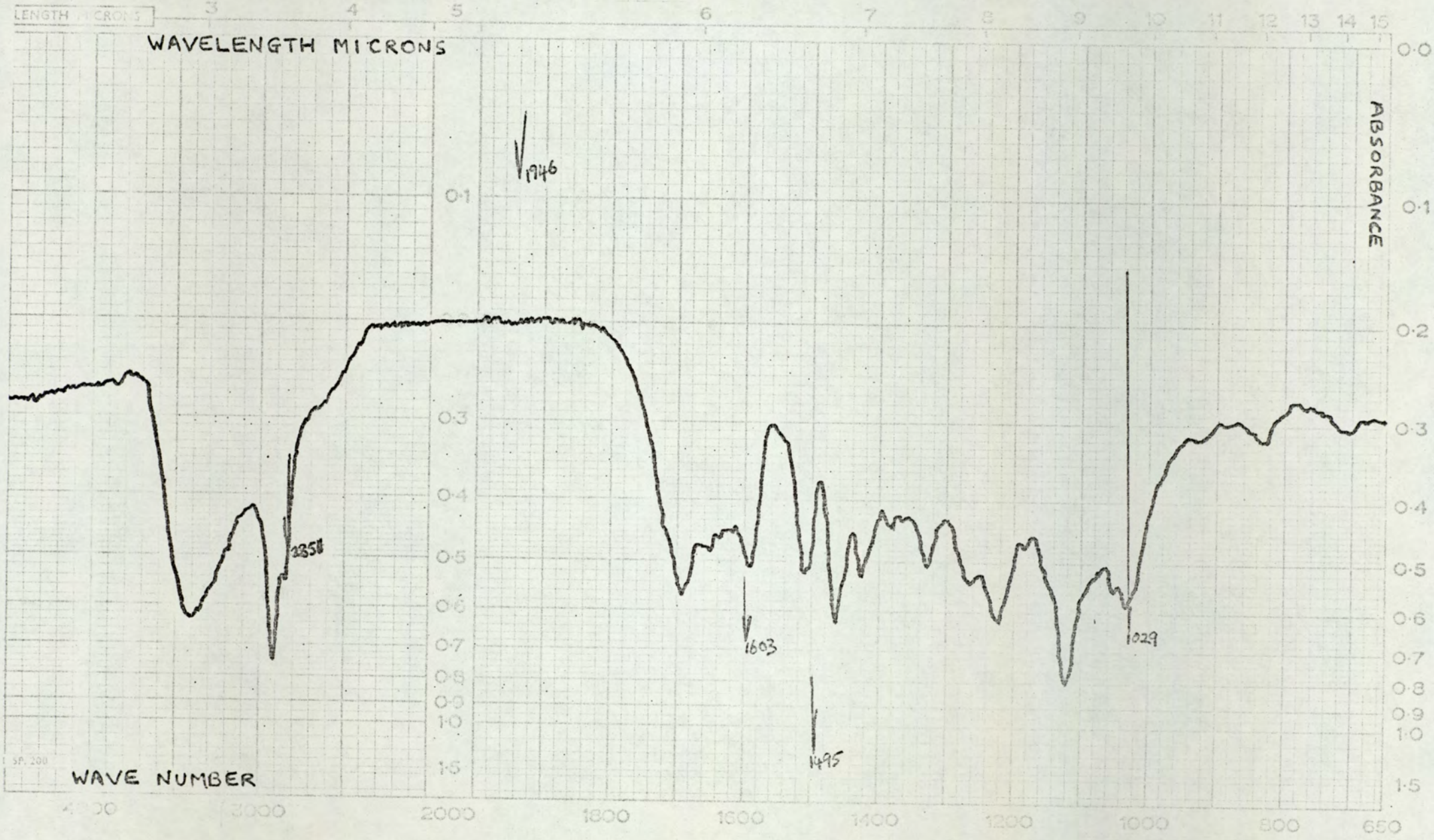

WAVELENGTH MICRONS
3
4
5
6
7
8
9
10
11
12
13
14
15
ABSORBANCE
0·0
0·1
0·2
0·3
0·4
0·5
0·6
0·7
0·8
0·9
1·0
1·5
1746
2851
1603
1495
1029
WAVE NUMBER
4000
3000
2000
1800
1600
1400
1200
1000
800
650

FIGURE 34

reports have been documented by Kononova (1966).

Discussion

Levels of "humic acids" over and above control values could be isolated from beechwood veneers which were supporting the growth of seventeen thermophilic fungi. In general, the more cellulolytic the fungus, in the perfusion systems, the more "humic acid" could be extracted with time. Cellulolytic and non cellulolytic fungi growing on the strips supported on YpSs agar appeared to be utilising these humus substances at certain times, whilst at other times their production was increased. It was apparent from the results that many factors other than the growth of the thermophilic fungi on the strips contributed towards the production of these humus substances, but nevertheless increased production of "humic acid", over control levels, was observed in all strips supporting the growth of thermophilic fungi.

Humus substances were produced in the town waste compost experiment of Chapter 2, and these substances were shown to have a similar composition to humus substances extracted from beechwood veneers. It would appear, from evidence presented in this chapter, that most of the thermophilic fungi are indeed able to contribute towards the production of humus substances, although it is still unknown what contribution this production makes to the overall production of humus substances within composting town waste. It would be interesting to pursue this topic further, if one could be sure of producing a model "town refuse" free of all micro-organisms with the exception of the thermophilic fungi.

CHAPTER 9

CHAPTER 9

CONCLUSION

"If composting of garbage and refuse should ever become widespread, detailed knowledge of the activities of thermophilic fungi would be of practical importance."

Cooney and Emerson (1964)

If the composting of town waste is to become an accepted method of the future, then an understanding of the complete process is required so that modifications might be made to improve upon present methods. Composting is a biological process which can be used to recycle waste material, and this factor has largely been ignored in previous composting ventures. The composting of town waste is basically a two stage process during which heat is generated within the mass of waste and complex organic molecules are degraded into simpler, more stable compounds. During the first, or thermophilic, stage of town waste composting, temperatures within the mass of waste rise to within the limits required for growth of the thermophilic micro-organisms. Previous work has indicated that a high percentage of the cellulosic material present within town waste is degraded during this phase. When temperatures within the heap start to fall during the second, or mesophilic, stage, the degradation of the remaining organic compounds is completed by the mesophilic micro-organisms. There appears to be a need, therefore, to investigate the actions of the groups of

micro-organisms present within composting town waste so that the process, as a whole, might be better understood. One group of organisms present within composting town waste are the thermophilic fungi, and this investigatio has attempted to outline the role played by these fungi in the biodegradation of certain components of town waste.

The changing composition of town waste (Table 1) indicates an increase in the cellulosic content, which would appear to make town waste an excellent substrate for the biological composting process. Biochemical studies on the degradation of cellulose present within a small town waste windrow confirmed previous results that the cellulose content decreased sharply during the thermophilic stage of composting, but that very few thermophilic cellulolytic fungi could be isolated from such material. Six cellulolytic thermophilic fungi were isolated from the windrow and from composting Birmingham town waste and it was demonstrated that these fungi viz: Chaetomium thermophile, Aspergillus fumigatus, Malbranchea pulchella, Humicola grisea, Humicola insolens and Torula thermophila, together with Sporotrichum thermophile, Cephalosporium sp., Myriococcum albomyces, Stilbella thermophila and Thielavia thermophila were all able to hydrolyse particles of cellulose in an agar medium.

If these twelve fungi (two varieties of C. thermophile) were all able to hydrolyse cellulose, why had only three of them (C. thermophile, A. fumigatus and M. pulchella) previous to this study, and also three isolated during this study (H. grisea, H. insolens, and Torula thermophila) ever been isolated from composting town waste?

A study of the relative cellulolytic activities of these twelve fungi was carried out using insoluble cellulose in a form similar to cellulose present within town refuse. Strength loss testing showed that most of these fungi were able to bring about a 100% loss in wet strength of the cellulose within twelve days at 48°C; and these results extend all previously published results. On the basis of weight loss results (a primary measure of cellulolysis), the cellulolytic thermophilic fungi were placed into three new arbitrary groupings depending upon the degree of weight loss of insoluble cellulose produced within twelve days at 48°C. From these groupin it could be seen that of the cellulolytic thermophiles isolated from composting town refuse, Chaetomium thermophile var. dissitum, Aspergillus fumigatus and Humicola insolens were strongly cellulolytic; Chaetomium thermophile var. coprophile and Humicola grisea were reasonably cellulolytic and Malbranchea pulchella and Torula thermophila were weakly cellulolytic. It appeared, therefore, that these fungi were in some way adapted to environmental parameters imposed by the process of town waste composting and that the remaining cellulolytic thermophiles viz: Sporotrichum thermophil Thielavia thermophila, Cephalosporium sp., Stilbella thermophila and Myriococcum albomyces were in some way suppressed.

It was decided to investigate two environmental factors in this study, although the limitations of these investigations were fully appreciated. The first factor studied was the effect of pH upon the cellulolytic activity of the cellulolytic thermophiles. Previous work, and work carried out here (Chapter 2), confirmed that pH values within a mass of composting town waste quickly rise to alkaline values within the thermophilic phase of composting. When the cellulolytic thermophiles isolated from such systems were grown on insoluble cellulose perfused with nutrients buffered at various

pH levels, it was observed that the majority of these fungi were able to produc hydrolysis of the cellulose at all pH values tested and that Chaetomium thermophile var. coprophile and variety dissitum, Malbranchea pulchella, Torula thermophila, Humicola insolens and Humicola grisea were all able to produce mycelial growth and in most cases sporulation also at alkaline pH values. The remaining cellulolytic thermophiles and Aspergillus fumigatus, produced better growth and sporulation at low, acidic pH values. When the cell free extracts of the twelve fungi were tested for their power to hydrolyse soluble cellulose in the form of carboxymethyl cellulose (CMC), it was shown that the C_x enzymes from the two varieties of Chaetomium thermophile, Thielavia thermophila, Torula thermophila, Malbranchea pulchella and Humicola insolens showed optima, or rises towards optima, in the alkaline region. With the exception of Thielavia thermophila, the other cellulolytic thermophiles have all been isolated from composting town waste. These results would tend to suggest that those thermophilic cellulolytic fungi which are able to grow, sporulate and maintain cellulase enzyme efficiency at alkaline pH values will be successful in colonising a substrate such as composting town waste. Of the six (seven) thermophilic cellulolytic fungi previously isolated from composting town waste, it would appear that the two varieties of Chaetomium thermophile together with Humicola insolens, Torula thermophila and Humicola grisea, (which also showed considerable C_x activity in the alkaline region), are the most important thermophilic fungi in the first stage of the composting of town waste. Malbranchea pulchella and Aspergillus fumigatus probably only play a secondary role in the degradation of cellulose within composting town waste since both fungi produced better growth, sporulation and cellulolysis at low, acidic pH values.

The second factor, which arose from this initial research, was a study of the interaction effects between the thermophilic cellulolytic fungi. Although individual thermophiles in isolation might be extremely cellulolytic, it was not known what effect one fungus would have upon another's cellulolytic activity when the fungi were grown in combination on cellulose - a situation similar to that existing in composting town waste.

The twelve cellulolytic thermophiles were grown in every combination on agar containing ball milled cellulose, and interaction effects such as inhibition of depth of clearing (i.e. cellulase inhibition), intergrowth and sporulation were noted. It was found that the notable depressors of cellulolytic activity were Humicola grisea, Humicola insolens, Torula thermophila and Malbranchea pulchella, and that these fungi greatly depressed the cellulolytic activity of the two varieties of Chaetomium thermophile. This effect appeared to be balanced by the ability of the two varieties of Chaetomium thermophile to reduce the growth and sporulation of Torula thermophila, Humicola insolens and Humicola grisea. The fact that the four depressors in combination in the Rautela-Cowling tubes produced excellent growth and sporulation under partial anaerobic conditions, might account for the success of these four fungi in composting town waste. These results indicated that the six species of fungi were, in general, able to compete together successfully on a cellulosic substrate. When the interaction effects of the above six species of fungi on the growth and cellulolytic activity of the highly cellulolytic Sporotrichum thermophile, Thielavia thermophila and Cephalosporium sp. were noted, it was observed that the depths of clearing of these three fungi were greatly depressed by interaction with Humicola grisea, Humicola insolens and Torula thermophila. The former fungi were all able to induce heavy sporulation in the latter fungi and

these observations would tend to indicate that large scale colonisation of composting town waste by Sporotrichum thermophile, Thielavia thermophila and Cephalosporium sp. might be suppressed, although the former two fungi show considerable C_x activity in the alkaline region.

To summarise this initial research, therefore, it appears that to be successful in colonising composting town waste, a thermophilic cellulolytic fungus must be able to grow, sporulate, compete with interacting fungi and produce a reactive cellulase enzyme under the environmental conditions present within composting town waste. It has been shown that the two varieties of Chaetomium thermophile, together with Humicola insolens, Humicola grisea, Torula thermophila, Malbranchea pulchella and Aspergillus fumigatus are able to fulfill some or all of these conditions, and that these fungi are the thermophilic cellulolytic fungi usually isolated from composting town waste.

As previously stated, the limitations of these investigations were fully realised and there are obviously many other factors which could be investigated so that a more complete understanding of the role of thermophilic fungi in the process of composting might be attained. Some factors which could be investigated in future research work are briefly outlined below.

The first factor could be a complete ecological study of the process of town waste composting to indicate whether thermophilic fungi are naturally present within such a material, or invade such a material at the collection, pulverisation or the composting stage. Recent work by Evans (1972) has shown that the dominant thermophiles in the air (Mucor pusillus, H. lanuginosa and Aspergillus fumigatus) were similarly

dominant in the upper layers of coal spoil tips, whilst Chaetomium thermophil Myriococcum albomyces and Thermophile aurantiacus were absent from the air but consistently isolated from the lower layers of these tips. Studies such as these could also be applied to composting town waste.

A second line of research could be the effect of physical parameter within composting town refuse upon the growth and activity of the thermophilic fungi. The effect of trace metals, other nutrients and the effect of temperature and pH upon the germination of thermophilic fungal spores could be studied to establish whether conditions within composting town waste are conducive to the germination and growth of the thermophilic fungi. Work by Mills (1968), Hedger and Hudson (1970), Fergus (1971) and Fergus and Amelung (1971) in this respect has demonstrated that thermophil fungi only sporulate in certain regions of a compost heap, and that their spores are extremely susceptible to thermal death. Since high temperature are reached initially within the central layers of composting town waste, a study of the recolonisation of these layers by thermophilic fungi when temperatures are more favourable to their growth is needed.

The last, and probably the most formidable factor to be mentione here, could be a study of the effects of other micro-organisms present with composting town waste on the activities of the thermophilic fungi. Initial studies presented here indicated that a several thermophilic actinomycetes were present in large numbers at all stages of the composting process. Work by Stutzenberger (1971) has shown the importance of a thermophilic actinomycete, Thermomonospora curvata, isolated from composting town waste in the degradation of cellulose; and similar research work could outline the role played by such organisms. Such work could probably best

be carried out in small scale, model town waste composting systems.

The conclusions outlined above have shown that at least twelve thermophilic fungi are able to actively degrade cellulose and that six species of cellulolytic thermophiles are able to compete together on cellulose under environmental conditions present within composting town waste. Town waste also contains increasing quantities (Table 1) of synthetic polymers or "plastics"; materials which have previously been shown to be resistant to mesophilic fungal degradation. Tests were, therefore, carried out on several synthetic polymers to establish whether these materials could be degraded by the thermophilic fungi. The results confirmed that the two non-biodegradable synthetic polymers, polyethylene and polyvinyl chloride, were indeed resistant to thermophilic attack, but that the biodegradable polymer, polyvinyl acetate, could be greatly modified, especially in thermophilic soil burial tests.

When the polyethylene polymer was gently oxidised in air at 200^{o}C, surface growth and some penetration by thermophilic fungi and actinomycetes was noted. These experiments suggested that the biodegradation of waste synthetic polymeric material might be achieved if some degree of initial oxidation of the recalcitrant molecule was effected. Such treatment would provide an oxidisable substrate for microbial utilisation whilst recycling elemental carbon locked up in the polymer. Air oxidation of polyethylene produced only surface growth of microorganisms on the very high molecular weight oxidation products, and so it was decided to oxidise the polymer more vigorously to produce low molecular weight oxidation products, in this case dicarboxylic acids, using nitric acid as oxidising agent.

Since the polyethylene polymer is largely responsible for the increases in plastics materials within town waste, it was decided to attempt to modify its chemical structure to produce chemical degradation products which might further be susceptible to microbiological degradation. This was achieved by oxidising polyethylene with nitric acid to produce low molecular weight dicarboxylic acids. It was demonstrated that these acids could support the growth of many of the thermophilic fungi. The use of similar methods to pretreat plastics waste from town refuse could yield many future benefits. Plastics could be separated off from town refuse after the composting stage and pretreated with one of the many oxidising agents available (Further research by the author and Dr. B. S. Brown at the University of Manchester has shown that polyethylene bags and wrappings, plastic milk bottles, egg cartons and washing up bottles can be treated in this way). The breakdown products could then be re-introduced int a biological system for further thermophilic utilisation, or separated off and purified to yield valuable chemical by-products. In this way recycling of the carbon, originally locked up within these recalcitrant molecules could be achieved, with the simultaneous production of a plastics-free compost.

When the nineteen thermophilic fungi were tested for their ability to degrade plasticisers i. e. synthetic molecules utilised to modify the physical properties of synthetic polymers, it was demonstrated that all nineteen thermophiles were able to grow upon and utilise several of these compounds. Many plasticisers are esters and it was found that all the thermophiles tested were able to either produce clearing zones in a plasticiser enriched agar, or to produce hydrolysis of an ester plasticiser in an enzymatic technique. These results demonstrated the existence of esterase enzymes in all nineteen thermophilic fungi tested. Six of the

plasticisers under test, viz: DNBT, TNBC, PE200, TCP, TEOP, and DOP (see text, Chapter 7) have previously been shown to be non biodegradable under mesophilic conditions. These six plasticisers, and one "unknown" plasticiser, TTP, could all be utilised by at least one thermophilic fungus at 48°C. In general, the thermophilic cellulolytic fungi did not produce as much growth on the plasticisers as did the non cellulolytic thermophiles. In this respect Talaromyces emersonii proved to be the most adaptive thermophile, and similar results were observed for growth of Thermoascus aurantiacus. It was also observed that several of the plasticisers which could not support thermophilic fungal growth when present as the sole carbon source, were able to support growth when supplemented with additional microbial nutrients. It was concluded that the thermophilic fungi must not be overlooked as a group of test micro-organisms by plastics manufacturers when testing their products for biodeterioration.

The end product of the biological process of the composting of town waste is "humus" which can be used as a highly effective soil conditioner. The erosion of arable soil in this and in other countries by the excessive use of inorganic fertilisers could be prevented by the simultaneous application of organic humus to replace that being gradually depleted. The use of town waste compost, in certain developed countries, has previously been avoided by farmers because of its unattractive nature i. e. incomplete breakdown of organic material and high content of plastics material. One suggestion for the removal of plastics material has been outlined above, and studies similar to those carried out here should elucidate the requirements of the particular groups of micro-organisms concerned in the biodegradation process. Research work outlined in Chapter 8 demonstrated that all the thermophilic fungi tested were able to produce "humic acids" (a measure

of the efficiency of the composting process) over and above control levels found in natural substrates. The cellulolytic thermophiles, in general, produced more "humic acids" than the non cellulolytic thermophiles, and these humus substances were shown to have a similar chemical composition to humus substances extracted from composted town waste.

It would appear, therefore, that the thermophilic fungi, as a group, could have an extremely important part to play in the initial stages of the composting of town waste if environmental parameters could be adjusted to encourage their development. This research has indicated several ways in which this might be achieved.

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